GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

PROGRESS AND CHALLENGES IN ATHEROSCLEROSIS AND PAIN

Habilitation Thesis

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Abbreviation list

ACS: Acute coronary syndrome

ANOVA: analysis of variance

Apo: apolipoprotein

ASCVD: atherosclerotic cardiovascular disease

AT: oxygen uptake at the anaerobic threshold

AUC: area under the curve

BCAAs: branched chain amino acids

BDI: beck depression inventory

BIPQ: illness perception questionnaire

BMI: body mass index

BP: blood pressure

CAD: coronary artery disease

CD: cluster of differentiation

CDS: cardiac depression scale

CG: λ-carrageenan

CI: confidence intervals

CP: cold plate

CR: cardiovascular rehabilitation

CRP: C-reactive protein

CV: cardiovascular events

CVD: cardiovascular disease

DdLV: diastolic diameters of the left ventricul

EAS: European Atherosclerosis Society

ELISA: enzyme-linked immunosorbent assay

EMLA: eutectic mixture of local anesthetics

ESC: European Society of Cardiology

FGF-2: basic fibroblast growth factor

GSH-Px: glutathione peroxidase

HADS: hospital anxiety and depression scale

HDL: high-density lipoprotein

HDL-C: high-density lipoprotein cholesterol

HE: haematoxylin and eosin

HFrEF: heart failure with reduced ejection fraction

HOMA: homeostasis model assessment

HP: hot plate

HR: maximal heart rate

HRR: heart rate reserve

ICAM: intercellular adhesion molecules

IL: interleukin

IPAQ – L: international physical activity questionnaire long form

IPAQ: international physical activity questionnaire

IR: insulin resistance

IVS: interventricular septum

LDL: low-density lipoprotein

LDL-C: low-density lipoprotein cholesterol

LVM: left ventricular mass

MACE: major adverse cardiovascular events

MET: total metabolic equivalent of task

MI: myocardial infarction

NSTEMI: myocardial infarction without ST-segment elevation

PA: physical activity

PAD: Peripheral artery disease

PANAS: positive and negative affect schedule

PDGF: platelet-derived growth factor

PE: pulmonary embolism

RER: maximal value of the respiratory exchange ratio

RWT: the relative thickness of the ventricular wall

SD: standard deviation

SOD: superoxide dismutase

STEMI: myocardial infarction with ST-segment elevation

T2DM: type 2 diabetes

TF: tissue factor

TG: triglycerides

UA: unstable angina

VCAM-1: vascular cell adhesion molecule 1

VLDL-C: very low-density lipoprotein cholesterol

VO2: max maximal oxygen uptake

VSMC: vascular smooth muscle cells

Rezumat

Teza de abilitare reflectă rezultatele semnificative obținute în activitatea personală desfășurată în perioada post-doctorală (2011-2022), precum și câteva dintre direcțiile viitoare de cercetare.

Lucrarea este elaborată în conformitate cu criteriile recomandate și aprobate de către Consiliului Național de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU). În cadrul tezei sunt prezentate cele mai relevante rezultate științifice cu privire la studiul bolilor cardio-vasculare, inițiate în cadrul cercetării doctorale, și continuate în perioada post-doctorală cu dezvoltarea aspectelor etiopatogenice, morfologice și de management în ateroscleroză. Demersul științific personal a fost orientat și către preocupările în domeniul extrem de actual al managementului durerii în practica medicală, o problemă globală care afectează atât pacientul pediatric cât și adultul. Ambele teme au fost și sunt cercetate atât în cadrul Disciplinei de Morfopatologie, cât și în colaborare cu alte discipline ale Universității de Medicină și Farmacie" Grigore T. Popa" Iași, printre care Histologie, Fiziologie, Medicină Internă, Cardiologie, Pediatrie, Neurologie.

Teza este structurată astfel:

Secțiunea I cuprinde o trecere în revistă a realizărilor academice, profesionale și științifice din perioada postdoctorală.

Secțiunea II sumarizează câteva direcții de cercetare asupra cărora mă voi focusa în viitor.

Secțiunea III conține lista de referințe bibliografice care stă la baza prezentei lucrări.

Rezultatele cercetării științifice desfășurate și a contribuțiilor personale au fost structurate pe două direcții:

Prima direcție de cercetare prezentată în **Capitolul 1** este direcționată către studiul aterosclerozei și reprezintă o continuare a studiilor efectuate în teza de doctorat intitulată *Modificări ale histofiziologiei arteriale consecutiv leziunilor de ateroscleroză*, susținută în anul 2011. Așa cum am menționat anterior, prin colaborarea cu colegii din Disciplina de Morfopatologie, dar și prin colaborări multidisciplinare a fost posibilă elaborarea de lucrări științifice care au fost publicate în reviste de specialitate cu vizibilitate internațională, cotate ISI sau BDI.

Subcapitolul 1 face referire la studiul factorilor de risc cu impact în dezvoltarea bolii aterosclerotice. Această patologie necesită o abordare riguroasă deoarece identificarea factorilor de risc favorizanţi, cu implicaţii certe în iniţierea şi progresia afecţiunii, precum şi modularea celor cu rol protectiv, pot avea un impact deosebit în găsirea unei atitudini terapeutice adecvate care să amelioreze evoluția şi consecinţele acesteia.

Subcapitolul 2 detaliază o preocupare constantă asupra studiului inițierii și dezvoltării plăcii de aterom – leziunea de bază în ateroscleroză. În acest sens am efectuat o serie de cercetări experimentale în care am urmărit evidenţierea modificărilor histopatologice care caracterizează ateroscleroza şi determinarea unor markeri biochimici cu o importantă valoare prognostică. De asemenea am studiat potenţialul antiaterogen al unor aminoacizi nonpolari în condiţiile unei hipercolesterolemii induse de o dietă bogată în lipide. Interesul meu a fost direcționat și către

cercetarea aspectelor anatomopatologice ale plăcii aterosclerotice pe fragmente tisulare recoltate prin endarterectomie de la pacienți simptomatici.

Subcapitolul 3 face referire la rezultatele unor colaborări multidisciplinare cu privire la managementul și reabilitarea pacienților cu boală coronariană. Studii științifice legate de ateroscleroză sunt abordate în mod constant în fluxul principal de publicații, odată cu creșterea morbidității și mortalității prin boală ischemică cardiacă. Astfel, comunitatea medicală se concentrează în mod justificat asupra tratamentului și prevenției bolilor cardiovasculare, urmărind controlul factorilor de risc implicați în etiologia acestor boli.

Contribuțiile personale în urma acestor cercetări au fost publicate în reviste de prestigiu precum: *Medicine (Baltimore).* IF:1.889, *Medicina.* IF: 2.430, *Applied Sciences.* IF: 2.679, *Diagnostics.* IF: 3.706, *Biomolecules.* IF: 4.879, *Journal of Personalized Medicine*. IF: 4.945.

Capitolul II prezintă a doua direcție de cercetare și anume studiul durerii, un fenomen complex, polimorf și multidimensional cu consecințe patofiziologice, psihosociale și emoționale covârșitoare asupra pacientului. Pornind de la faptul că administrarea transdermală a unor compuși analgezici oferă multe avantaje față de tratamentele orale sau injectabile, preocupările personale au fost direcționate către abordarea experimentală a acestei idei. În mod concret am studiat efectul analgezic la diferite intervale de timp al unor substanțe active încorporate în diferiți carrieri asupra sensibilității nociceptive la animalele de laborator.

Contribuțiile personale în urma acestor cercetări au fost publicate în reviste de prestigiu precum: *Rom J Morphol Embryol*. IF: 1.411, *Farmacia*. IF: 1.607, *Medicina.* IF: 2.430, *Curr Pharm Des*. IF: 3.052.

Secțiunea II evidențiază direcțiile principale ce vor constitui suportul dezvoltării personale în planul activităților didactice, medicale și de cercetare. Cu referire la activitatea didactică am discutat perspectivele de evoluție privind parteneriatul cu studenții și medicii rezidenți. În ceea ce privește activitatea medicală, aceasta se va axa pe acumularea de noi cunoștințe, tehnici și dezvoltarea de noi competențe. Temele de cercetare viitoare, fundamentate pe convergența carierei didactice cu cea de coordonator de doctorat, vor avea în vedere o dimensiune europeană și un impact ridicat al rezultatelor în planul dezvoltării cunoașterii teoretice și practice. În mod concret, mă voi concentra pe continuarea cercetărilor atât în domeniul aterosclerozei, cât și al durerii, completate cu studiul fenomenului de tranziție epitelio-mezenchimală în patologia tumorală și non-tumorală.

Secțiunea III include o selecție a referințelor bibliografice citate în această teză de abilitare.

Summary

The habilitation thesis reflects the significant results obtained in the personal activity carried out in the post-doctoral period (2011-2022), as well as some of the future research directions that I aim to approach.

The paper has been conducted in accordance with the criteria recommended and approved by the National Council for Attestation of University Degrees, Diplomas and Certificates (CNATDCU). Overall, the thesis presents the most relevant scientific results regarding the study of cardiovascular diseases, which were initiated in the doctoral research and continued over the postdoctoral period, focusing on the development of etiopathogenic, morphological and management aspects in atherosclerosis. During my scientific journey, the research that I have conducted was also directed towards the concerns in the current field of pain management in the clinical practice, a global problem that affects both the pediatric and the adult patient. Both topics have been and are being researched within the Discipline of Morphopathology, and in collaboration with other departments from the University of Medicine and Pharmacy "Grigore T. Popa" Iasi, including Histology, Physiology, Internal Medicine, Cardiology, Pediatrics, Neurology.

The thesis is structured as follows:

- **Section I** includes an overview of the academic, professional and scientific achievements during the postdoctoral period.
- **Section II** outlines several areas of research that I intend to develop in the future.
- **Section III** contains the list of academic references used to conduct this paper.

The results of my scientific research carried out and personal contributions are structured in two directions:

First and foremost, **Chapter 1** focuses on the study of atherosclerosis and it is a continuation of the studies conducted in the doctoral thesis entitled ***Changes in arterial structure and function related to atherosclerotic lesions*** that was completed in 2011. As previously mentioned, due to my collaboration with colleagues from the Morphopathology Department, but also through multidisciplinary ones, it was possible to conduct several scientific papers, which were published in specialized ISI or BDI journals with international visibility.

Subchapter 1 covers the study of some risk factors that influence the development of atherosclerotic disease. This particular disease requires a rigorous approach because identifying potential risk factors with definite implications for the onset and progression of this medical condition, as well as modulating those with a protective role, can have a significant impact in finding an appropriate therapeutic attitude to improve its evolution and consequences.

Subchapter 2 details a constant concern in relation to the study of the development and progression of atheroma plaque – the typical lesion of atherosclerosis. In this regard, we conducted a series of experiments with the purpose of highlighting the histopathological changes that characterize atherosclerosis as well as the identification of biochemical markers

with significant prognostic significance. In addition, we investigated the antiatherogenic potential of several nonpolar amino acids in conditions of induced hypercholesterolemia by a high-fat diet. My research interest was also directed towards investigating the anatomopathological aspects of the atherosclerotic plaque on tissue fragments, that were collected by endarterectomy from symptomatic patients.

Subchapter 3 shows the results gathered from multidisciplinary collaborations in relation to the management and rehabilitation of patients suffering from coronary heart disease. As the morbidity and mortality from ischemic heart disease rises, scientific studies on atherosclerosis are constantly addressed in the main stream of publications. Therefore, the medical community justifiably focuses on the treatment and prevention of cardiovascular diseases, seeking to control the risk factors involved in the etiology of these diseases.

My personal contributions to this research have been published in prestigious journals, such as *Medicine (Baltimore).* IF:1.889, *Medicina*. IF: 2.430, *Applied Sciences.* IF: 2.679, *Diagnostics.* IF: 3.706, *Biomolecules.* IF: 4.879, *Journal of Personalized Medicine*. IF: 4.945.

Chapter II presents the second direction of research, namely the study of pain – a complex, polymorphic and multidimensional phenomenon with overwhelming pathophysiological, psychosocial and emotional consequences on the patient. Given that the transdermal administration of some analgesic compounds offers many advantages over oral or injectable treatments, personal concerns were focused towards the experimental approach of this idea. More precisely, we studied the analgesic effect at different time intervals of some active substances incorporated in different carriers on the nociceptive sensitivity in laboratory animals.

My personal contributions to this research have been published in prestigious journals, such as *Rom J Morphol Embryol*. IF: 1.411, *Farmacia*. IF: 1.607, *Medicina.* IF: 2.430, *Curr Pharm Des*. IF: 3.052.

Section II highlights the main directions that will support my personal development in terms of teaching, medical and research activities. With regard to didactic activity, I reviewed the future evolution directions regarding the partnership with medical students and residents. In terms of my medical activity, it will focus on the accumulation of new knowledge, techniques and the development of new skills. Future research topics, based on the convergence of the teaching career with that of a PhD coordinator will allow me to consider the European dimension and a high impact of the results in terms of theoretical and practical knowledge development. Specifically, I will focus on continuing research in both the field of atherosclerosis and pain, in addition to the study of the epithelial-mesenchymal transition phenomenon in tumour and non-tumour pathology.

Section III includes a selection of the references cited in this habilitation thesis.

SECTION I. SHORT REVIEW OF ACADEMIC, MEDICAL AND SCIENTIFIC ACTIVITIES

Professionalization, particularly in the early phases of training, is an extremely significant aspect of the teaching vocation, as it should assure a genuine, well-directed, motivated, and open development for those who choose this path. The pedagogical practice activity, which aims to highlight the ability of teachers to operate with the information and skills acquired in specialized disciplines and in the field of education sciences, plays an important role in achieving high levels of performance and efficiency in accordance with modern standards of the profession.

In this context, I consider that the advancement of a contemporary university career must be founded on prior steps done in this area and attempt to maximize the prior beneficial elements while continually adapting to new and modernity in the future.

I consider my work during the last two decades as a collection of meaningful insights obtained in the medical, educational, and research domains. Therefore, it is reasonable to want to look back in time and memorize the key events that set the basis of my professional training in all of the fields where I worked.

Academic activity

Any teacher, regardless of expertise, participates in ongoing training to ensure that he is always up to date, capable of answering any student's query, and to provide them with the most up-to-date information in the field. In this regard, competencies in pedagogical analysis of contents and curricular documents, as well as skills in information accessibility, didactic activity design, and so on, are required, but so are the capacity to sympathize with students, creativity, and communication. Professional standards and competences are strongly intertwined to professionalization and career advancement. The teaching staff is trained using a competency-based concept and the fundamental principle of cumulative development of the teaching staff's level of competence. Thus, the main goals are to improve the teaching career, to place the training system in the European context of ongoing professional learning, and to guide the training system toward mobility, career advancement, and professional growth.

I started my teaching career in 2002 as Teaching Assistant in Discipline of Histology, Faculty of Medicine, under the guidance of Professor PhD Coriolan Cotuțiu. Three years later in 2005 I became University Assistant in the same discipline and in 2012 University Assistant by competition in Discipline of Morphopathology. In the following years, I passed all of the didactic qualifications which I earned also through competition: Senior Lecturer (2016), and Associate Professor (2020). In my position I conducted lectures, practical works and final examination for 3rd year students in Medicine, Romanian and French sections, for 2nd year students in Dental Medicine, French section and for 2nd year students General Nursing, as well as lectures for residents in Pathology.

During the early years of my teaching career, my interested focused on improving the level of knowledge in the field of normal and pathological morphology, as well as on teaching

methodology, through continuous updating of academic concepts, aiming the acquisition of high-quality information in the fields of education, communication, and the most appropriate dissemination of medical data as possible.

As a result, in 2003 I participated and graduated the Psychopedagogy Course for the training of the teaching staff, organized by the Teacher Training Department of the Faculty of Psychology and Educational Sciences, University "Al.I. Cuza" Iaşi. I participated in the *PRIME course for medical teachers getting the most out of teaching* in 2010 and I attended the course *Modern technologies in medical education*, within the E-Medical Summer School, organized by the University of Szeged, Hungary, in 2011. Last but not least, I participated in 2014 in the *Academic Teaching Excellence (ATE)* course, organized by the British Council Romania in support of the academic staff who carry out their teaching activity through the English language, which allowed me a better communication with the students.

All the information I accumulated are set up into practice. In addition, I have continuously improved my teaching skills by constantly interacting with students in a variety of settings aside from those required by the teaching standard of the university qualification at the time. In this regard, I guided 3rd year students to develop, communicate and publish scientific papers on pathology, some of them awarded. I coordinated annually bachelor's degree papers of the graduates of the Faculty of Medicine and General Health Care of "Grigore T. Popa" University of Medicine and Pharmacy Iasi. As a tutor for 3rd year students – English section, I attended meetings with students and I got involved in training and professional counselling issues. As a member of the Medicine Faculty Council and in my position of Vice-Dean of the Faculty of Medicine of "Grigore T. Popa" University of Medicine and Pharmacy, I am permanently interested in improvement of the teaching act in accordance with the students' requests. I also applied my teaching skills in the field of postgraduate courses in various areas of pathology where I interacted with dozens of resident doctors, specialists and senior pathologists, gynecologists, family doctors, nutritionists, and other specialties.

I consider that staying informed on the most recent knowledge, recommendations, and research findings is essential. Therefore, I attended various national and international conferences either as passive participant or as lecturer (invited lecturer, commented posters, oral presentations).

All of the medical information I gathered over time allowed me to design interactive courses that underlined the unique aspects of the cases I encountered with evocative illustrations that integrated into the course material and enabled the student retain the information properly. In addition, the complexity of cases in the Pathology Laboratory from the Emergency Hospital for Children "St Mary" from Iasi, where I work, allows the observation of different pathologies (respiratory, cardiovascular, hematological, renal, gastrointestinal) and helps me in the interaction with the students.

A doctor's communication and interpersonal skills include the ability to gather information to aid proper diagnosis, counsel appropriately, deliver therapeutic instructions, and create caring relationships with patients. Consequently, medical students' communication skills should improve as they progress through school, and doctors in training should focus more and more on patient management. Furthermore, the emotional and physical rigor of medical education, particularly during internship and residency, should develop empathy rather than replace communication with techniques and procedures.

In addition to aspects related to the teaching act, the didactic activity demands organizational attributes. In this regard, I participated on various examination committees for acquiring the titles of specialty doctor and primary care physician, as well as fulfilling open jobs in the health network for pathologists, biologists, and chemists. In the past years, I have participated, as president or as a member in promotion committees for occupying positions of academic career. In latest years, I've also been involved in the administrative activities of the discipline and of the Faculty of Medicine by participating in various working commissions such as the commission for compiling the student schedule, the working commission for preparing and submitting ARACIS accreditation files, admission commission, undergraduate exam, residency exam simulations as well as residency competitions, and in other commissions and work teams in recent years.

My commitment to the ongoing improvement of the didactic act at the university was recognized by my colleagues' confidence in me when they voted me for the Departmental Council of the Morphofunctional Sciences I Department and also for the Medicine Faculty Council of "Grigore T. Popa" University of Medicine and Pharmacy, which will allow me to participate more fully in the process of organizing the didactic activity.

I have positively responded to the requests of the department, faculty and university. The privilege of being appointed as Vice-Dean in 2020 by Prof. Dr. Lăcrămioara Șerban, Dean of the Faculty of Medicine, occurred at a time when I needed new insights on my personal development. It is an honour to be a part of the large, administrative family of the University and to actively participate in organizational and decision-making activities at the level of the Faculty of Medicine and of the University.

Medical activity

Altogether, the foregoing activities occurred concurrently with my training as a doctor, initially resident, later specialist and senior physician. In my opinion, it is unconditionally necessary to reach a certain threshold of knowledge in order to teach the students and residents. Thus, in 2009 I completed the residency in the specialty of Pathology. After obtaining the title of specialist doctor I received integration in the Pathology Laboratory from the Emergency Hospital for Children "Sf Mary" from Iasi. Five years later, in 2014, I was able to sustain and achieve the title of senior specialist in the speciality of Pathology.

I participated in numerous workshops, training courses, POSDRU projects, which allowed me to continuously update my knowledge in the field of interest and to constantly improve my training level: *Molecular histopathology of archived tissue,* organized by "Victor Babeş" National Institute of Pathology, Bucharest, within the project System of professional training of medical staff in the field of new health technologies (molecular diagnosis)(2011); *Training and prevention for a healthy life,* training Session in the field of Screening for Cervical, Colorectal and Breast Cancer, Iaşi (2012); *The East European Network of Excellence for Research and Development in Chronic Diseases CHRONEX-RD* (2014-2015); *Evidence-Based Policies and the Impact on the Labor Market (INFO-HE*), partner University of Medicine and Pharmacy "Grigore T. Popa" Iasi (2014); *The importance of the first 1000 days of life in the prevention of chronic diseases*, Iasi (2020); *National Symposiums of Clinical Cytology* (2005-2018) and other medical training courses.

In my position of secretary of the *Society of Clinical Cytology*, I also organized a series of scientific events during the *National Symposiums of Clinical Cytology* attended by residents, specialists and senior, pathologists, biologists and other specialties.

I developed a series of teaching and educational materials and I published some books and books chapters in "Grigore T. Popa" UMF Iasi and in other publishing house.

In addition, I was a member in an educational project: *Innovative education project for cancer pain management in the second largest oncology hospital in Romania (INECAPOR),* funded by the International Association for the Study of Pain. Contractor: UMF „Grigore T. Popa“, Iași. Project manager: Dr. Maria Magdalena Leon (2013-2014), completed with the publication of a book - Leon MM, Mungiu O (eds). *Pain therapy - current issues*, "Grigore T. Popa" Iasi Publishing House, 2014.

By getting the credential in Health Services Management offered by the Romanian Ministry of Health in 2020, I was also concerned about the administrative components of the medical act.

Research activity

Scientific research is a fundamental component of any university's educational-formative development, and it is both a duty and a privilege for any teacher.

The beginning of the research activity was made with the admission to doctorate under the guidance of the scientific coordinator Professor PhD Coriolan Cotuțiu, the doctoral topic being focused on the study of histophysiological changes that are evident at the arterial level during atherosclerotic disease. The doctoral research activity included a retrospective study of a group of patients with clinical manifestations of atherosclerosis, as well as an investigation of the role of two nonpolar essential amino acids, valine and leucine, in modulating the experimentally induced atherosclerotic process in animal models. The research findings were disseminated through a series of published articles, which was made possible by the development of practical abilities in scientific writing in the medical area.

Scientific research, in my opinion, can only be carried out in well-organized groups in which members support one another and each individual is critical to the team's success. As a result, throughout the preceding activity, I attempted and I believe that I was successful in integrating into teams, as evidenced by scientific publications, research funding, and participation and graduation from a variety of postgraduate courses, trainings, and workshops.

The major scientific landmarks that have emerged from the start of my university career and continue to this day are represented by papers communicated at various scientific events, ISI articles or BDI indexed articles published in journals in the country and abroad, publication of medical book chapters and specialized books. Thus, I contributed to the writing and publication of a number of 41 articles *in extenso* in ISI journals (28 as main author and 13 as co-author), 35 articles *in extenso* in BDI journals (16 in as principal author and 19 as co-author) whose visibility in the main stream of publications is reflected by a Hirsh index of 7, .

I was a member in the research teams of national and international projects, within which we approached new research directions: *Phenothiazine hybrids - heterocultures containing nitrogen and sulfur atoms with potential pharmacological effects (FENHETFARM)*, a CEEX project, funded by the National Authority for Scientific Research. Coordinator: "Al.I.Cuza"

University. Partner: UMF „Grigore T. Popa“, Iaşi (2006-2008); *Innovative education project for cancer pain management in the second largest oncology hospital in Romania (INECAPOR),* an educational project funded by the International Association for the Study of Pain. Contractor: UMF „Grigore T. Popa“, Iaşi (2013-2014); *Complex formulations based on liposomes and cyclodextrin for transdermal pain therapy (NANODERMA)* (2014-2017), funded by UEFISCDI. Project coordinator: "Petru Poni Institute of Macromolecular Chemistry", Iași. UMF partner "Grigore T. Popa", Iasi (2014-2017). This grant was completed with the publication of articles in prestigious ISI journals and with the submission of the documents necessary to obtain a patent for the new compound entitled CX001 which contains lidocaine and opens new perspectives for pain treatment by a local administration, being the first one described in Romania; *Detection des erreurs d'analyse medicale – un defi d'entreprenneuriat social et d'optimisation de la qualite de vie*, funded by the Agence Universitaire de la Francophonie (AUF). Contractor: UMF „Grigore T. Popa“, Iaşi. Project director: Prof. Univ Dr Liliana Foia (2020-2021); Increasing the visibility and the impact of the “Grigore T. Popa” University of Medicine and Pharmacy in Iași on an international educational level - iNTERmEDis 4.0. Contractor: UMF „Grigore T. Popa“, Iaşi Project director: Prof. Univ Dr Liliana Foia (1.09.2021- 17.12. 2021).

I also attended a postdoctoral research program "Program of excellence in multidisciplinary doctoral and postdoctoral research in chronic diseases" (2014-2015). The postdoctoral project aimed to study the pattern of immunohistochemical expression of E-cadherin, β-catenin and vimentin in renal carcinomas in surgically treated patients, considering them as important factors in regulating malignant transformation processes at the subcellular level and conferring them the status of possible prognostic factors and possible targets in diagnosis and treatment. In medium to long term, the predicted outcomes aim to improve personal skill in tumour pathology research. The complexity of the epithelio-mesenchymal transition events that characterize the malignant transformation generated at the kidney level inspired the choice of this study topic. During epithelio-mesenchymal transition, epithelial cells acquire a fibroblast-like phenotype, which allows them to direct migration and detachment from one another, as tumours progress. These events are the basis for the invasion and metastasis of malignant cells. The resumption of mesenchymal features by malignant epithelial cells evokes a return to the mesenchymal-epithelial pattern present in normal kidney development.

I am a member of national and international scientific societies in the medical field. In recent years I have been part of the organizing committees of national scientific events, I have given oral presentations at national congresses and with international participation. Because I have always enjoyed teamwork and collaboration, I constantly promoted interdisciplinary interaction, the consequence being the multidisciplinary approach of children, adolescents and adult’s pathology and the subsequent dissemination of research results.

The acknowledgment of my performance in scientific research was confirmed by the awards granted in 2018, 2019, 2020 and 2021 by UEFISCDI for articles published in specialized journals with an important impact factor.

A medical university career requires a continuous process of training and improvement in all of the aforementioned areas, which is augmented by the capacity and availability to disseminate the gained information.

Chapter I. LEARNING ABOUT ATHEROSCLEROSIS

State of the art

Cardiovascular (CV) disease is the major cause of morbidity and death in the world, with major social and economic consequences. According to recent statistics, heart disease is the leading cause of death in the United States of America, as well as the leading cause of high costs in terms of hospitalization, medication, and medical services provided [Arnett et al., 2019]. According to the European Cardiovascular Disease Report 2017, CV disorders are responsible for 1.8 million fatalities in the European Union (EU) and account for 37% of all deaths in EU nations [Wilkins et al., 2017; Simionescu et al., 2019]. The latest statistics from the World Health Organization prove that Romania ranks first in the world in terms of the rate of deaths caused by CV diseases and especially mortality from ischemic coronary heart disease and stroke. Many of these deaths could be avoided given that most people who die from myocardial infarction have at least one major risk factor that can be modified by lifestyle changes or therapy [WHO, 2018; Simionescu et al., 2019].

The most common cause of mortality is coronary artery disease (CAD), followed by cerebrovascular disorders. Stroke has a significant influence on public health in Europe, as it is the largest cause of long-term disability and the third leading cause of death. INTERHEART and INTERSTROKE, two case-control studies that included a majority of patients from developing countries, were pivotal in finding common risk factors for acute myocardial infarction and stroke, respectively [Teo, Dokainish, 2017]. Stroke can be caused by several mechanisms. Thus, ischemic stroke may be due to rupture of the atherosclerotic plaque (being called atherothrombotic stroke) or the atherosclerotic process may stimulate the onset of an embolus (cerebral embolism). Because the atherosclerotic process determines the appearance of the disease, the most affected are the elderly. Stroke can also be hemorrhagic, produced by a rupture of a vessel, but in this case, the atherosclerotic process intervention is minimal, the category most often involved being the young individuals [Barquera et al., 2015; Kobiyama, Ley, 2018].

The atherosclerosis process is very common in the lower extremities. Peripheral artery disease (PAD) is smoking dependent, respectively dose dependent [Campia et al.; 2019]. Venous thromboembolism is the third leading cause of CV disease, including deep vein thrombosis and pulmonary embolism (PE). PE is a major cause of mortality, morbidity and hospitalization in Europe and is most frequently the consequence of deep vein thrombosis [Mach et al., 2020].

Unfortunately, atherosclerosis is clinically "dumb" until midlife or even later, when arterial lesions precipitate organic damage. Between the ages of 40 and 60, the incidence of myocardial infarction increases significantly [Raygor, and Khera, 2020]. Extensive studies highlight and draw attention to some markers that signal the damage of the CV system more and more frequently in children. The atherosclerotic process occurs very early in childhood, according to some studies right from the fetal stage [Hayman et al., 2020]. Histopathological examination of tissue fragments collected from children who died in various accidents revealed the presence of lipid streaks and fibrous plaques on the walls of the coronary arteries, more

commonly in children who smoked or had obesity, dyslipidemia, hypertension [McCloskey et al., 2014].

In this context, the identification of risk factors at an early age proves to be extremely useful in the arsenal of prevention of the occurrence of frequently fatal complications, such as myocardial infarction [Pederiva et al., 2021]. The rare causes of myocardial infarction in adolescents in a seemingly perfect state of health have usually been correlated with the identification of proaterogenic factors. Therefore, knowing the risk factors is extremely important for the health of adolescents and includes assessing family history, measuring blood pressure and body mass index, cholesterol dosing in those who are obese and last but not least whether they are smokers or not [Hayman et al., 2020; Pederiva et al., 2021].

Multiple risk factors accumulate their effects. Thus, the presence of 2 risk factors increases the risk about 4 times and if 3 risk factors are present, the rate of myocardial infarction increases 7 times [Meha et al., 2020]. Also, the level of exposure to risk factors causes considerable variations in the evolution of the atherosclerotic process and therefore its early determination by different methods could be extremely useful in the assessment of cardiovascular risk. However, even in individuals who have lived a healthy lifestyle and have no apparent genetic predisposition, atherosclerosis and its consequences can develop in the absence of any risk factor.

In recent times, the view of who is at risk for a heart attack has changed dramatically. A heart attack used to bring up images of a white man middle-aged smoker, with high cholesterol levels and high blood pressure, but these traditional notions of what adds to risk have shifted in recent years. Therefore, new thinking on the following topics is included in these updated perspectives: the main cause of death worldwide nowadays is atherosclerotic cardiovascular disease (ASCVD); in addition, women, younger people, and patients from all categories of life are increasingly affected by atherosclerosis; the importance of high-density lipoprotein (HDL) cholesterol in heart disease prevention has been highlighted, while triglycerides have risen as a prospective target for lowering cardiovascular risk. Last but not least, inflammation may have a role in the relationship between recognized risk factors such as abnormal lipids, smoking, and diabetes, as well as atherosclerotic complications such as heart attack and stroke [Meha et al., 2020].

Cases in which atherosclerosis is accompanied by a single risk factor are rare. The identification of these risk factors that predispose to atherosclerosis and therefore to ischemic heart disease was initiated years ago with the help of prospective studies of well-defined population groups, the most notable being "The Framingham Heart Study", "Multiple Risk Factor Interventional Trial", "Seven Country Study" [Di Napoli et al., 2002]. These studies also drew attention to the cumulating effects of risk factors. At the same time, the level of exposure to risk factors produces notable variations in the evolution of the atherosclerotic process and therefore its early quantification by different methods could be extremely useful in assessing cardiovascular risk [Andersson et al., 2021]. However, atherosclerosis and its consequences can occur in the apparent absence of any risk factor, even in people who lead a prudent life and without an apparent genetic predisposition. Therefore, atherosclerosis, or artery stiffening, is now responsible for the vast majority of fatalities globally, and breakthroughs in our knowledge of the disease's biology are redefining old perspectives in order to offer new therapeutic options.

Essentially, atherosclerosis is an irreversible process characterized by a narrowing of the arterial lumen due to deposits of fat, cholesterol, calcium, and other substances, and it is the primary cause of the emergence and progression of heart disease [Skilton et al., 2019]. According to new research, atherogenesis begins with the accumulation of low-density lipoprotein (LDL) cholesterol (LDL-C) and other cholesterol-rich apolipoprotein (Apo) B carrying lipoproteins within the arterial wall [Skilton et al., 2019].

The blood lipids cholesterol and triglycerides have the biggest impact on atherosclerosis and ischemic heart disease. Patients with plasma cholesterol levels above 260 mg/dL have a 3 to 4 times increased risk of atherosclerosis than those with values below 200 mg/dL, according to numerous prospective studies. LDL-cholesterol, which is necessary for the supply of cholesterol to peripheral tissues, is the primary component of total serum cholesterol linked to an elevated risk [Gidding, Allen, 2019]. HDL-cholesterol, on the other hand, has anti-inflammatory, antioxidant, and antithrombotic properties, helping to maintain a normal physiological status of the endothelium and implicitly inhibiting atherosclerosis by mobilizing cholesterol from atheromas in the process of formation or those that have already formed and transporting it to the liver to be excreted in the bile. As a result, the higher the amount of HDL-cholesterol, the lower the risk of atherosclerosis, which is why special attention is devoted to diets, drugs, and behavioral patterns that lower serum LDL and increase serum HDL [Lee Y, Siddiqui, 2021]. Obesity and smoking have antagonistic effects on HDL levels and the distribution of LDL-cholesterol subfractions, whereas regular physical exercise, moderate ethanol use, and carbohydrate restriction enhance HDL levels and modify the distribution of LDL-cholesterol subfractions. A diet high in cholesterol and saturated fats raises plasma cholesterol levels, whereas a diet low in cholesterol and polyunsaturated fats lowers them [Gidding, Allen, 2019].

Previous data showed that serum levels of lipids and lipoproteins vary depending on age, gender and race [Dai et al., 2014; Linton et al., 2019]. Patients can associate moderate to severe raised triglycerides (TG), normal to slightly increased LDL-cholesterol, and lowered HDL-cholesterol readings as primary pattern of dyslipidemia. Both dyslipidemia patterns have been linked to the development and progression of atherosclerotic plaques [Linton et al., 2019].

Some research showed that lipid levels are lower during puberty (total and LDL-cholesterol levels can be lowered by 10% to 20% or more), and they rise again following this period. In addition, with the onset of puberty, girls' total and LDL-cholesterol levels increase [Linton et al., 2019].

Numerous studies conducted over the last three decades have suggested that atherosclerosis is an inflammatory disease, with the immune system and inflammatory processes playing a significant role in the development and progression of atherosclerosis [Wu et al., 2017]. Atherosclerosis is defined by the accumulation of monocytes/macrophages, smooth muscle cells, and lymphocytes in the artery wall, as previously stated. The intake of lipids by monocytes and macrophages enhances the development of these cells into massive, lipid-laden foam cells in the artery wall. The generation of reactive oxygen species and cytokines is triggered by the accumulation of inflammatory cells [Wu et al., 2017]. As a result, the prior belief that atherosclerotic lesion development is solely dependent on lipid deposition has been modified by the current belief that activated immune and inflammatory responses play a key role in plaque onset and progression. Consequently, in addition to existing lipid-lowering therapies, various anti-inflammatory therapeutic techniques have developed as prospective

treatments for atherosclerotic disease. Thus, ongoing research is focusing on a variety of inflammatory molecules and targets for treatment, but further studies are still needed. Because of the limited number of participants involved or the short duration of several of these research, no definitive results can be drawn. In the near future, many therapeutic compounds targeting various inflammatory pathways / chemicals will be tested in clinical trials. Further larger clinical trials will be required to confirm these hopeful findings and to explore the idea that an anti-inflammatory approach could improve therapeutic efficacy in atherosclerosis patients.

Essentially, the atherosclerotic disease requires a rigorous approach because the identification of favourable risk factors, with definite implications in the initiation and progression of this disease, as well as the modulation of those with a protective role, can have a special impact in finding an appropriate therapeutic attitude to alleviate CV disease and their consequences.

In this context, my own research has focused on the etiopathogenic and anatomo-clinical study of atherosclerosis lesions in experimental and clinical studies, with the goal of highlighting the atherosclerotic process while also improving and preventing it by supplementing the diet with certain compounds (essential amino acids) that may influence some risk factors.

I.1. Current knowledge on atherosclerosis associated risk factors

I.1.1. Scientific context

More and more specialists are now joining the view that the prevalence of cardiovascular disease will reach epidemic levels in the near future, due to the increase in high blood pressure, diabetes and obesity prevalence. Most epidemiological studies indicate that we face a multiplication of risk factors, an increase in their genetic conditioning, as well as an acceleration of the effects generated by non-genetic factors [Boudoulas et al., 2016; Libby et al., 2019; Lechner et al., 2020].

In this context, there is an increasing interest to reconsider the atherosclerosis approach - both as etiopathogenesis, which refers to the stages of damage and degradation of the vascular wall, and also as therapeutic modalities [Libby et al., 2019; Raygor, and Khera, 2020].

According to the preventive guidelines, the prevalence and severity of atherosclerotic disease varies a lot depending on the individual, with specific features regarding the race, gender, age or genetic background. If these constitutional risk factors are considered unchangeable, there are added modifiable risk factors, which can diminish or counteract the atherosclerotic process.

If age, gender, ethnicity, and family history of early cardiovascular disease, are among the unmodifiable factors, obesity, diabetes mellitus, hypertension, and elevated cholesterol and triglyceride levels are some of the modifiable one [Deng et al., 2018; ESC, 2019]. Compliance to rehabilitation programs reduces the number of risk factors, which improves the patient's prognosis and improves their quality of life. These results are observed over time, as the number of patients with cardiovascular disease is significantly reduced and the number of risk factors decreases [Libby et al., 2019; Raygor, and Khera, 2020].

Epidemiological studies proved that **age** has a major influence on the development of the atherosclerotic process. As in other organic diseases, in atherosclerosis the vulnerability to

the disease increases with age, the number and severity of lesions growing with it. For this reason, the prevention of atherosclerosis even in the 7th and 8th decades of life is very important [Libby et al., 2019].

With regard to **the gender**, epidemiological studies prove that the male gender is a risk factor in the onset of atherosclerotic disease, being more affected than the female [Man et al., 2020]. According to an INTERHEART study, women had their first acute myocardial infarction 9 years later than men [Anand et al., 2008]. Women are initially shielded against the occurrence of cardiovascular disease due to hormonal protection. The incidence of cardiovascular disease rises rapidly after menopause, eventually equalling and then surpassing the number of male patients almost in the sixth decade of life. Atherosclerotic complications in premenopausal women are uncommon and are caused by the interaction of various risk factors such as hypertension, diabetes mellitus, obesity, smoking, and others [ESC, 2019]. Various studies proved that estrogenic replacement therapy has been shown to reduce risk by increasing HDL levels and decreasing LDL levels [Vautrin et al., 2016]. In addition, steroid hormonal contraceptives raise the risk of atherosclerosis, particularly of coronary artery disease, by 2-3 times, especially in women over 35 who smoke. Hormones like estradiol and androgens are thought to be responsible for some circulatory alterations that occur long before atherosclerosis develops. Many endocrine variables involved in atherosclerosis are modulated by long-term exposure to sex hormones [Budak et al, 2019; Lechner et al, 2020]. As a result, a thorough understanding of the pathophysiology is essential.

The notion of **genetic conditioning** in the development of coronary heart disease is supported by the majority of epidemiological research. Cardiovascular disease is most likely polygenic, but it occurs as a result of risk factors acting on a susceptible condition. the guidelines. Thus, according to recent guidelines, patients with a family history of early cardiovascular disease (men before 55 years of age and women before 65 years of age) have a higher chance of getting the condition [ESC, 2019; Raygor, and Khera, 2020]. Although several genetic markers have been linked to an elevated risk of this disorder, their use in clinical practice is not supported. Currently, several disorders characterized by familial aggregation, such as familial hypercholesterolemia, polygenic hypercholesterolemia, and polygenic hypoalfalipoproteinemia, are thought to play a key role in the development of atherosclerotic disease. Genetic tests are performed on this group of patients, and genetic risk scores are determined [Semaev et al., 2020]. Many genes involved in different signalling pathways and in modulation of the extracellular matrix have the potential to influence the structure and function of the arterial wall. Their study is critical, as it will provide new biomarkers for detecting arterial compliance as well as novel treatment targets for reducing vascular rigidity. Because low arterial compliance has such a high prognostic value for cardiovascular events, determining it has become a key priority in the study of arterial function. As a result, the guidelines propose measuring arterial stiffness in individuals with extensive atherosclerosis, which provides us with more information [Pazoki et al., 2018; Raygor, and Khera, 2020].

There are four significant risk factors involved in atherosclerosis considered to be potentially reversible: hyperlipidemia, high blood pressure, smoking and diabetes mellitus [Deng etal., 2018].

Genetic, observational, and interventional research have all demonstrated the importance of **dyslipidemia**, particularly hypercholesterolemia, in cardiovascular disease

[Patzoki et al., 2018; Khatana et al., 2020; Semaev et al., 2020]. The presence of cholesterol and cholesterol esters in the atheroma plaque has been demonstrated by morphological studies. Experimentally, rats fed a high-fat diet developed a generalized form of atherosclerosis, providing the first evidence that lipids play a role in the development of atherosclerosis [Emini et al., 2017]. LDL-cholesterol, which plays an important physiological role in the delivery of cholesterol to peripheral tissues, is the major cholesterol fraction involved in the atherogenic process. HDL-cholesterol, on the other hand, is responsible for mobilizing cholesterol from atheromas in the process of forming or those that have already developed and transferring it to the liver to be excreted in the bile. HDL-cholesterol exhibits anti-inflammatory, antioxidant, and antithrombotic characteristics in addition to its ability to eliminate cholesterol from the cellular level, all of which contribute to enhanced endothelial function and atherosclerosis prevention [Robbins, 2018; Souilhol et al., 2020].

In both men and women under the age of 40 who have acute coronary syndrome, **smoking** is the most major modifiable risk factor. It has been found in nearly comparable amounts in people with normal coronary arteries as well as those with simple or multivascular coronary lesions. Essentially, smoking does not appear to be the sole risk factor, but rather functions in concert with additional risk factors like hypertension or diabetes [Campagna et al., 2019; Libby et al., 2019]. Ischemic heart disease mortality is greatly increased by smoking a pack of cigarettes per day or many packs per year for several years [Arnett et al., 2020]. It has been proved that complete removal of this risk factor significantly reduces the risk of developing the disease [Libby et al., 2019].

It is known that **diabetes mellitus** is another key risk factor, since it causes hypercholesterolemia, which is a support for an increased tendency to atherosclerosis, even in the absence of other risk factors. According to statistical research, diabetics have a 2-fold increased risk of myocardial infarction than healthy people and there is also a higher risk of stroke. In addition, diabetics who smoke have a 100-fold greater risk of atherosclerosis-induced lower extremities gangrene [Robbins, 2018].

Diabetes has been shown to affect the elasticity of the arterial wall, regardless of the presence of other risk factors or the presence of intimal damage in patients with peripheral vascular disease [Makita et al., 2010]. Acute hypoglycemia causes important physiological changes, affecting the cardiovascular system and some hematological parameters, mainly as a consequence of sympathetic - adrenergic activation. Cardiovascular symptoms in healthy persons are transitory and have no significant effects, but in diabetic patients with endothelial dysfunction, they can become pathological. Previous data showed that acute hemodynamic and hematological alterations might raise the risk of regional tissue ischemia, and acute hypoglycemia is able to cause myocardial or cerebral ischemia [Choi et al., 2021].

Prior research supports the concept that insulin resistance syndrome and high circulating insulin levels play a role in the development of ischemic heart disease. Patients with non-insulin-dependent diabetes or obesity are more likely to develop insulin resistance. Obesity and non-insulin-dependent diabetes are more common in hypertensive people than they are in normotensive people [Poznyak et al., 2020]. Hyperinsulinemia disrupts the normal metabolism of omega 6 fatty acids, causing the production of PGE2, a prostaglandin that induces inflammation in artery walls and hence contributes to atherosclerosis. The effects of hyperglycemia in the blood are comparable to those of smoking. It seems that hyperglycemia

in the blood works in a similar way to smoking in that it contributes to the body's saturation with oxidizing chemicals that harm the artery walls. Glycosylation is the most common cause of arterial damage in persons with type 2 diabetes, a condition in which insulin resistance is so severe that blood glucose levels can no longer be controlled [Bellis et al., 2021].

At any age, **high blood pressure** is a key risk factor for atherosclerosis. Men between the ages of 45 and 62 whose blood pressure surpasses 169/55 mmHg, have a 5 times higher risk for ischemic heart disease than those with a blood pressure ≤ 140/90 mmHg [Oliveros et al., 2020]. Both systolic and diastolic blood pressure values are important in increasing risk [Arnett et al., 2020].

Diabetes and hypertension are strongly associated. Increased blood pressure, macrovascular problems, and reduced renal function occur as glucose tolerance declines [Pazoki et al., 2018]. The two risk factors produce pathophysiological changes both in the large vessels and in the microvascularization; an increase in arterial stiffness causes an increase in systolic and pulse pressure, which results in a decrease in coronary perfusion. The occurrence of arterial hypertension and the overexpression of the negative hemodynamic effects of reduced arterial compliance are determined by the remodelling of the resistance arteries and thinning of the capillaries; therefore, therapeutic interventions to stop these vascular changes should aim to reduce central systolic pressure and increase vascular bed perfusion [Robbins, 2018].

Obesity is another risk factor that raises the risk of myocardial infarction in men by two times and in women by 2.5 times. According to the Framingham study, the risk of cardiovascular disease is 2.5 times higher in obese males under 50 than in obese women of the same age [Andersson et al., 2021]. Obesity and atherosclerosis are linked because atherosclerosis affects obese individuals at least ten years earlier than it does normal-weight people. Dylipidemia is one of the side consequences of unhealthy eating. Obesity additionally induces insulin hypersecretion due to a persistent overload of beta cells in the pancreas, resulting in hypoglycemia, functional depletion of these cells, and the development of diabetes, which promotes atherosclerosis [Arnett et al., 2019]. During the active gonadal cycle, obesity takes down women's immunity to atherosclerosis. Consequently, obesity and atherosclerosis should be viewed as a coordinating link rather than a cause-and-effect relationship.

The underlying mechanisms by which cardiovascular disorders develop in overweight people is unknown, however abnormal intracellular lipid metabolism is a key factor in metabolic syndrome pathogenesis [ESC, 2019]. Adipose tissue is now recognized as an endocrine and paracrine proinflammatory secretory organ, as well as a source of fat accumulation. It's known for being high in proinflammatory mediators, which can contribute to vascular injury, insulin resistance, and atherogenesis. As a result, inflammation of this tissue can play a role in the onset of a variety of symptoms linked to the metabolic syndrome's clinical characteristics, including diabetes and atherosclerosis [La Sala and Pontiroli, 2020].

Identifying adipocytokines, biomarkers that assess the metabolic activity of adipose tissue, can help define an obesity phenotype linked to cardiovascular risk. Metabolic syndrome has been linked to biochemical and inflammatory indicators that alter vascular physiology in obese children [Recinella et al., 2020; Jackson et al., 2021]. Impaired endothelial function associated with obesity severity and insulin resistance is considered a condition that conveys a premature atherogenic status and is linked to other common risk factors in adults [Libby et al., 2019].

Numerous researches have been conducted on the role of excess or deficiency of certain dietary components in the etiology of several chronic diseases caused by partial NO regulation. As a result, too much fructose or saturated fat causes insulin resistance and heart disease. Similarly, protein, vitamin A, C, and Fe deficiencies in the diet impacts immune function and increases susceptibility to infections [Mozaffarian, Wu, 2018; Astrup et al., 2020]. Any substance that prevents the oxidation of other compounds can be considered an antioxidant from a chemical point of view. Mechanisms of defence against oxidative stress include enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase, catalase and non-enzymatic vitamins such as vitamin C, coenzyme Q10 E, flavonoids, carotenoids. These antioxidants can be endogenous or exogenous through nutritional intake [Mozaffarian et al., 2018].

Endothelial dysfunction has been demonstrated in numerous trials to be improved by lifestyle changes, such as a strict diet. Regular exercise, quitting smoking, drinking alcohol, and eating a high-fat diet should all contribute. Supplementing the diet with antioxidant vitamins and folic acid, omega-3 fatty acids, or other anti-atherogenic chemicals has been linked to a lower incidence of cardiovascular events and may also impact other coronary risk factors [Libby et al, 2019; Souilhol et al., 2020]. Pharmacological therapy targeted toward pathophysiological processes that cause endothelial dysfunction can be added to these measures. As a result, hypolipidemic, hypoglycemic, anti-inflammatory, and hormone replacement therapy promotes vasodilation, lipid peroxidation, and leukocyte adherence to the endothelium [ESC, 2019; Souilhol et al., 2020; Raygor, and Khera, 2020].

The infectious etiology of atherosclerosis is also of special concern. Various pathogens can contribute directly and indirectly to chronic inflammation. Bacterial agents as *Chlamydia pneumoniae*, *Mycoplasma pneumoniae, Helicobacter pylori, Enterobacter hormaechei*, multiple periodontal organisms (eg. *Poryphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella intermedia, Tanerella forsreptocytea, Streptococcus*) and viruses (cytomegalovirus, hepatitis C virus, human immunodeficiency virus, herpes simplex virus, Epstein-Barr Virus, enteroviruses and parvovirus) may interfere with endothelial cell function, leading to increased leukocyte and platelet adhesion in the affected vascular segment [Pothineni et al., 2017].

Stress also represent an independent risk factor for cardiovascular disease and increases morbidity and death in individuals with pre-existing pathology. Psychic or emotional stress, as well as anxiety, have been linked to the development of ischemic heart disease and sudden death. Chronic stress triggers plenty of nonspecific systemic responses, all of which shape the development of atherosclerosis. One possible mechanism is that stress damages endothelial cells, activates macrophages, promotes adipose cell production, and eventually results in atherosclerotic plaque formation [Yao et al., 2019].

Sedentarism is a significant contributor to the onset of cardiovascular disease. Physical activity is emphasized in all guidelines, with non-pharmacological management which involves movement being the first option for all cardiovascular diseases. In any cardiovascular disorders, changing one's lifestyle and participating in personalized kinetotherapy programs (aerobic, fitness, cardio, etc.) are critical for patient evolution [Lazaros et al., 2019].

Early detection of risk factors at an early age is particularly valuable in the arsenal of prevention against the occurrence of commonly fatal complications, such as myocardial

infarction [Pederiva et al., 2021]. The rare causes of myocardial infarction in adolescents in an apparently perfect state of health have usually been correlated with the identification of proaterogenic factors. Therefore, knowing the risk factors is extremely important for the health of adolescents and includes assessing family history, measuring blood pressure and body mass index, cholesterol dosing in those who are obese and last but not least whether they are smokers or not [Hayman et al., 2020; Pederiva et al., 2021].

Personal contribution related to atherosclerosis risk factors was synthesized in the following papers:

ISI ARTICLES

1. Trandafir LM, Russu G, Moscalu M, Miron I, Lupu VV, Leon Constantin MM, **Cojocaru E**, Lupu A, Frasinariu OE. Waist circumference a clinical criterion for prediction of cardio-vascular complications in children and adolescences with overweight and obesity. *Medicine (Baltimore*) 2020; 99(30):e20923. **IF: 1.889**
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7387051/

2. Trandafir LM, **Cojocaru E**, Moscalu M, Leon Constantin MM, Miron I, Mastaleru A, Teslariu O, Datcu ME, Fotea S, Frăsinariu O. Predictive Markers of Early Cardiovascular Impairment and Insulin Resistance in Obese Pediatric Patients. *Diagnostics*. 2021; 11(4):735. **IF: 3.706**
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8074748/

3. Mitu F, Leon MM, **Ștefanachi (Cojocaru) E**. Alcohol and cardiovascular disease - a social impact analysis. *Revista de Cercetare si Interventie Sociala* 2013; 40: 180-187. **IF: 1.141**
   http://www.rcis.ro/images/documente/rcis40_13.pdf

BDI ARTICLES

1. Mitu F, **Stefanachi (Cojocaru) E**, Leon MM. The incidence of essential hypertension in elderly patients with metabolic syndrome. *Rev Med Chir Soc Med Nat Iasi* 2013; 117 (3): 630-634.
   http://www.ncbi.nlm.nih.gov/pubmed/24502027

2. Leon MM, **Stefanachi (Cojocaru) E**, Cobzaru R, Mitu F. Impact of metabolic syndrome on the development of cardiovascular disease. *Rev Med Chir Soc Med Nat Iasi* 2013; 117 (3): 635-640.
   http://www.ncbi.nlm.nih.gov/pubmed/24502028

3. Ifrim S, Amălinei C, Azoicai D, **Cojocaru E**, Butcovan D, Ifrim M, Matei MC. The assessment of the risk factors for atherosclerosis among population from the north-eastern region of Romania. *Rev Med Chir Soc Med Nat Iasi* 2017; 121(2):381-390.
   https://www.revmedchir.ro/index.php/revmedchir/issue/archive?issuesPage=3#issues

BOOK CHAPTERS

1. **Cojocaru E**, Mastaleru A, Tamba B, Vasile R, Tudor RC, Ripa CV, Cobzaru R, Leon MM. Overview of some risk factors in cardiovascular disease. In: Kumar A (ed). Recent Trends in Cardiovascular Risks. London: IntechOpen, 2017; 37-56. DOI: 10.5772/65843. ISBN 978-953-51-3328-5, Print ISBN 978-953-51-3327-8.

2. Mitu F, Iliescu R, **Cojocaru E,** Leon MM. Stopul cardiac la femeia gravidă. În: Mitu F, Pop D, Zdrenghea D. Particularităţi ale bolilor cardiovasculare la femei. Iași: Editura Pim, 2012; 233-243. ISBN 978-606-13-0782-1.

I.1.2. The link between waist circumference and cardiovascular outcome in obese children

I.1.2.1. Introduction

Obesity has become more common in developed and developing countries in recent decades, affecting both children and adults [Ng et al., 2014; Ogden et al., 2012]. Obesity prevalence in youths has risen from 0.7 percent in 1975 to 5.6 percent in 2016 in females and 0.9 percent to 7.8 percent in boys as stated by NCD Risk Factor Collaboration data [NCD-RisC, 2017]. According to Atlas of childhood obesity, this disorder would affect 254 million children aged 5 to 19 years by 2030, according to some estimations [World Obesity. Atlas of childhood obesity, 2019. World Obesity Federation].

Despite the fact there are significant variances among countries and social categories, numerous WHO European Region have developed and introduced projects and strategies to combat overweight and obesity, but more measures, larger actions, and financial forces are needed to reach the targets indicated by the WHO Global Action Plan for the Prevention and Control of Non-Communicable Diseases 2013–2025 in all children of all countries [Breda et al., 2021]. Along with the increasing prevalence of obesity in pediatric age, the number of obesity-related comorbidities has risen as well: dyslipidemias, type 2 diabetes, fatty liver disease, sleep apnea, and microalbuminuria, all being recognized risk factors for the onset of cardiovascular diseases [Cozzolino et al., 2015; Alpert et al., 2016; Elshorbagy et al., 2016].

Childhood obesity and overweight are linked to early cardiovascular impairment and a higher risk of cardiovascular morbidity and mortality as an adult. Significantly higher arterial blood pressure, changes in the structure and function of the myocardium, and the presence of long-term epicardial fat are all signs of cardiovascular dysfunction in obese children [Cozzolino et al., 2015; Alpert et al., 2016]. Heart failure, acute coronary syndrome, and unexpected premature death in adult life are all major consequences of cardiovascular diseases in childhood [Zalesin et al., 2008].

Children and adults have varied diagnostic criteria for obesity. Obesity in children cannot be diagnosed just on the basis of a high body mass index (BMI). The amount and distribution of adipose tissue should be assessed and compared to percentiles for age and gender. Thus, the initial child evaluation is vital for determining the accurate diagnosis. With this regard, the entire anamnesis should consider the prenatal data, personal antecedents, family history, and behavioral peculiarities in order to assess the personal and family history of obesity. Following the anamnesis, a comprehensive physical examination should be performed, which includes anthropometric indices such as weight and height, BMI, waist circumference (WC), and skin-fold thickness; in addition, body fat distribution, blood pressure measurement, identification of clinical signs of potential comorbidities, and endocrine disturbances, etc. should be noted [Pereira et al, 2015].

All overweight and obese individuals over the age of five must undergo WC. The WC is a simple-to-determine clinical criterion for monitoring a child's nutritional state that is independent of BMI. Also, according to Lee et al. [2020], WC is one of the criteria for diagnosing the metabolic syndrome and the cardiovascular risk. Because WC is linked to visceral obesity, obese children with high WC should be closely managed in order to avoid long-term cardiometabolic consequences [Romero-Velarde et al., 2013; Umer et al., 2017].

I.1.2.2. Aim

WC is used to assess the risk of cardiometabolic comorbidities in obese people. The purpose of the current research was to establish if WC represents a clinical criterion for predicting vascular and cardiac injury in obese children.

I.1.2.3. Materials

Study Design

We performed a retrospective study on 160 children with overweight and obesity, hospitalized during 2016 - 2018 in the "Saint Mary" Emergency Children Hospital Iași, Romania. Overweight and obesity diagnosis without associated pathologies and the signed informed consent (paternalistic consent) were the main inclusion criteria. We divided the patients in two groups according to age: group A included 97 children between 6–11 years old; group B included 63 adolescents between 12–18 years old.

Anthropometric and Biochemical Measurements

All the patients were evaluated anthropometrically, biologically and imagistic. The anthropometric data included height, weight, WC and body mass index (BMI). Interpretation of BMI values was based on BMI Z score and BMI percentile, applicable for age and sex, according to WHO standards, using WHO AnthroPlus software [WHO, 2007]. Depending on the BMI Z score, the patients were classified into overweight (BMI Z score >+1SD or BMI percentiles between 85-97th), obese (BMI Z score >+2SD or BMI percentiles between 97-99,9th) and severe obesity (BMI Z score > +3 SD or BMI percentiles> 99.9th) [de Onis M, and Lobstein, 2010; Rolland-Cachera, 2011].

For WC determination we measured halfway between the coastal rim and the iliac crest, at the end of the expiration. Tables with specific percentiles for age and sex, developed based on NHANES III data [Fernandez et al.,2004] used for WC assessment. Because WC provides an indication of visceral adipose tissue, visceral obesity was defined by values over the 90th percentile of WC [Brambilla et al., 2006; Lee and Song, 2020].

Biological profile included: total cholesterol (TC), low-density lipoprotein cholesterol (LDL_c) and high-density lipoprotein cholesterol (HDL_c), triglycerides (TG), glucose levels, alanine aminotransferase values, urea and creatinine values. The reference standards were used to interpret the values of biological parameters.

We also evaluated the blood pressure (BP) value according to the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents. Children. Hypertension was defined as systolic BP and/or diastolic BP greater than the 95th percentile, adjusted for height, age, and sex, at least three separate determinations. The values between the 90-95 percentile values were considered normally high blood pressure. In our study, BP values ≥ 90th percentile were defined as "elevated BP" or vascular impairment [NIH, 2004]. An echocardiographic evaluation was performed in all patients, following parameters as thickness of the interventricular septum (IVS), diastolic diameters of the left ventricul (DdLV), left ventricular mass (LVM), the relative thickness of the ventricular wall

(RWT), the presence of epicardial fat. Diastolic dysfunction was evaluated through the E/A ratio and the pulmonary venous flow through the S / D ratio.

The analysis of the LVM was made according to LVM index or LVM-for-height Z score (LVM divided by height raised to a power of 2.7) [Foster at al., 2008]. Left ventricular hypertrophy (LVH) was defined as LVM index greater than the 95th percentile for normal children and adolescents [De Simone et al., 1995]. RWT was measured to assess the LV geometric pattern [De Simone at el., 1992]. The RWT value above > 0.41 is considered pathological. Patients with increased LVM index and elevated RWT (>0.41) had concentric LVH; those with increased LVM index and normal RWT (<0.41) had eccentric LVH. Concentric remodelling was defined as elevated RWT, but with normal LVM index. Pathological epicardial fat quantified echocardiographic was considered over 4.1 mm [Abaci et al., 2009]. We considered cardiac impairment the concentric or eccentric LVM hypertrophy, concentric remodelling, and/or epicardial fat with pathological values.

Statistical Analysis

Continuous type variables were reported as mean with standard deviation using a SPSS software v.20. Comparisons between the analysed groups were performed using Student's t-test or Mann-Whitney U Test for continuous variables. The qualitative variables were presented as absolute (n) and relative (%) frequencies, and the comparisons between groups were made based on the results of McNemar, Yates Chi-square or Fisher's exact test. Univariate and multivariate analysis of prognostic factors regarding cardiovascular complications was performed using the Logistic regression model. The significance level calculated in the used tests (P-value) was considered significant for P values <0.05.

I.1.2.4. Results

All clinical, biological, and imagistic data of all 160 pediatric patients included in this study were evaluated and we obtained the following results: in group A the mean age was 9.82 ± 2.2 years, and in group B 14.7 ± 1.6. We noticed the predominance of the male sex in both groups (59.8% in group A compared with 60.3% in group B) (Table I).

Obesity was more prevalent than overweight in both groups (53.61% in children and 49.21% in adolescents). 32.99% of patients in study group A had severe obesity, whereas in group B, 44.44% of adolescents had overweight (Table I). Obesity and severe obesity were associated (P <.001) with the age of fewer than 12 years, and at this age category, BMI had significantly higher mean values (Table I). BP values shows that at obese adolescents (8.3% vs. 30.2%; P = .0003) pre-hypertension and hypertension are more frequent (20.6% vs. 14.4%; P = .0003). Only triglyceride levels showed statistically significant variations between children and adolescents (p = .0142) (Table I). In addition, we evaluated the correlation between epicardial fat and visceral obesity in children and adolescents as seen in Table II.

The results showed that visceral obesity was associated (χ^2 = 11.72, P=0.0006) with the presence of pathological epicardial fat in 21.74% of 92 cases. The statistical data proved that pathological epicardial fat has an increased predictive power (AUC = 0.668, 95% CI: 0.562-0.775, P = 0.014) in the presence of visceral obesity (Figure 1).

Table I. Baseline characteristics

Baseline characteristics†	Study lot (n = 160)		Statistical test	P-value
	Group A: 6-11 years (n = 97)	**Group B: 12-18 years (n = 63)**		
Age: years	9.82±2.2	14.7±1.6		
Gender, (men/women)	58/39 (59.8%/40.2%)	38/25 (60.3%/39.7%)	0.0041‡	.9473
Environment (urban/rural)	47/48 (49.5/50.5%)	36/26 (58.1%/41.9%)	1.1142‡	.2911
BMI (kg/m2)	24.4±3.5	27.5±3.8	27.3245†	<.001*
Percentiles BMI	98.67±2.16	96.80±3.14	-5.1565†	<.0001*
WC (cm)	80.7±11.6	93.6±9.8	34.2075†	<.001*
Percentiles WC (median)	99.6	97.7		
Visceral obesity (No/Yes)	39/58 (40.21%/59.79%)	23/40 (36.51%/63.49%)	0.2207‡	.6385
Nutritional status				
Overweight	13 (13.44%)	28 (44.44%)	28.4990‡	<.001*
Obesity	52 (53.61%	31 (49.21%)		
Severe obesity	32 (32.99%)	4 (6.35%)		
Cholesterol (mg/dl)	166±29.4	177.7±43.5	3.0882†	.0814
Triglyceride (mg/dl)	96.05±63.03	125.87±73.69	-2.4507†	.0142*
Vascular impairment (No/Yes)	**76/21 (78.35% /21.65%)**	**32/31 (50.79%/49.21%)**	**13.1127‡**	**.0002***
Systolic BP	126.94±24.32	133.89±26.74	0.8198†	.3700
Diastolic BP	80.31±15.39	85.25±12.33	1.4810†	.2299
Normal value BP	75 (77.3%)	31 (49.2%)		
Pre-hypertension	8 (8.3%)	19 (30.2%)	16.1997‡	.0003*
Hypertension	14 (14.4%)	13 (20.6%)		
Cardiac impairment (No/Yes)	**37/60 (38.14%/61.86%)**	**22/41 (34.92%/65.08%)**	**0.1710‡**	**.6792**
IVS thickened (normal/>9/>1.2)	90/7/0 (92.8%/7.2%/0%)	56/6/1 (88.9%/9.5%/1.6%)	2.1738‡	.3372
IVS (cm)	0.76±0.16	0.85±0.17	12.4642†	.0005*
Pw (cm)	0.80±0.17	0.92±0.23	14.8411†	.0001*
DdLV (cm)	3.98±0.461	4.62±0.573	59.1216†	<.001*
RWT	0.39±0.08	0.38±0.09	0.3550†	.5521
RWT>0.42	31 (31.96%)	17 (26.98%)	0.4535‡	.5006
Epicardial fat (yes)	54 (55.67%)	35 (55.56%	0.0002‡	.9886
Epicardial fat (cm)	3.06±1.53	3.24±1.5	0.2885†	.5925
LVM (g)	118.4±37.12	180.9±52.95	76.5654†	<.001*
LVM index ($g/m^{2.7}$)	1.30±1.04	1.24±1.04	0.1002†	.7522
Concentric LVH	87 (89.69%) 10 (10.31%)	53 (84.13%) 10 (15.87%)	1.0599	.3032
Concentric remodeling LV	75 (77.32%) 22 (22.68%)	55 (87.30%) 8 (12.70%)	2.6027	.1066
Eccentric LVH	79 (81.44%) 18 (18.56%)	47 (74.60%) 16 (25.40%)	1.0540	.3045

Continuous variables were expressed as: mean ± standard deviation; categorical variables: number (%)
† Student's t-test or Mann-Whitney U Test for continuous variables; (*) Marked effects are significant at P <.05;‡ Chi-square test (McNemar Chi-square/Yates) or Fisher's exact test;

Table II. Evaluation of the association of epicardial fat *vs.* visceral obesity

	Visceral obesity			
	Absent	**Present**	**Statistical test‡**	**P-value**
Pathological epicardial fat				
No (n/%)	45/97.83%	72/78.26%	11.7281	0.0006*
Yes (n%)	1/2.17%	20/21.74%		
Visceral obesity vs. pathological epicardial fat		**Asymptotic 95% Confidence Interval:Lower Bound-Upper Bound**		
Area Under the Curve	0.668	0.562-0.775		0.014*

‡ Chi-square test (McNemar Chi-square/Yates)

The evaluation of the area under the curve (AUC) revealed that BMI is not a significant predictor for vascular impairment for either children or adolescents (AUC = 0.57, P = 0.327 vs. AUC = 0.54, P = 0.53) but is a predictive factor for the occurrence of cardiac impairment in children (AUC= 0.62, P =0 .041) and adolescent (AUC = 0.66, P = 0.036) (Table III).

For adolescents, visceral obesity is an important predictive factor for the occurrence of both vascular (AUC = 0.669, P = 0.021) and cardiac (AUC= 0.697, P = 0.037) impairment. Also, for adolescents, increased levels of TG and LDLc are predictable for the occurrence of cardiac impairment (AUC = 0.67, P = .044; AUC = 0.66, P = 0.038) (Table III).

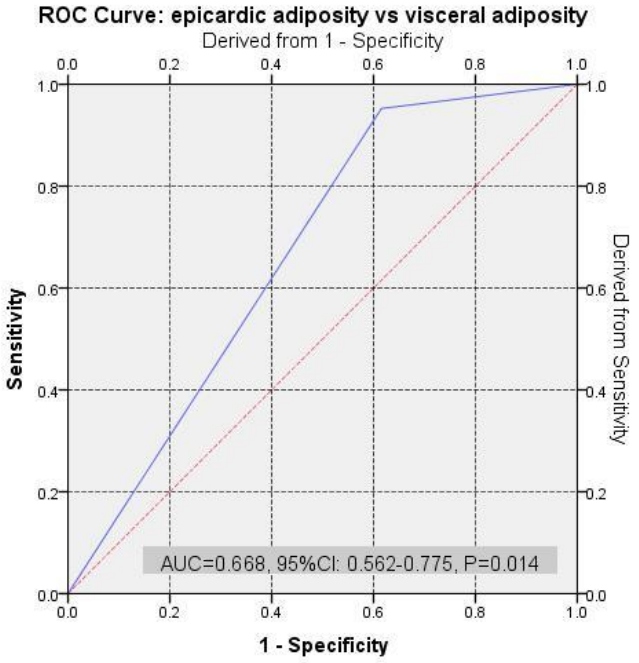


Figure 1. *AUC: Epicardial fat vs visceral obesity*

The findings reveal that visceral obesity is associated with higher LMV index values in both children and adults (AUC = 0.594, P = 0.024) and adolescents (AUC copil = 0.53, P = .035) and concentric LV hypertrophy is influenced by the presence of visceral obesity (AUC= 0.664, P = 0.013 children: AUC= 0.716, P = .026 adolescents) (Table IV).

Table III. The estimated parameters in the evaluation of the predictability of the clinical and biological parameters on the vascular and cardiac impairments

	Study cohort (n = 160)			
	Group A: 6-11 years (n = 97)		**Group B: (12-18 years) (n = 63)**	
	AUC (95%CI)	**P-value**	**AUC (95%CI)**	**P-value**
	Vascular impairment			
BMI (kg/m^2)	0.570 (0.426-0.714)	.327	0.547(0.389-0.695)	.530
Visceral obesity	**0.635(0.508-0.762)**	**.059**	**0.669(0.534-0.804)**	**.021***
Cholesterol (mg/dL)	0.518 (0.352-0.683)	.837	0.485(0.316-0.655)	.864
Triglyceride (mg/dL)	0.558 (0.386-0.731)	.488	0.637 (0.477-0.797)	.107
LDL_c (mg/dL)	0.533 (0.291-0.776)	.799	0.650 (0.350-0.950)	.329
HDL_c (mg/dL)	0.489 (0.242-0.735)	.932	0.636 (0.323-0.950)	.366
	Cardiac impairment			
BMI (kg/m^2)	0.620 (0.505-0.735)	.041*	0.666 (0.529-0.802)	.036*
Visceral obesity	**0.559 (0.419-0.700)**	**.432**	**0.697(0.533-.861)**	**.037***
Cholesterol (mg/dl)	0.427 (0.290-0.563)	.296	0.572 (0.400-0.743)	.419
Triglyceride (mg/dl)	0.447 (0.313-0.581)	.453	0.677 (0.513-0.842)	.044*
LDL_c (mg/dl)	0.364 (0.158-0.569)	.198	0.667 (0.565-0.851)	.038*
HDLc (mg/dl)	0.556 (0.335-0.778)	.595	0.308 (0.006-0.609)	.258

Area Under the Curve – AUC; 95%CI - Confidence Interval);
(*)Marked effects are significant at P < 0.05

Table IV. Estimated parameters in evaluating the predictability of visceral obesity on cardiac impairment

	Group A: 6-11 years (n = 97)			
	LVM index (g/m$^{2.7}$)	**Concentric hypertrophy of LV**	**Concentric remodeling of LV**	**Eccentric hypertrophy of LV**
Visceral obesity				
AUC (95%CI)	0.594 (0.521-0.767)	0.664 (0.621-0.806)	0.408 (0.232-0.585)	0.576 (0.434-0.719)
P-value	.024*	.013*	.327	.314
	Group B: (12-18 years) (n = 63)			
	LVM index (g/m$^{2.7}$)	**Concentric hypertrophy of LV**	**Concentric remodeling of LV**	**Eccentric hypertrophy of LV**
Visceral obesity				
AUC (95%CI)	0.53 (0.509-0.721)	0.716 (0.695-0.836)	0.630 (0.349-0.911)	0.451 (0.285-0.617)
P-value	.035*	.026*	.286	.564

Area Under the Curve – AUC; 95%CI - Confidence Interval); (*)Marked effects are significant at P <0 .05

I.1.2.5. Discussion

Childhood obesity and overweight are linked to early cardiovascular dysfunction, which raises the risk of cardiovascular morbidity and mortality in adulthood. The consequences of cardiac structural and functional changes that affect the myocardium in obese patients are represented by so-called "obesity cardiomyopathy" [Abaci et al., 2009]. The persistence of cardiovascular risk factors determines a cardiac dysfunction that will progress into adulthood, and studies suggest that heart failure develops 10 years faster in overweight and obese adults than in patients with a normal BMI. [Abaci et al., 2009; Umer et al., 2017]. For this reason it is extremely important to identify the clinical and biological parameters for predicting cardiovascular risk from childhood.

BMI and WC were the first clinical parameters to be examined in childhood and adolescence related to cardiovascular risk factors. Janssen and colleagues collected information from the Bogalusa Heart Study, which included 2597 youngsters aged 5 to 18. Investigators observed that, despite heterogeneity in origin environments, ethnicity, and other factors, both BMI and WC indicate a higher cardiovascular risk in children and adolescents with visceral obesity [Katzmarzyk et al., 2004; Janssen et al., 2005; Csige et al., 2018; Lee at al., 2020]

WC represents a marker for visceral adipose tissue. Abdominal computed tomography, magnetic resonance imaging, and dual-energy X-ray absorptiometry are imaging modalities that offer reliable measurements of visceral adipose tissue, although they are costly and difficult to employ in the clinical environment. WC offers a nearly similar measurement of visceral adipose tissue in adults, according to various studies [Schneider et al., 2007; Harrington et al., 2013], and can be used to identify persons at risk of cardiovascular disease and type 2 diabetes mellitus. In obese 12-14-year-old youngsters, Flodmark and colleagues have found that WC is linked to a potentially atherogenic lipoprotein profile [Flodmark et al., 1994; Lee at al., 2020]

The link between visceral adiposity and cardiovascular risk is well understood, making fat distribution assessment a mandatory technique in obese children. WC has a crucial role in the early detection of metabolic syndrome and cardiometabolic risk in overweight children [Flodmark et al., 1994; Maffeis et al., 2008]. The research of Chen et al. in 2011 proved that higher WC was an indication of elevated blood pressure in preschool children, particularly boys. Additionally, WC was independently associated with high BP in this category of children [Chen et al., 2011]. Consequently, high BP in children has been associated with increased WC, and childhood obesity is associated with high risk of adult hypertension [Chen et al., 2011; Matsushita et al., 2015; Mehta et al., 2015]. Concerning the predictive power of BMI and WC on elevated BP, literature studies have shown that increased WC is associated with elevated BP even when BMI is normal [Pazin et al., 2017].

Our results demonstrated that WC is a significant predictor of vascular damage (pre-hypertension and hypertension) in teenagers, but not in children under the age of 12. In our research, BMI was found to be a poor predictor of vascular impairment in both children and adolescents.

Prior research highlighted that visceral obesity causes a number of structural changes in the heart's structure and function. Abnormal LV geometry (LV hypertrophy or relative wall thickness) and cardiac dysfunction are more common in children with morbid obesity [Alpert et al., 2016]. The pathways that lead to a change in LV diastolic function are extensive and

regulated by a number of factors. Additionally, the risk of left ventricular hypertrophy increases in obese children with hypertension [Šileikienė R et al., 2021].

Obese children had a higher LVM index, according to most research [Abaci et al., 2009; Jing et al., 2016; Abdul-Raheem et al., 2021]. In addition, in children and adolescents with essential hypertension, the LVM index was found to be substantially linked with BMI [Abdul-Raheem et al., 2021]. In both children and adolescents, WC above the 90th percentile was found to be a predictor of increased LVM index and concentric hypertrophy. Furthermore, the presence of visceral obesity had no effect on concentric remodelling or the presence of eccentric hypertrophy [Lee et al., 2015; Jing et al., 2016].

Previous studies showed that obese children have more epicardial fat deposition than sex- and age-matched children with a healthy BMI. Furthermore, epicardial adipose tissue measurement by echocardiography has been linked to visceral adipose tissue deposition [De Simone et al., 1992]. As a result, the prevalence of visceral obesity was found to be substantially linked with the existence of pathological epicardial fat in our study.

Study limitations

There are a few limitations in this study. In the absence of abdominal magnetic resonance imaging or dual-energy X-ray absorptiometry, the definition of visceral obesity is solely based on waist circumference. Furthermore, we could not perform an arterial ultrasound and determine intima media thickness for the assessment of subclinical atherosclerosis, a sign of morphological vascular impairment.

I.1.2.6. Conclusions

Childhood obesity and overweight are linked to early cardiovascular disease. In teenagers, visceral obesity is a significant predictor of vascular impairment (pre-hypertension and hypertension), especially when compared to children. In both children and adolescents, WC above the 90th percentile is a predictor of increased LVM index and concentric hypertrophy. To avoid long-term cardiovascular impairments, obese pediatric patients with increased WC must be commonly followed.

I.1.3. Markers of cardiovascular impairment in obese pediatric patients

I.1.3.1. Introduction

The rising obesity epidemic diagnosed since childhood and adolescence has been linked to an elevated risk of numerous comorbidities and mortality at these age groups [Shivpuri et al., 2012] and it has been associated with impaired adult health in numerous studies. For instance, obesity has long been linked to an increased risk of various diseases, such as cardiovascular disease (CVD), type 2 diabetes (T2DM), and neoplasia. [Syrenicz et al., 2006; Bruyndonckx et al., 2013; Head, 2015; Elnashar et al., 2017; Genovesi, and Parati, 2020]. In the etiology of obesity-related comorbidities, insulin resistance (IR) and chronic inflammation play critical roles [Sinaiko et al., 2005; Lopez-Sandoval et al., 2018]. The early detection of cardiovascular impairment and insulin resistance (IR) in obese children and adolescents is critical for the early implementation of lifestyle corrective strategies and the long-term health of these age categories. As a result, early childhood obesity management attempts to not only reduce BMI but also to delay the onset of early CVD and T2DM [Marson et al., 2016; Vukovic et al., 2019].

BMI, hypertension, dyslipidemia, IR, and increased circulating inflammatory chemicals are all early CVD predictors [Raj, 2012]. During the early phases of childhood obesity, inflammatory changes damage metabolic and cardiovascular health [Kim et al., 2010]. Several inflammatory mediators are released by adipose tissues, predisposing to a pro-inflammatory state and oxidative stress.

Interleukin 6 (IL-6) is a pro-inflammatory adipocytokine that contributes to IR and is one of the hallmarks of chronic inflammation. In adulthood, elevated levels of IL-6 have been linked to metabolic or cardiac comorbidities, according to studies. According to Boutagy et al. systemic, low-level increases of gut-derived endotoxin have a role in metabolic alterations in obesity [Boutagy et al., 2016; Hirano, 2021]. Because metabolic endotoxemia is linked to both systemic and local inflammation, it may have a role in the risk of cardiometabolic illness associated with obesity, at least in part [Kallio et al., 2015; Määttä et al., 2021].

Detecting children with higher cardiovascular risk at an early age is one of the most important goals in preventing the comorbid diseases linked with obesity [Varda et al., 2020]. [Varda et al., 2020].

I.1.3.2. Aim

The purpose of the study was to evaluated the presence of early markers of cardiovascular risk represented by IL-6, Intercellular Adhesion Molecules (ICAM) and endotoxemia and their relation to IR metabolic indicators such as insulinemia, HOMA index, and plasma cortisol.

I.1.3.3. Material and methods

Study Design

Between January 1st and December 31st, 2019, 85 obese pediatric patients aged 6 to 18 years old with obesity but no accompanying diseases were followed at the Children's Hospital " Saint Mary " Iaşi. Thirty pediatric patients with a normal BMI made up the control group.

Patients with newly diagnosed obesity who did not respond to dietary and/or pharmacological treatment were eligible. Smoking, pregnancy, secondary and genetic causes of obesity, cardiovascular disease in treatment and other chronic diseases, autoimmune diseases, hormonal abnormalities (thyroid diseases, polycystic ovarian syndrome, secondary amenorrhea), or administration of any chronic therapy in the previous three months were all considered exclusion criteria. Only children and adolescents with an informed consent (paternalistic consent) were included in the study. The study was approved by the Ethical Committee of the "Saint Mary" Children's Hospital.

Anthropometric and Biochemical Measurements

Body weight, height, waist circumference, and BMI were the anthropometric parameters that were measured. According to CDC regulations, BMI values were interpreted using the BMI percentile, which is appropriate for age and sex. For children and teenagers of the same age and sex, overweight is defined as a BMI above the 85th percentile but below the 95th percentile. Obesity is defined as a BMI of 95th percentile or higher in children and teenagers of the same age and gender. Patients were divided into two categories based on their BMI percentiles

adjusted for age and gender: obese (BMI percentiles 95-99th) and severe obesity (BMI percentiles > 99th).

WC values over the 90th percentile were used to define visceral obesity. The blood pressure (BP) was measured in all of the patients, and the results were compared to percentiles for age and gender. The blood pressure (BP) value was evaluated according to the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents. Children's hypertension was defined as systolic and/or diastolic blood pressures exceeding the 95th percentile on at least three distinct occasions, after adjusting for height, age, and sex. High blood pressure was formerly defined as readings between the 90th and 95th percentiles (National Institute for Health, 2004). In our study, blood pressure readings above the 90th percentile were classified as "high blood pressure" or "vascular impairment."

We evaluated total lipid profiles, liver function tests, total protein, blood glucose, creatinine. The values of biological parameters were interpreted using the reference standards. Age and gender were used to define the cardiovascular risk markers. Total cholesterol levels of 180-199 mg/dl were considered borderline for cardiovascular risk. According to SINUPE 2000, a medium cardiovascular risk was classified as a cholesterol value of 200-249 mg/dl or an LDL-cholesterol (low density lipoprotein - cholesterol) value of 130-159 mg/dl. Cholesterol levels of more than 250 mg/dl or LDL cholesterol levels of more than 160 mg/dl were considered high cardiovascular risk.

The inflammatory and metabolic status were assessed using IL 6, ICAM, endotoxemia, insulinemia, plasma cortisol, and HOMA-IR (Homeostasis model assessment). For the quantitative detection of human IL-6, sICAM-1, and cortisol, we utilised enzyme-linked immunosorbent assay (ELISA) kits. Cortisol in serum had reference values of 60-230 ng/ml. BioVendor kits were used to determine Il-6 and ICAM-1 levels. OD readings were extrapolated against Il-6/ICAM-1 standard concentrations using a standard curve to quantify Il-6 or ICAM-1. The minimum detectable concentrations were 0.10 pg/mL for IL-6, 2.2 ng/mL for ICAM-1 and the interassay coefficient of variation was 7.0% for all kits. Abbexa ELISA kit was used to measure endotoxin (ET). The sensitivity of the Abbexa ET kit is 0.005 EU/ml, while the concentrations of ET in serum vary from 0.015 EU/ml to 1.0 EU/ml.

Statistical analysis

The STATA 16 software was used to perform statistical analysis of data (StataCorp LLC, Texas 77845-4512, USA). Continuous variables were given as medians with lower and upper quartiles (Q1; Q3) or as mean values and standard deviation. We used the ANOVA test or the Mann-Whitney U test, to compare the values of the continuous type parameters corresponding to the two groups of patients. The results of the Pearson Chi-square test were used to compare qualitative variables. The Pearson univariate correlation test was used to assess the correlations between the continuous type variables. Based on the results of multiple linear regression, a quantitative study of the role of each biochemical parameter and BMI in the change of inflammatory indicators and metabolic markers of insulin resistance was done. Simultaneously, the receiver operating characteristic (ROC) curve and the AUC value were used to measure their predictive power (area under the ROC curve). In the statistical analysis, the reference threshold for the level of significance p was 0.05. A p value < 0.05 indicated with 95% confidence that there was statistical significance.

I.1.3.4. Results

A total of 115 pediatric patients, ranging in age from 6 to 18, were enrolled in the study. There were no significant differences between the two research groups in terms of the children's age and gender. In the control group, the mean age was 13.4 ± 2.47 years and for the study group, the mean age was 12.1 ± 3.4 years. Table V outlines the main features of the patients who were included in the study.

Table V. Comparison of clinical and biochemical parameters between the group of obese pediatric patients and the control group.

Baseline Characteristics	Study Group (*n* = 115)				Statistical Test	*p*-Value
	Control Group (*n* = 30)		Obese Pediatric Patients (*n* = 85)			
	Mean ± SD	Std.Err.	Mean ± SD	Std.Err.		
Age: Years †	**13.4 ± 2.47**	0.34	12.1 ± 3.4	0.29	2.40	0.142
Gender, (boys/girls) ‡	12/18 (40%/60%)		44/41 (51.8%/48.2%)		1.24	0.267
BMI (kg/m^2) †	19.2 ± 1.8	0.28	29.2 ± 5.2	0.47	27.64	<0.001*
Percentiles BMI †						
mean ± SD	40.6 ± 2.3	0.75	97.52 ± 2.4	0.21	65.16	<0.001*
median (Q1; Q3)	40.5 (17; 58)		98 (97; 99)			
Percentiles W C †						
mean±SD	42.8 ± 4.6	0.50	93.9 ± 4.6	0.41	35.78	<0.001*
median (Q1; Q3)	44.5 (34; 50)		95 (92; 97)			
Lipid profile and liver function tests †						
Total serum cholesterol (mg/dl)	152.3 ± 14.1	2.57	165.7 ± 30.7	2.63	5.29	0.023*
Triglycerides (mg/dl)	44.1 ± 5.6	1.02	104.1 ± 51.96	4.33	39.67	<0.001*
LDL cholesterol	64. 3± 5.5	0.98	79.9 ± 24.4	2.03	12.16	0.0006*
HDL cholesterol	79.2 ± 11.7	1.65	64.9 ± 15.2	1.34	22.03	<0.001*
ALT	13.6 ± 8.7	1.58	24.1 ± 15.9	1.39	11.58	<0.001*
AST	18.7 ± 7.2	1.31	22.4 ± 8.9	0.78	4.34	0.039*
TP	70.8 ± 4.1	0.62	73.3 ± 3.4	0.30	10.27	0.001*
Glucidic profile and insulin resistance †						
Plasma glucose level	85.3 ± 9.5	1.03	89.3 ± 11.2	1.82	0.65	0.438
Hb A1c	4.1 ± 0.4	0.07	4.8 ± 0.5	0.04	14.06	0.0002*
Insulin, μU/mL	13.8 ± 9.2	1.57	23.3 ± 14.8	1.36	10.95	0.001
HOMA index	3.17 ± 2.3	0.32	4.94 ± 2.9	0.21	8.85	0.003*
Inflammatory markers and the hormones profile †						
IL6	1.68 ± 1.3	0.22	9.08 ± 15.1	1.25	7.14	0.008*
ICAM 1	385.6 ± 71.7	8.09	481.6 ± 79.7	6.94	33.80	<0.001*
Endotoxemia	3.98 ± 0.1	0.01	3.83 ± 0.2	0.01	24.08	<0.001*
Plasma cortisol	184.7 ± 108.4	9.78	176.1 ± 105.9	9.63	0.145	0.704
Blood pressure †						
SBP, mm Hg	102.3 ± 5.2	0.94	117.7 ± 14.1	1.22	34.13	<0.001*
DBP, mm Hg	60.7 ± 1.6	0.30	73.7 ± 12.5	1.07	32.3	<0.001*
Blood pressure value ‡						
Normal	30 (100%)		46 (54.1%)		20.83	0.0003*
Borderline hypertension	0 (0%)		16 (18.8%)			
Hypertension	0 (0%)		23 (27.1%)			

* Marked effects are significant at $p < 0.05$.

In terms of lipid profiles, we found that obese patients had significantly higher triglyceride levels and lower HDL cholesterol levels. Obese patients had significantly higher levels of HbA1c, insulinemia, and the HOMA IR index. Furthermore, obese patients had considerably greater levels of inflammatory markers such as IL-6, ICAM 1, and endotoxemia than the control group.

Correlations between the BMI Percentile and Biochemical Markers with Inflammatory Markers of Early Cardiovascular Risk (IL-6, Endotoxemia and ICAM 1).

We observed strong correlations between BMI and changes in inflammatory markers in obese patients included in the study. Accordingly, IL-6 correlates significantly with blood glucose (r = -0.334, p = 0.001) and BMI percentile (r = 0.252, p = 0.031) (Table VI). We also established a link between ICAM and serum TG levels (r = 0.253, p = 0.001), plasma glucose level (r = -0.145, p = 0.044) and with BMI (r = 0.302, p = 0.037). As well, in the context of obesity, the results indicated a significant correlation between endotoxemia and plasma glucose level (r = 0.346, p = 0.024) but also with BMI percentile (r = -0.255, p = 0.001) (Table VI).

Table VI. Univariate analysis showing correlations between inflammatory markers and biochemical parameters and BMI percentiles.

Dependent Variable	Independent Variable	Correlation Coefficient (Pearson Correlations)	*p*–Value
IL6 vs.	Total serum cholesterol	−0.039	0.318
	LDL-cholesterol	−0.0633	0.427
	Triglycerides	−0.034	0.341
	Plasma glucose level	−0.334	0.001*
	BMI percentile	0.252	0.031*
ICAM vs.	Total serum cholesterol	0.121	0.072
	LDL-cholesterol	0.208	0.008*
	Triglycerides	0.253	0.001*
	Plasma glucose level	−0.145	0.044*
	BMI percentile	0.302	0.037*
Endotoxemia vs.	Total serum cholesterol	0.082	0.166
	LDL-cholesterol	−0754	0.343
	Triglycerides	−0.035	0.335
	Plasma glucose level	−0.346	0.042*
	BMI percentile	−0.255	0.001*

* Marked effects are significant at $p < 0.05$.

Consequently, we noticed that IL-6 was correlated with blood glucose and BMI percentile, both of which are important predictors of cardiometabolic syndrome. Obesity and low-grade inflammation, IR, and endothelial dysfunction are currently being studied in details. In this investigation, we measured both IL-6 and ICAM in order to determine low-grade inflammation (Figure 2).

We used a multivariate analysis to assess the impact of each biochemical parameter, as well as the BMI percentile, in the change of inflammatory markers, starting with the results of the univariate analysis.

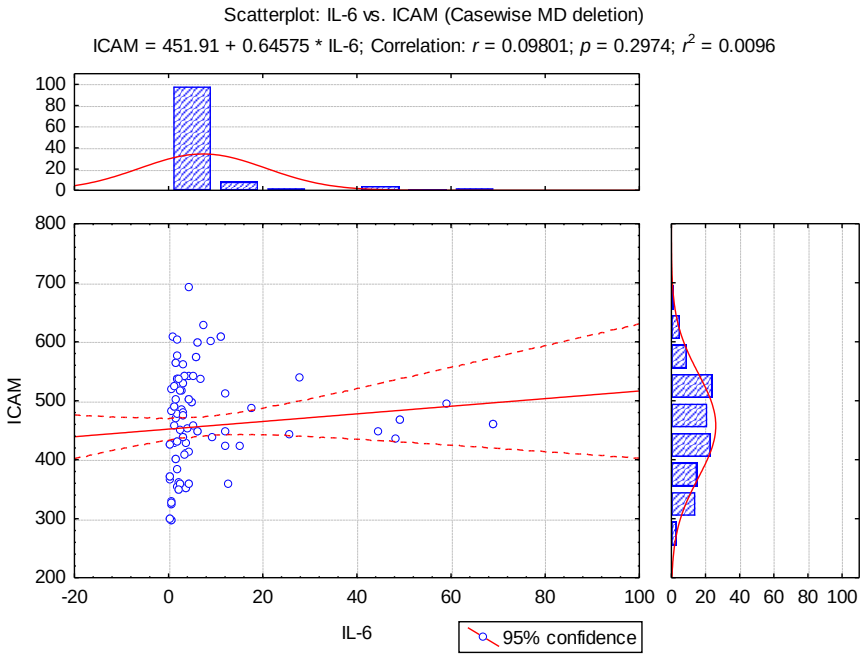


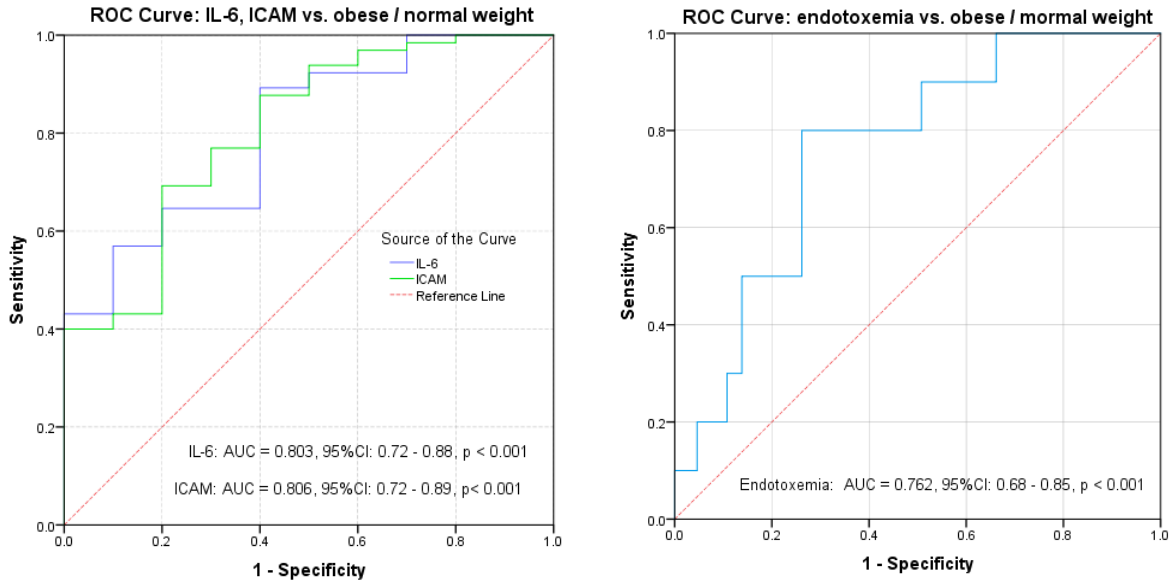
Figure 2. *The regression line in the correlation of IL-6 and ICAM values.*

Table VII. The coefficients of multiple linear regression regarding the correlations between early inflammatory markers of cardiovascular risk and BMI percentile and biochemical parameters

Multiple Linear Regression	Unstandardized Coefficients		Standardized Coefficients	t–Value	*p*–Value
	B	Std. Error	Beta		
Dependent Variable: IL-6					
Total serum cholesterol	0.026	0.042	0.054	0.607	0.545
LDL-cholesterol	−0.126	0.016	−0.207	−1.192	0.235
Triglycerides	−0.050	0.026	−0.184	−1.898	0.060
Plasma glucose level	−0.413	0.091	−0.254	−3.520	0.024*
BMI percentile	0.631	0.054	0.421	2.411	0.017*
Model verification: ANOVA, F = 4.815, p = 0.001*					
Dependent Variable: ICAM					
Total serum cholesterol	0.095	0.168	0.032	0.355	0.723
LDL-cholesterol	1.053	0.154	0.267	1.610	0.109
Triglycerides	0.429	0.064	0.250	2.621	0.010*
Plasma glucose level	−0.726	0.177	−0.100	−1.257	0.211
BMI percentile	0.787	0.142	0.244	2.883	0.005*
Model verification: ANOVA, F = 4.098, p = 0.003*					
Dependent Variable: Endotoxemia					
Total serum cholesterol	0.001	0.001	0.110	1.347	0.180
LDL-cholesterol	0.021	0.001	−0.012	−0.149	0.881
Triglycerides	−0.002	0.001	−0.014	−1.174	0.083
Plasma glucose level	−0.032	0.001	−0.013	−0.195	0.694
BMI percentile	0.452	0.001	0.276	3.185	0.038*
Model verification: ANOVA, F = 5.873, p = 0.028* * Marked effects are significant at $p < 0.05$.					

We utilized a ROC curve analysis to investigate the predictive potential of BMI percentile, plasma glucose level, and serum triglycerides on IL-6, ICAM, and endotoxemia,

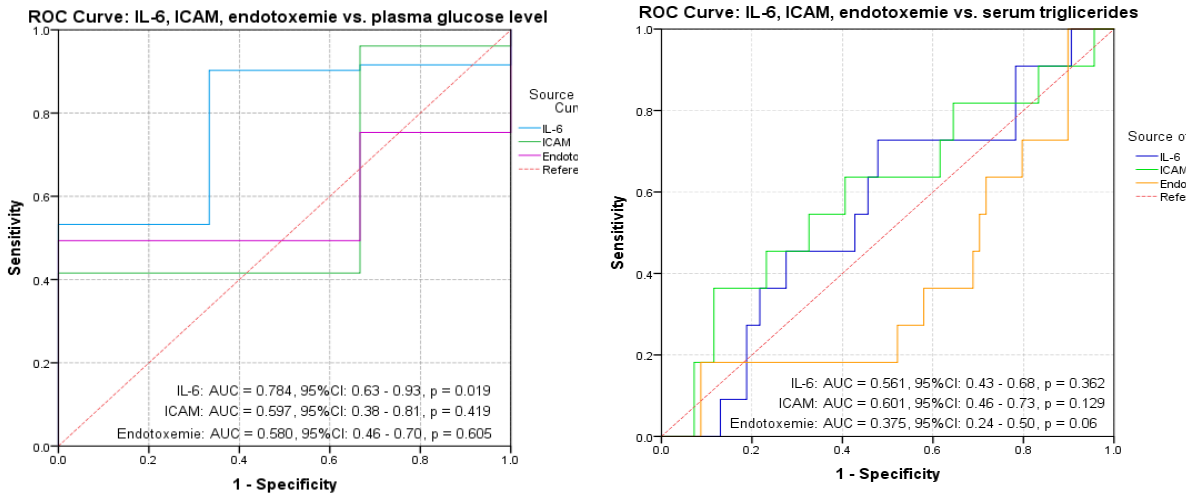
considering the correlations between inflammatory markers and the BMI percentile. The results indicated a significant predictive power of BMI percentile on inflammatory markers: IL-6 (AUC = 0.803, 95% CI: 0.72–0.88, $p < 0.001$), ICAM (AUC = 0.806, 95% CI: 0.72–0.89, $p < 0.001$) and endotoxemia (AUC = 0.762, 95% CI: 0.68–0.85, $p = 0.019$) (Table VII, Figure 3 a,b).



(**a**) (**b**)

Figure 3. *ROC curves for BMI vs. (**a**) IL-6 and ICAM and (**b**) endotoxemia.*

Plasma glucose level shows a significant prediction for IL-6 (AUC = 0.784, 95% CI: 0.63–0.93, p = 0.019) (Table VII, Figure 4a). Although a significant correlation with ICAM was observed in the case of serum TG (p = 0.01), the results did not indicate a significant predictive power on any inflammatory marker (AUC = 0.60; 95% CI: 0.46–0.73, p = 0.129) (Table VII, Figure 4b).



(**a**) (**b**)

Figure 4. *ROC curves for IL-6 and ICAM and endotoxemia vs. (**a**) plasma glucose level and (**b**) serum triglycerides.*

Identifying Cut-Off Values for Inflammatory Markers in Obese Children and Adolescents

We established baseline cut-off values for IL-6, ICAM, and endotoxemia for obese children and adolescents with early vascular impairment, confirming the predictive potential of BMI percentile and plasma glucose levels (Figure 5).

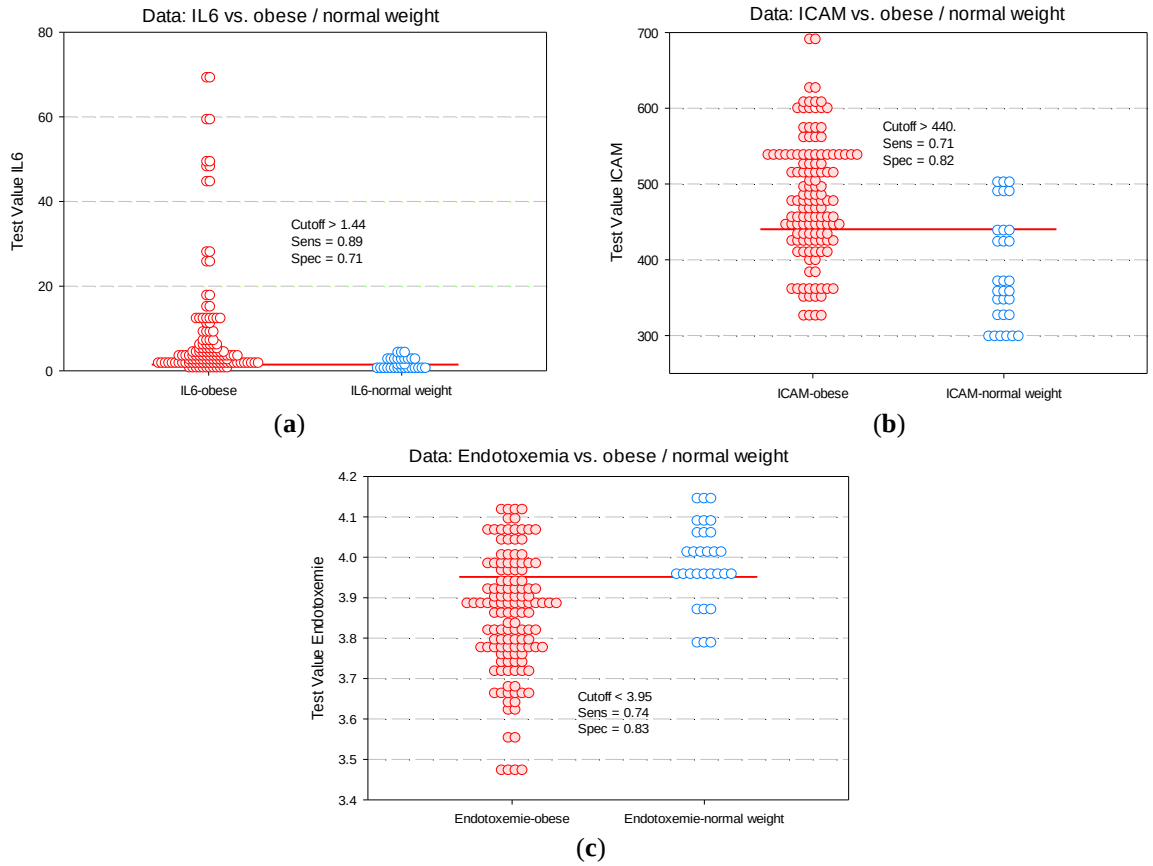


Figure 5. *Identifying cut-off values predictive for vascular impairment in obese children (dot histogram) for: (**a**) IL-6; (**b**) ICAM; (**c**) endotoxemia.*

Correlation of BMI Percentile and Biochemical Markers with Metabolic Markers for Insulin Resistance (Insulin, HOMA Index and Hormones Profile).

When we analysed the correlation between BMI percentile, metabolic markers, and the biochemical parameters, we noticed that insulin value had a strong relationship with BMI (r = 0.52, p = 0.001), TC (r = 0.265, p = 0.022) and TG level (r = 0.228, p = 0.006) (Table VII).

The HOMA index correlates significantly with BMI (r = 0.516, p = 0.001), which is a significant predictive factor for the value of the HOMA index. Accordingly, the significant correlation between the HOMA index and the BMI percentile confirms that obesity is a major risk factor for the development of IR. The HOMA index also shows a significant correlation with TC (r = 0.273, p = 0.017) and with serum TG (r = 0.205, p = 0.009) (Table VIII).

Only the BMI percentile has a significant predictive potential for metabolic markers of insulin resistance (insulin value: AUC = 0.72, p 0.001 and HOMA index: AUC = 0.68, p = 0.003), according to the results of the multivariate analysis (Table IX, Figure 6).

Although univariate analysis found other correlations between biochemical markers (total cholesterol and triglycerides levels) and insulin value or HOMA index, this aspect was noticed. We found that the risk of vascular injury in obese children increased at insulin cut-off values of 14.3 (Figure 7a) and HOMA index cut-off of 3.32 (Figure 7b), respectively.

Table VIII. Univariate analysis showing correlations between metabolic markers of insulin resistance and biochemical parameters and BMI percentile.

Dependent Variable	Independent Variable	Correlation Coefficient (Pearson Correlations)	*p*–Value
Insulin value vs.	Total serum cholesterol	0.265	0.022*
	Triglycerides	0.228	0.006*
	Plasma glucose level	−0.126	0.142
	BMI percentile	0.522	0.001*
HOMA index vs.	Total serum cholesterol	0.273	0.017*
	Triglycerides	0.205	0.009*
	Plasma glucose level	0.132	0.092
	BMI percentile	0.516	0.001*
Plasma cortisol vs.	Total serum cholesterol	0.037	0.326
	Triglycerides	0.027	0.372
	Plasma glucose level	0.042	0.596
	BMI percentile	0.144	0.067

* Marked effects are significant at $p < 0.05$.

Table IX. The coefficients of multiple linear regression regarding the correlations between metabolic markers of insulin resistance and BMI percentile and biochemical parameters.

Multiple Linear Regression	Unstandardized Coefficients		Standardized Coefficients	t–Value	*p*–Value
	B	Std. Error	Beta		
Dependent Variable: Insulin Value					
Total serum cholesterol	0.061	0.049	0.115	1.231	0.220
Triglycerides	−0.018	0.030	−0.060	0.603	0.548
Plasma glucose level	−0.105	0.106	−0.081	−0.992	0.323
BMI percentile	1.217	0.063	0.512	6.820	<0.001*
Model verification: ANOVA, F = 14.712, $p < 0.001$*					
Dependent Variable: HOMA Index					
Total serum cholesterol	0.011	0.010	0.101	1.085	0.280
Triglycerides	−0.004	0.006	−0.072	−0.718	0.474
Plasma glucose level	0.031	0.021	0.119	1.475	0.142
BMI percentile	0.430	0.013	0.520	7.019	<0.001*
Model verification: ANOVA, F = 16.186, $p < 0.001$*					
Dependent Variable: Plasma Cortisol					
Total serum cholesterol	0.111	0.370	0.029	0.301	0.764
Triglycerides	−0.013	0.228	−0.006	−0.441	0.660
Plasma glucose level	0.339	0.796	0.036	0.425	0.571
BMI percentile	2.767	0.472	0.060	0.668	0.062
Model verification: ANOVA, F = 4.815, $p = 0.001$*					

* Marked effects are significant at $p < 0.05$.

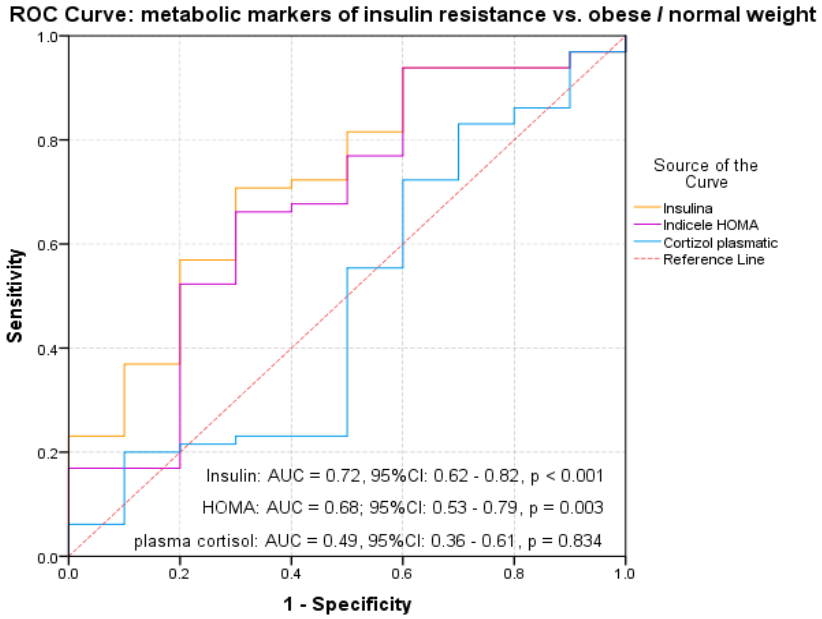
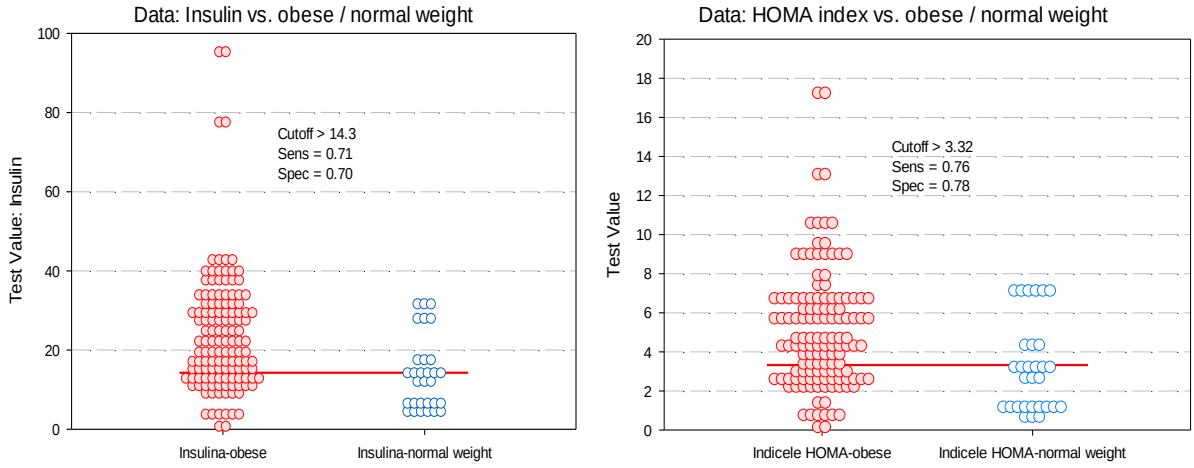


Figure 6. *ROC curves for metabolic markers of insulin resistance and obesity in children and adolescents.*



(**a**) (**b**)

Figure 7. *Identifying cut-off values predictive for vascular impairment in obese children and adolescents (dot histogram) for metabolic markers of IR: (**a**) insulin; (**b**) HOMA index.*

We found no correlation between all of these markers since the evolution of obesity in our pediatric patients' group is quite short (HOMA index and IL-6, ICAM and endotoxemia) (Figure 8a–c).

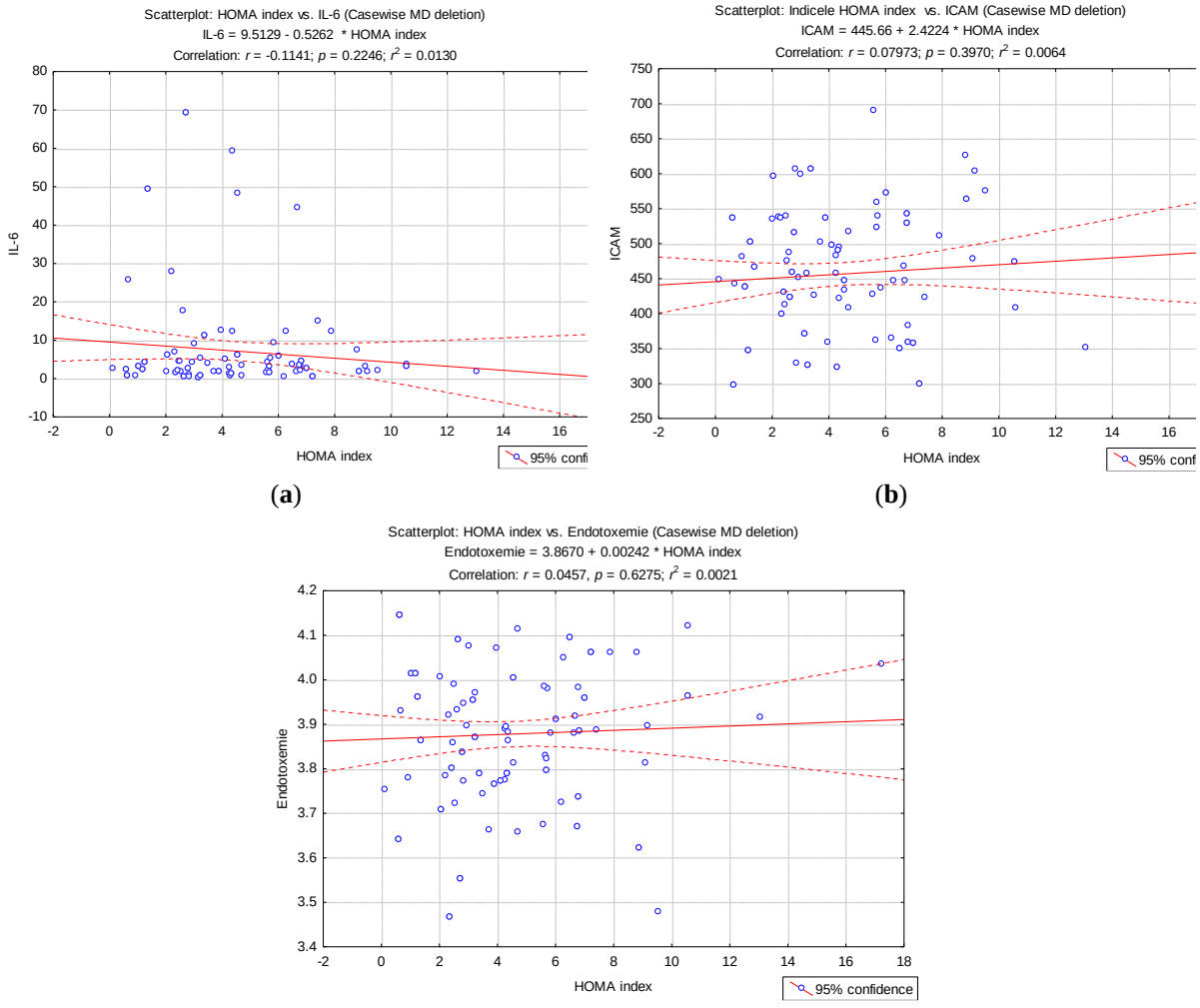


Figure 8. *The regression line in the correlation of the HOMA index and **(a)** IL-6 values, **(b)** ICAM and **(c)** endotoxemia.*

I.1.3.5. Discussion

Several studies highlighted the importance of identifying obese children at high risk of becoming obese adults [Simmonds et al., 2016; Jankowska et al., 2021] and to avoid this real problem by changing their lifestyle. The development of IR and atherosclerosis is linked to chronic inflammation in the context of obesity. Without considerable measures, the evolution from obesity to IR and diabetes, as well as atherosclerosis, hypertension, and CVD, leading to early mortality, is unavoidable [Trandafir et al., 2020]. A clear understanding of this basic processes of obesity-induced inflammation is critical for therapeutic; moreover, preventive programs to avert or delay the onset of these comorbidities by intervening early in life, throughout infancy and adolescence are required [Calcaterra et al., 2020].

Detecting children with higher cardiovascular risk and IR at an early stage is one of the most important goals in preventing pediatric obesity consequences [Varda, Medved, Ojsterek, 2020]. BMI percentile and WC are two clinical measures that are often used in practice, however they have some limitations [Trandafir et al., 2018]. Despite the fact that BMI is

commonly used to assess obesity, it is insufficiently sensitive to detect early fat storage and CVD risk [Varda, Medved, Ojsterek, 2020; Trandafir et al., 2018; Simmonds et al., 2015].

Low-grade inflammation is increasingly being considered in the literature as a link between obesity, IR, and endothelial dysfunction. In this research, we measured both IL-6 and ICAM in order to determine low-grade inflammation. As a result, we noticed that IL-6 was correlated with blood glucose and BMI in a significant way. It has been proven that an elevated level of IL-6 correlates with the emergence of metabolic or cardiac comorbidities in adulthood. Low-grade inflammation in obese children is linked to changes in blood pressure, particularly systolic pressure. Blood pressure is known for causing inflammation in the arterial wall [Syrenicz et al., 2006]. IL-6 promotes the proliferation of vascular smooth muscle tissue, a precursor to hypertension and atherosclerosis. Moreover, increased IL-6 levels are linked to inflammation [Todendi et al., 2015].

ICAM-1 is a molecular marker of endothelial dysfunction, together with soluble adhesion molecules such as E-selectin and vascular cell adhesion molecule 1 (VCAM-1). The onset of atherosclerosis is supported by increasing the levels of circulating adhesion molecules in obese individuals [Nirmalkar et al., 2018]. sICAM-1 appears to reflect the extent of atherosclerotic lesions and is likely to be a predictive factor for future cardiovascular events in adulthood [Elnashar et al., 2017]. In the present study, ICAM had a substantial correlation with serum lipids, blood glucose, and BMI percentile, with the BMI percentile having significant predictive value over ICAM. In the studied groups, there was no evidence of a positive association between IL-6 and ICAM values. A previous study found a link between central obesity/insulin resistance and sICAM-1 levels, with sICAM-1 being regarded as a prototype of inflammatory marker [Elnashar et al., 2017].

The link between low-grade inflammation and high blood pressure in obese children and adolescents was studied by Polish researchers. They analysed serum concentrations of C-reactive protein, IL-6, IL-1β, ICAM-1, VCAM-1, glucose and insulin in 281 overweight children aged between 6 and 18. During 24 hours, C-reactive protein (CRP), IL-6, IL-1, and ICAM-1 were found to have a significant correlation with mean systolic blood pressure. Following 24 hours, inflammatory markers such as CRP and IL-6 were correlated to mean diastolic blood pressure. The scientists found no link between the ICAM-1 cell adhesion marker and the blood pressure of the patients studied, but they did find that low-grade inflammation may have a role in blood pressure modulation as early as childhood [Syrenicz et al., 2006].

Another study performed by researchers in Saudi Arabia analysed markers of inflammation in obese children in the pre-pubertal period, trying to find a correlation between them and the metabolic syndrome. In two groups of 25 obese and normal-weight children, weight, height, BMI, systolic and diastolic pressure, blood glucose, C-reactive protein, IL-6, and sICAM-1 were examined. The results showed elevated levels of insulin, CRP, IL-6 and sICAM-1 in obese children. In none of the groups, the glucose level was modified. Additionally, a correlation was discovered between sICAM-1 and insulin, CRP, and IL-6 levels. Obese pre-pubertal patients display changes that signal insulin resistance, endothelial dysfunction, and the existence of an inflammatory condition, all of which may raise the risk of evolving cardiovascular disease and type 2 diabetes in maturity, according to the researchers [Elnashar et al., 2017].

Endotoxemia was found to be significantly correlated with BMI in our study. Endotoxins (lipopolysaccharides) from the intestine cause a small increase in plasma levels of endotoxins (lipopolysaccharides) with a pro-inflammatory effect in metabolic endotoxemia. The presence of lipopolysaccharides leads to increased lipid absorption and development of obesity, correlated with the value of BMI [Eworo, Egbe, Okhormhe, 2020]. A 0.5 up to 2-fold increase in endotoxins may be a reliable predictor of metabolic endotoxemia. Dietary and pharmacological interventions on the gut microbiota may be essential in decreasing inflammation and endothelial dysfunction [Nirmalkar. et al., 2018].

Current study aims to analyse the relationship between endotoxemia and obesity, diabetes and metabolic syndrome, as well as the link between the value of endotoxemia and other in-creased markers. For instance, African researchers studied endotoxemia in obese children and adolescents and its possible relationship with insulin, lipid profile and C-reactive protein. They compared the results of 20 patients with a normal weight with those of 30 obese children and adolescents, aged between 5 and 18 years. The evaluated parameters were lipid profile, liver function, endotoxin, C-reactive protein, glycemia and IR. The results showed that endotoxin and CRP value were significantly higher in the obese group compared to the other group and a positive correlation between serum endotoxin and BMI, WC, TG, TC and insulin was identified. Thus, they settled that endotoxin may play a role in cardiometabolic risk factors associated with obesity in children and adolescents [Omar et al, 2020].

A study conducted by Nigerian experts on 90 patients separated into two groups: 47 persons with metabolic endotoxemia and 43 people who served as controls, shows a relationship between endotoxemia and obesity in patients. The patients in the first group were separated into three subgroups: normal weight patients, overweight patients, and obese patients. The researchers noted significant correlations between BMI and endotoxemia, total cholesterol and endotoxemia, and triglycerides and endotoxemia in the first group of patients. They reached the conclusion that the value of endotoxemia may precede the development of obesity [Eworo et al., 2020].

Regarding our research, insulin value was investigated among children and adolescents, trying to find a correlation with excess weight. Our results found that the values of insulin are correlated significantly with TG and BMI percentile, but only the BMI percentile has predictive power over insulin.

German researchers have discovered possible links between insulin levels, glycemic index, and blood sugar levels in children throughout puberty and adult body composition. The study was founded on the idea that frequent meal consumption in young people causes higher levels of postprandial blood sugar, and that high insulin levels can have an adverse influence on body composition in maturity. The study involved 262 people who were given two dietary measurements every three days during puberty and anthropometric measures at the ages of 18–25 years. There was no link between glycemic index or glycemic load during puberty and adult body composition, however a high insulin value during puberty was linked to a higher percentage of body fat in maturity. As a result, it was established that the postprandial increase in insulinemia, which began in the pubertal period, contributes to the abnormal development of body composition in adulthood [Joslowski et al., 2012].

A study was undertaken in the United States to see if there was a link between blood insulin levels and insulin resistance and the increase of body fat in African-American and

Caucasian children. The study group included 249 children ages 6 to 12 who were healthy but at high risk of becoming obese as adults due to childhood obesity or due to overweight parents. Anthropometric and psychological measurements were taken on the subjects until they were 15 years old. Anthropometric and biochemical measurements were taken on the subjects on a regular basis until they reached the age of 15.

At the beginning of the trial, 39% of the children had a BMI percentile that was higher than the 95th percentile. There was no link between insulin value, glycemic index, fat percentage, or BMI percentile, implying that BMI value and adipose tissue amount play a significant part in the development of an obese adult during childhood. They found no link between plasma insulin levels and obesity, implying that this relationship needs to be investigated further [Sedaka et al., 2017].

Previous data proved that HOMA index has significantly correlations only with BMI percentile, which is a significant predictive factor for the value of the HOMA index. According to this correlation, obesity is a major risk factor for the development of insulin resistance. This aspect was also validated in the study of Elnashar et al. [Elnashar et al, 2017], who showed that insulin and the HOMA index have a significant increase in obese children. Insulin and HOMA were found to have positive correlations with BMI percentile, systolic BP, LDL cholesterol, and TG as metabolic parameters, as well as CRP, IL-6, and ICAM 1 as an inflammatory parameter. In this regard, we noticed a strong correlation between cardiovascular risk variables and HOMA-defined insulin resistance in our research. In the pathophysiology of obesity, some research state that gender differences must be considered.

Based on the findings, we plan to conduct a more in-depth investigation of the changes in inflammation and insulin resistance markers that occur in the occurrence of cardiovascular problems in obese children and adolescents in the future. Our tests revealed no significant correlations between plasma cortisol and cholesterol, TG, or glycemia levels, nor with BMI percentile, a finding supported by Abraham's study [Marson et al, 2016], which found no correlation between BMI percentile or weight and cortisol (either salivary or urinary/24 h), and no correlation between cortisol and the values of TG, HDLc, or blood pressure.

Our study's limitations included the small number of patients in both groups and the incapacity to assess fat mass and fat free mass using densitometry or dual-energy X-ray absorptiometry. Despite its widespread use as a surrogate for adiposity, the BMI is a measure of excess weight rather than excess body fat [Freedman et al., 2005]. The parameters related to body mass composition will be evaluated in future investigations. In a meta-analysis of four studies, overweight and obese children who became normal in adulthood were no different in terms of additional cardiovascular disease risk parameters than those patients who were never obese [Juonala et al., 2011]. Consequently, early nutritional interventions instituted in obese children that lead to weight loss will contribute to the health of future adults.

I.1.3.6. Conclusions

A thorough comprehension of the inflammatory mechanisms that characterize obesity is critical for preventing the disease and its consequences in obese pediatric patients. Inflammatory markers, such as IL-6, ICAM 1 and endotoxemia, show significantly higher values in pediatric obese patients, leading to chronic and systemic inflammation. These markers can be considered major predictors of cardiometabolic disorders in these patients, according to

the findings of our research. The relationship between low-grade inflammation, IR, and endothelial dysfunction, as well as obesity, is particularly interesting. Obesity is a key risk factor for the development of insulin resistance, as evidenced by a strong correlation between the HOMA index and the BMI percentile. The BMI percentile has a lot of predictive power for IR metabolic biomarkers. Nevertheless, our findings serve as a basis for more research into the age-related dynamics of obese pediatric patients.

I.2. Histological assessment of atherosclerotic lesions – from experimental to clinical studies

I.2.1. Scientific context

The role of arteries in vascular hemodynamics is closely correlated with the functionality of each structural component. The primary components of the arterial wall, endothelial cells and smooth muscle cells, play a vital role in vascular biology and pathophysiology.

In 1980, Furghott and Zawadzki revealed the importance of the endothelium in acetylcholine-induced vasodilation, redefining cardiovascular physiology and triggering a large interest in research in this area [Furchgott, Zawadzki, 1980]. According to subsequent research, endothelial cell dysfunction is the key to the onset and progression of cardiovascular disease, and its amelioration seems to be a treatment option. Maintaining arterial wall homeostasis and appropriate circulatory function is essential for the endothelium's structural and functional integrity [Vanhoutte et al., 2017; Konukoglu, Uzun, 2017; Kwaifa et al., 2020; Sun et al., 2020].

The phenotypic plasticity of the vascular endothelium is linked to its anatomical position and dynamic response to local environment. Endothelial cells limit the leukocyte adherence, inhibit platelet aggregation and the proliferation of vascular smooth muscle fibers, and are involved in the relaxation of smooth muscle cells. Any change in its function induces a disrupted vasodilator response, due to an imbalance between vasoconstrictor and vasodilator factors, inadequate intervention in fibrinolysis control, wider availability of leukocytes with disturbance of the inflammatory process, and changes in adhesion molecule expression [Monteiro et al., 2019; Kwaifa et al., 2020].

Endothelial dysfunction is an early indicator of atherosclerosis, as it is the underlying cause of increased vascular permeability, leukocyte adhesion, thrombosis risk, and smooth muscle cell proliferation, all of which are key features in the progression of the disease. Simultaneously, because endothelial dysfunction is a reversible condition, identifying patients at risk for endothelial dysfunction and monitoring the effectiveness of therapy to reduce this phenomenon should become a goal in the future, with the end result being lower cardiovascular disease morbidity and mortality [Daiber et al., 2017; Monteiro et al., 2019; Marchio et al., 2019].

Assessment of the severity of endothelial dysfunction is not currently working as a routine procedure, owing to the considerable fluctuation in endothelial cell function from day to day. Furthermore, on the same day, factors such as hormone status, sleep quality, and physical exercise might produce major variations [Sun et al., 2020].

Endothelial function assessment methods, in theory, aim to assess the endothelium's vasodilator response following the administration of NO release compounds (acetylcholine, bradykinin), and may comprise invasive or non-invasive imaging techniques, as well as related biological biomarkers, correlated with endothelial dysfunction [Verma et al., 2003; Tousoulis et al., 2005; da Silva et al., 2021].

Endothelial function appears to be a significant predictive factor in the assessment of individuals with cardiovascular risk or coronary heart disease, according to new research. Endothelial dysfunction is an independent prognostic factor in these patients and may predict significant cardiovascular events. Simultaneously, the persistence of endothelial dysfunction in patients with advanced atherosclerosis necessitates a more aggressive approach to risk factor management and, most likely, a re-evaluation of therapy. Additionally, non-invasive endothelial function testing could be used to screen for subclinical atherosclerosis and could be integrated into global cardiovascular risk scores [Daiber et al., 2017; Monteiro et al., 2019; Marchio et al., 2019].

Atherosclerosis is a chronic disease that begins with a sequence of dysfunctions in the endothelium of the arterial wall that is now recognized by an increasing number of experts as the main event in the inflammatory process that precedes the formation of atherosclerotic plaque [Monteiro et al., 2019; Sun et al., 2020].

The peroxide degradation of the lipids of the intima, the release of proinflammatory mediators and growth factors, followed by an accelerated proliferation of smooth muscle cells, with subsequent collagen formation, are the steps that follow endothelial injury [Kumar, Abbas, Aster, 2018].

Several theories about the pathophysiology of atherosclerosis have been proposed over time [Capron, 1989; Wissler, 1992; Bertrand, Tardif, 2017]. One of them considers that the "organization" of thrombi at the arterial wall level to be the reference point of the atherosclerotic process [Gaudio et al., 2006; Steiner, Laco, 2008]; another one is connected to a damage of the intima to which macrophages, platelets, lymphocytes can attach, leading to degenerative atherosclerotic lesions [McManus, 1958].

The development and growth of atherosclerotic plaque was explained by many theories [Aziz et al., 2016].

According to *trombogenic theory of Rokitansky*, atherosclerosis is the result of repeated episodes of intramural thrombosis, which leads to accumulation of platelet and fibrin deposits, which then contribute to thrombus formation. The platelet membranes present in the formed lesion would be the provider of lipids in this scenario; in addition, smooth muscle cell proliferation is performed under the influence of platelet-derived growth factors. Furthermore, wall thrombosis becames the most important factor in determining the blockage of a vessel, particularly of the coronary arteries, which causes atherosclerosis [Schwartz et al., 1988; Golia et al., 2014].

Theory of lipid infiltration has the most proponents, and the main premise is that atherosclerosis is caused by a localized aggregation of lipids originating in plasmatic lipoproteins in the artery walls. Consequently, the recommendations in the preventive guidelines are to lower the lipids levels in the vascular network. Although the process by which this lipid build-up occurs is not completely understood, the theory is widely accepted. Nevertheless, it is not only the storage of lipids that contributes to the formation of atheroma

plaques, but also the proliferation of smooth muscle cells and thrombosis, etc. [Capron, 1989; Wong et al., 2016; Geovanini et al., 2018].

According to *monoclonal proliferation theory*, under the influence of environmental stimuli, atherosclerotic plaques demonstrate monoclonal proliferation of smooth muscle cells, with aberrant increase of the diameter of these cells comparable to that of smooth muscle neoplasms. [Benditt, 1977; Harangi et al., 2009].

The currently accepted theory is *theory of endothelial damage or response to aggression*, which was first hypothesized to understand the mechanism of atherosclerotic lesion smooth muscle cell accumulation. Growth factors generated by endothelial cells, macrophages, and even injured smooth muscle cells initiate the proliferation. Many of these alterations result in a change in the function of the vascular endothelium, determining a chronic inflammatory response. As long as the damning components are present at the injury site, the inflammation will persist [Capron, 1989; Libby, 2019]. Essentially, this theory considers that the essential element in atherogenesis is a type of endothelium injury manifested as endothelial dysfunction. [Reneman et al., 2006; Mannarino, Pirro, 2008; Libby et al., 2019].

Given the high blood pressure and shear pressures applied on the endothelial cells at this level, the lesion frequently arises near the bifurcation sites of the arteries. The start of chronic inflammation in the arterial wall lesion triggers the formation of atherosclerosis. At that level, hemodynamic stresses cause the expression of atherosclerosis-promoting factors like basic fibroblast growth factor (FGF-2), tissue factor (TF), plasminogen activator, and endothelin. Furthermore, the interaction between changed lipoproteins, macrophages produced from blood monocytes, T lymphocytes, and the normal cellular elements of the artery wall aids the propagation of the lesions. Disrupted endothelial cells enable subendothelial tissue to be exposed to various plasma constituents at the level of the vulnerable segments of the artery wall [Libby et al., 2010; Libby et al., 2019; Minelli et al., 2020].

Monocytes, together with mitogens produced from cells or plasma and mitogenic regulators, penetrate the subendothelial area of the intima in people with high cholesterol levels. Blood platelets may also interact with the exposed intimal collagen, promoting aggregation on the lesion's denuded surface. They discharge platelet-derived growth factor (PDGF), which has been shown to be chemotactic for smooth muscle cells, implying that PDGF may play a key role in inducing smooth muscle cell migration through the internal elastic limiting fenestrae, giving rise to focal invasion of the intima. As a result of these changes, the thickness of the intima grows, the vascular lumen narrows causing an even larger risk to endothelial cells, and the amount of blood passing through the vessel decreases, contributing to the individual's incapacity to exert effort. Therefore, the existence of a tiny thrombus completely obstructs the vessel due to its small diameter [Libby et al., 2019].

The Bogalusa Heart Study found atherosclerotic plaque in the coronary arteries of children aged 2 to 15 years, indicating that the atherosclerosis begins early in life [Berenson et al., 1998; Cheung, Cheung, 2021]. All vessels are affected by atherosclerosis, although the coronary arteries are the most examined being the first to show signs of alteration. According to previous research, the most common cause of type 1 myocardial infarction is atherosclerotic coronary artery disease, which is triggered by the rupture or erosion of the atheroma plaque. Coronary angiography reveals the presence of atherosclerotic plaque in the coronary arteries in

the majority of patients with type 2 myocardial infarction, its presence being associated with a poor prognosis [Cheung, Cheung, 2021].

The study of Nikolai N. Anichkov in 1913, which proved the function of cholesterol in atherosclerosis on animals fed an atherogenic diet, is considered one of the most significant scientific discoveries of the twentieth century. He developed human-like atherosclerotic lesions on the rabbit aorta, an animal in which total cholesterol levels and excretion are generally lower, allowing the plasma level of this biochemical marker to be easily boosted through diet [Anitschkow, Chalatow, 1913; Konstantinov et al., 2006]. Following this, numerous morphological and experimental studies have given further, unique data, which has been confirmed in significant histophysiological studies, demonstrating linkages between hyperlipidemia, specifically hypercholesterolemia, and atherosclerosis.

Personal contribution related to morphology of atherosclerotic lesions was synthesized in the following papers:

ISI ARTICLES

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   http://www.rjme.ro/RJME/resources/files/540213399404.pdf
2. **Cojocaru E,** Trandafirescu M, Leon M, Cotuţiu C, Foia L. Immunohistochemical expression of anti-CD68 antibody in atherosclerotic plaque. *Rom J Morphol Embryol* 2012; 53(1): 61–66. **IF: 0.620**
   http://www.rjme.ro/RJME/resources/files/530112061066.pdf
3. Ifrim S, Amalinei C, **Cojocaru E**, Matei MC. Administration of valine, leucine, and isoleucine improved plasma cholesterol and mitigated the preatherosclerotic lesions in rats fed with hypercholesterolemic diet. *Rev Romana Med Lab*. 2018; 26(1):65-75. **IF: 0.800**
   http://www.rrml.ro/articole/2018/2018_1_7.pdf
4. Strobescu-Ciobanu C, Giusca SE, Caruntu ID, Amalinei C, Rusu A, **Cojocaru E**, Popa RF, Lupascu CD. Osteopontin and osteoprotegerin in atherosclerotic plaque - are they significant markers of plaque vulnerability? *Rom J Morphol Embryol* 2020; 61(3):793–801. **IF: 1.033**
   https://rjme.ro/RJME/resources/files/610320793801.pdf
5. **Cojocaru E**, Magdalena Leon-Constantin M, Ungureanu C, Trandafirescu MF, Maștaleru A, Mihaela Trandafir L, Dumitru Petrariu F, Viola Bădulescu O, Filip N. Hypolipemiant Actions and Possible Cardioprotective Effects of Valine and Leucine: An Experimental Study. *Medicina* 2021; 57(3):239. **IF: 2.430.**
   file:///C:/Users/Elena/Downloads/medicina-57-00239%20(5).pdf

BDI ARTICLES

1. **Cojocaru E,** Zamfir CL, Lupuşoru CE, Cotuţiu C. The effects of some nonpolar aminoacids--valine, leucine-administration on the arterial wall already exposed to a hypercholesterolemic diet. *Rev Med Chir Iaşi* 2010; 114(2): 504-509.
   http://www.ncbi.nlm.nih.gov/pubmed/20700993

2. **Cojocaru E**, Zamfir C, Butcovan D, Cotuţiu C. Studiu retrospectiv al particularitatilor morfologice si evolutive ale leziunilor aterosclerotice. *Jurnalul de Chirurgie Iaşi* 2010; 6(3): 295-304. http://jurnaluldechirurgie.ro/jurnal/docs/jurnal310/art%2008_vol%206_2010_nr%203.pdf
3. **Cojocaru E,** Zamfir C, Zamosteanu N, Trandafirescu M, Cotuţiu C. The effects of branched chain aminoacids on HDL-cholesterol in experimental animals subjected to dietary hypercholesterolemia. *Rev Med Chir Iaşi* 2012; 116(1): 200-206. http://www.ncbi.nlm.nih.gov/pubmed/23077896
4. **Cojocaru E**, Leon M, Zamfir C, Amihăesei C, Trandafirescu M, Mitu F. The influence of branched amino acids on LDL-cholesterol levels in a model of experimental atherosclerosis. *Revista Română de Anatomie funcţională şi clinică, macro- şi microscopică şi de Antropolgie* 2012; 11 (1): 35-40. http://revanatomie.ro/ro/abstract.php?an_rev=2012&nr_rev=1&nr_art=6
5. **Cojocaru E**, Filip N, Ungureanu C, Filip C, Danciu M. Effects of Valine and Leucine on Some Antioxidant Enzymes in Hypercholesterolemic Rats. *Health* 2014; 6 (17): 2313-2321. http://www.scirp.org/journal/PaperInformation.aspx?paperID=50368
6. Butcovan D, Dumitrescu C, **Stefanachi (Cojocaru) E**, Arsenescu-Georgescu C. Vulnerability index assessment of the erosive atherosclerotic plaque on endarterectomy specimens. *Romanian Journal of Oral Rehabilitation* 2014; 6(3): 8-13. http://www.rjor.ro/wp-content/uploads/2014/10/08-13.pdf

BOOK CHAPTERS

1. **Cojocaru E,** Leon MM, Vasile R, Iurciuc M, Mitu F. Actualităţi în configuraţia moleculară a endoteliului vascular. În: Societatea Română de Cardiologie (ed). Progrese în cardiologie 2014. București: Editura Media Med Publicis, 2014; 39-50. ISSN: 1843-3731.

I.2.2. Essential amino acids impact in the evolution of the atherosclerotic process

I.2.2.1. Introduction

Hypercholesterolemia has a key role in the onset and development of atherosclerosis, which is the major cause of death worldwide among cardiovascular disorders [Mc Nair et al., 2016]. As previously stated, hyperlipidemia, defined as a high level of triglycerides, total cholesterol, low-density lipoprotein – cholesterol (LDL-C), very low-density lipoprotein (VLDL-C) – cholesterol, and a low level of high-density lipoprotein (HDL-C) – cholesterol, is the most significant risk factor for atherosclerosis [Li et al., 2016]. HDL-C is shown to be inversely connected with atherosclerosis in the scientific literature, indicating that it plays a substantial preventive function over the onset and progression of the disease [Vergeer et al., 2010; Zhao et al., 2016]., while LDL-C and atherosclerosis have a positive correlation [Badimon, Vilahur, 2012; Zhao et al., 2016].

Dietary essential amino acids are branched-chain amino acids (BCAAs) such as valine, leucine, and isoleucine. BCAA have been linked to lipolysis, lipogenesis, and cholesterol metabolism in current epidemiological reports [Zhang et al., 2017]. The results of previous studies have indicated that valine and leucine raise HDL-C levels in the blood, contributing to atherosclerosis regression [Cojocaru et al., 2012; Yang et al., 2014]. In addition, they were also related to a decreased of LDL-C serum level suggesting that they could be used as lipid-lowering compounds [Yang et al., 2014; Cojocaru et al., 2012].

I.2.2.2. Aim

The purpose of this study was to see how valine, leucine, and isoleucine affected the onset and progression of atherosclerosis in rats fed a hypercholesterolemic diet.

I.2.2.3. Material and methods

Animals and diets

In this investigation, 50 male Wistar rats weighing 250-300 g (supplied by the Cantacuzino Institute in Bucharest) were studied over a period of 2 months (60 days). The rats were divided into five groups, 10 rats for each group, and fed the following diets: group I (control) received standard diet; group II (C) received 0.4g/kgc/day cholesterol; group III (C + V) received 0.4g/kgc/day cholesterol and 62.5mg/kgc/day valine amino acid; group IV (C + L) received 0.4g kgc/day cholesterol and 69.985mg/kgc/day leucine amino acid; group V (C + iL) received 0.4g/kgc/day cholesterol and 69.985mg/kgc/day isoleucine amino acid. Other metabolic disorders were not assessed in the study's animals.

Biochemical Measurements and Histopathological analysis.

Blood samples were collected from the retro-orbitary plexus of each animal, in order to evaluate the serum levels of total cholesterol, at the beginning of the experiment (R0), after 1 month (30 days) (R1), and after 2 months (60 days) (R2). The current research was carried out using the instructions provided by the "*Guide for the Care and Use of laboratory animals*" [NRCNA, 2011]. The study complied with the national and international ethical regulation [http://www.nc3rs.org.uk/page.asp?id=1357; Festing et al., 2002]. Diagnosticum Zrt's kits were used to measure total cholesterol levels in the blood [Richmond, 1973].

The animals were euthanized after receiving 75 mg/kgc intraperitoneal ketamine for anesthesia. The collected samples (heart and aorta tissue fragments) were processed for optical microscopy using the paraffin embedding procedure. Hematoxylin and eosin (HE) staining and Goldner-Szekely trichrome stains were performed to stain the sections.

Statistical analysis

All data were analysed with Microsoft Excel statistical functions and Statistical Package for Social Sciences (SPSS) version 12 software. Mean (M), standard deviation (SD), coefficient of variation (CV), and confidence intervals (CI) were used to depict the recorded values. To compare means between groups, a one-way ANOVA was performed. A statistically significant p value <0.05 was used.

I.2.2.4. Results

Each group's total cholesterol levels were measured three times: at the start of the investigation (R0), after 30 days (R1), and after 60 days (R2). The most homogeneous set of values was documented at the end of the experiment (R2) for all 5 groups (control, C, C+V, C+L, C+i-L: CV = 4.0 - control group; CV = 4.8 - cholesterol group; CV = 3.3 - cholesterol

and valine group; CV = 3.9 – cholesterol and leucine group; CV = 4.1 - cholesterol and isoleucine group (Table X).

Total cholesterol levels in the control group varied from 33.1 to 41.2 mg/dl at the start of experiment (R0), with a mean of 36.9 mg/dl, followed by a minor increase in the average level during the next period to 37.7 mg/dl for R1 and 38.2 mg/dl for R2 (p=0.399).

Regarding the second group (C), total cholesterol levels varied between 29.3 and 41.2 mg/dl (with a mean of 36.9 mg/dl). After the initial moment of the assessment, the trend was ascending with significantly increasing values: a mean of 50.3 mg/dl for the second measurement (R1) (ranged from 42.2 to 54.3 mg/dl) and 76.9 mg/dl for the final moment of the research (R2) (ranged from 70.8 to 80.4 mg/dl) (p=0.001).

We evaluated the total cholesterol value for the next group (C+V). The values ranged from 35.1 to 38.5 mg/dl (mean of 37.0 mg/dl) at the start of the study (R0), then increased to a mean of 40.9 mg/dl (range 37.2 to 42.6 mg/dl) for the next evaluation (R1), and 44.3 mg/dl (range 42.3 to 46.1 mg/dl) for the final evaluation (R2) (p=0.001).

The fourth group (C+L) had a total cholesterol fluctuation between 33.1 mg/dl and 40.1 mg/dl (with a mean of 36.55 mg/dl) at the start of the study (R0).

Until the end of the investigation, variations in total cholesterol levels were seen. Therefore, after one month (R1), the mean values were 46.2 mg/dl (with a minimum of 40.4 mg/dl and a maximum of 49.1 mg/dl), and after two months (R2), the mean values were 49.7 mg/dl (with a minimum of 45.5 mg/dl and a maximum of 52.7 mg/dl) (p=0.001).

Table X. The evaluation of total cholesterol among the study groups

Group	R0	R1	R2
Group I: control			
M	36.9	37.7	38.2
SD	2.2	2.0	1.8
CV	6.0	5.3	4.0
Group II: C			
M	36.9	50.3	76.9
SD	3.4	3.5	3.1
CV	9.3	6.9	4.8
Group III: C+V			
M	37.0	40.9	44.3
SD	0,9	1. 6	1.5
CV	2.6	3.8	3.3
Group IV: C+L			
M	36.6	46.2	49.7
SD	2.4	2.5	1.9
CV	6.6	5.3	3.9
Group V: C+i-L			
M	36.8	46.7	49.9
SD	2.3	2.8	2.1
CV	6.4	6.1	4.1

M = mean, SD = standard deviation, CV = coefficient of variance

The first evaluation of the total cholesterol levels (R0) for the last group (C+i-L) recorded a mean of 36.8 mg/dl, with values ranging from 33.3 to 40.1 mg/dl. Following the initial measurement, significant increases in total cholesterol mean levels were found, as follows: 46.7 mg/dl after 30 days (R1) (range: 40.4 to 50.1 mg/dl) and 49.9 mg/dl after 60 days (R2) (range: 45.5 to 52.2 mg/dl) (p=0.001).

In comparison to the other two amino acids, valine caused a statistically significant decrease in cholesterol levels (p=0.002).

For the second evaluation (R1) and the final moment of the experiment, the most increased values were reported among rats from group C (cholesterol) (p=0.001) (R2). In the initial assessment, there was no statistical significance (p=0.993) between the mean values of total cholesterol across the studied groups (R0) (Table XI).

Table XI. Statistical indicators of total cholesterol values compared by study groups

Lot	N	Mean	Standard Deviation	Standard Error	95% IC		Mean		p value (FANOVA test)
					Min	Max	Min	Max	
R0									
Lot Martor	10	36.9	2.2	0.7	35.4	38.6	33.1	41.3	
Lot C	10	36.9	3.4	1.1	34.4	39.3	29.3	41.2	
Lot C+V	10	37.0	0.9	0.3	36.3	37.7	35.1	38.5	0.993
Lot C+L	10	36.6	2.4	0.8	34.83	38.3	33.1	40.1	
Lot C+i-L	10	36.8	2.3	0.7	35.1	38.5	33.3	40.1	
R1									
Lot Martor	10	37.7	2.0	0.7	36.3	39.1	34.4	40.1	
Lot C	10	50.3	3.5	1.1	47.8	52.8	42.2	54.3	
Lot C+V	10	40.9	1.6	0.5	39.9	42.1	37.2	42.6	**0.001**
Lot C+L	10	46.2	2.5	0.8	44.5	47.9	40.4	49.1	
Lot C+i-L	10	46.7	2.8	0.9	44.7	48.7	40.4	50.1	
R2									
Lot Martor	10	38.2	1.8	0.6	36.9	39.5	35.9	40.9	
Lot C	10	76.9	3.1	0.9	74.7	79.2	70.9	80.4	
Lot C+V	10	44.3	1.5	0.5	43.2	45.3	42.2	46.1	**0.001**
Lot C+L	10	49.7	1.9	0.6	48.3	51.1	45.5	52.7	
Lot C+i-L	10	49.9	2.1	0.7	48.4	51.3	45.5	52.2	

Individual total cholesterol values were higher in the second group, which received only cholesterol (Figure 9).

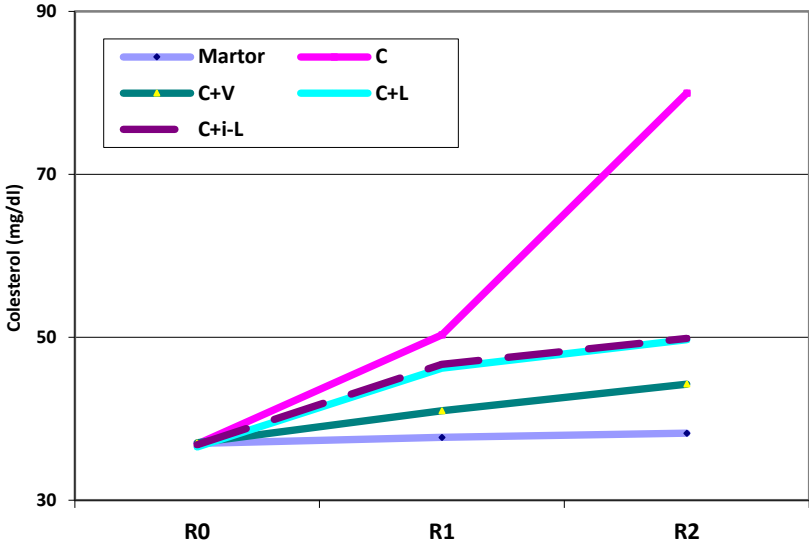


Figure 9. *The trend of total cholesterol values for experimental groups*

The purpose of the histopathological investigation was to analyse the onset of atherosclerotic lesions and to assess the relationship between pathological alterations associated with a hypercholesterolemic diet and the supply of some essential amino acids (valine, leucine, and isoleucine). The aorta and coronary arteries walls were found to have superficial erosions of the endothelium, variable adhesion of leukocytes, erythrocytes, and platelets to the arterial intima correlated with the degree of wall damage, rare foamy macrophages at the level of the arterial internal layer, and mild thickening of the vascular wall (Table XII).

Table XII. The histopathological changes related to early atherosclerotic process by study groups

Group	**Adhesions of leucocytes (number of rats)**	**Adhesions of erythrocytes and platelets (number of rats)**	**Superficial erosions of the endothelium (number of rats)**
Control	− (10)	− (10)	− (10)
C	++ (10)	++ (10)	++ (10)
C+V	+ (10)	+ (10)	− (10)
C+L	+ (10)	+ (10)	+ (6) /− (4)
C+i-L	+ (10)	+ (10)	+ (4) /− (6)

++ = intense; + = reduced/ slight; +/− = either present or absent; − = absent

The histological analysis of the artery walls in the control group revealed a normal morphology using both HE and Goldner-Szekely trichrome stains. A delicate simple squamous epithelium separated from the internal elastic lamina by a loose subendothelial connective tissue including some fibroblasts, smooth muscle cells, and thin collagen fibers was found in the artery wall sections. The tunica media has been found to have multiple concentrical layers of muscle fibers. The tunica adventitia was quite thin, with fibroblasts, collagen fibers in longitudinal bundles, and a loose network of elastic fibers. The optical microscopy analysis revealed distinct endothelium features associated with the prelesional stage of atherosclerosis, demonstrating a degree of endothelial dysfunction, such as erythrocyte and platelet adhesions, as well as

endothelial discontinuity in the tissue fragments of the second group (rodents fed a hypercholesterolemic diet) (Table XII). The leukocyte margination and isolated macrophages in the subendothelial tissue have also been noted (Figure 10).

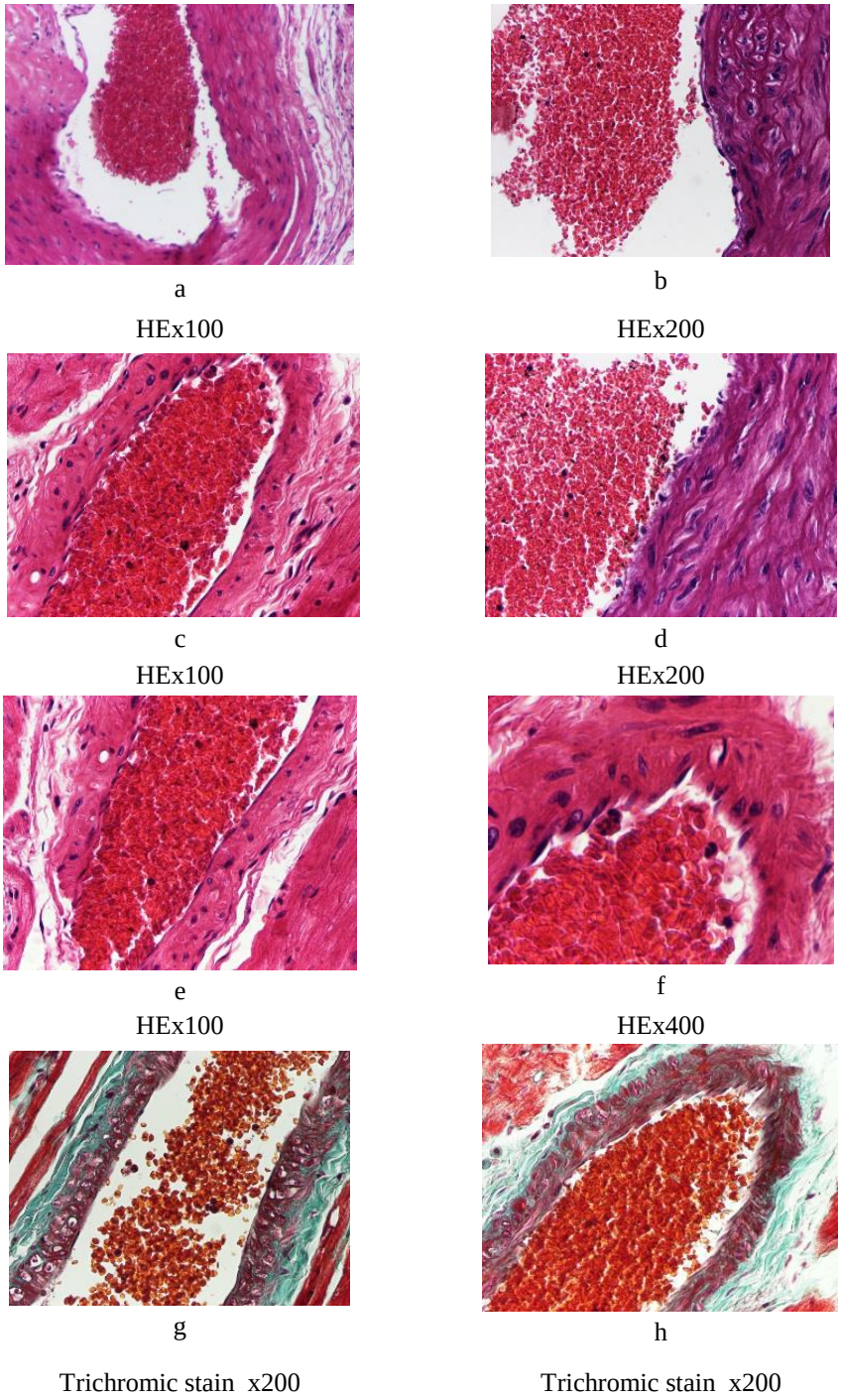
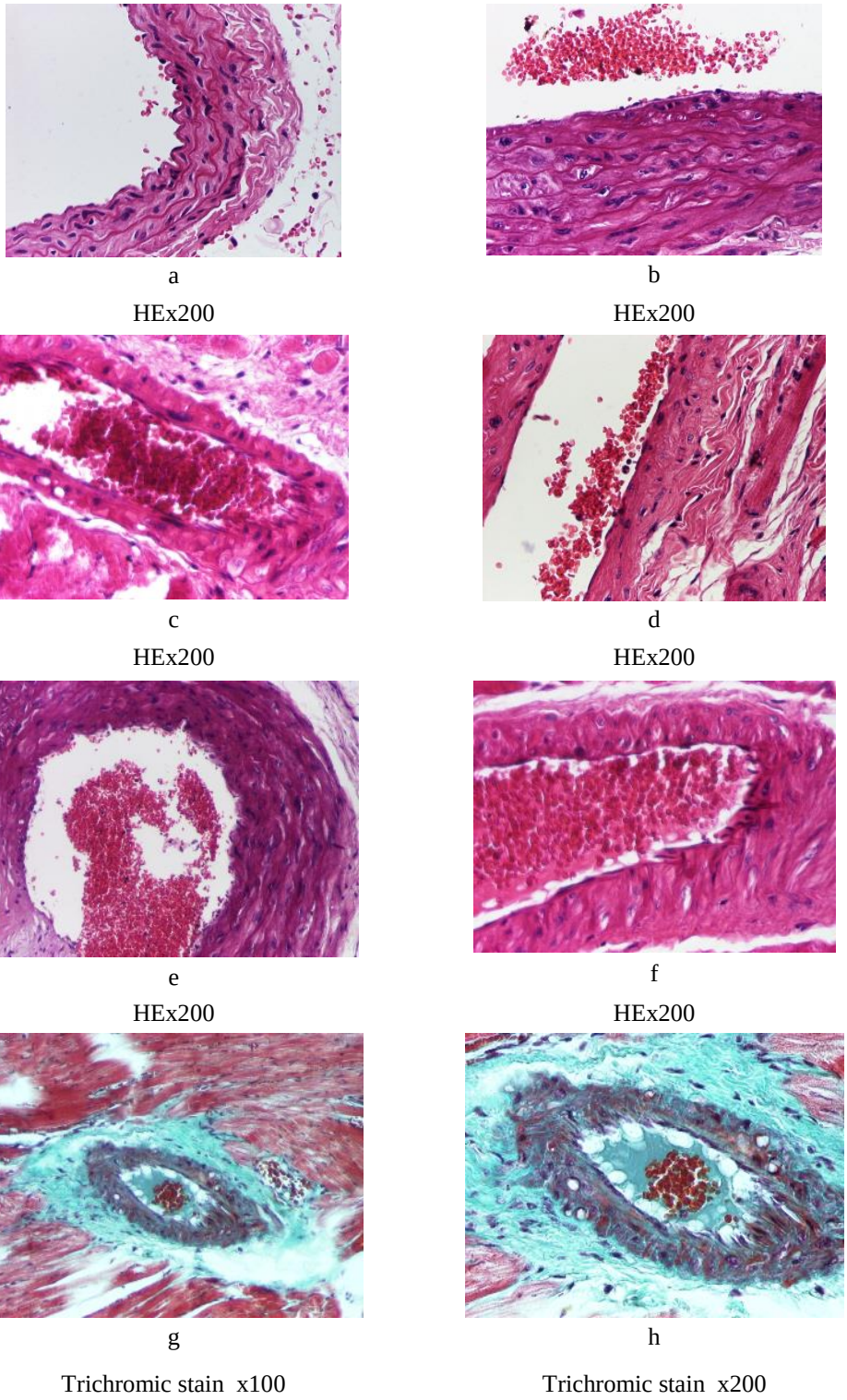


Figure 10. *Vascular wall with early atherosclerotic changes – group II (C): adhesions of erythrocytes and platelets associated with endothelial discontinuity; leukocyte margination and rare macrophages at the level of the subendothelial tissue (HE stain - a, c, e x100; b, d x 200; f x400; Goldner-Szekely trichrome stain - g, h x200)*

When compared to the group that received cholesterol, the above-mentioned alterations were reduced in the valine, leucine, and isoleucine groups, suggesting the beneficial effect of

the three amino acids associated with such a hypercholesterolemic diet. In HE and Goldner-Szekely trichrome stains, the internal elastic lamina was intact, and the elastic layers of the coronary wall structure had a structure that was within normal histological boundaries (Figure 11).



a HEx200 b HEx200

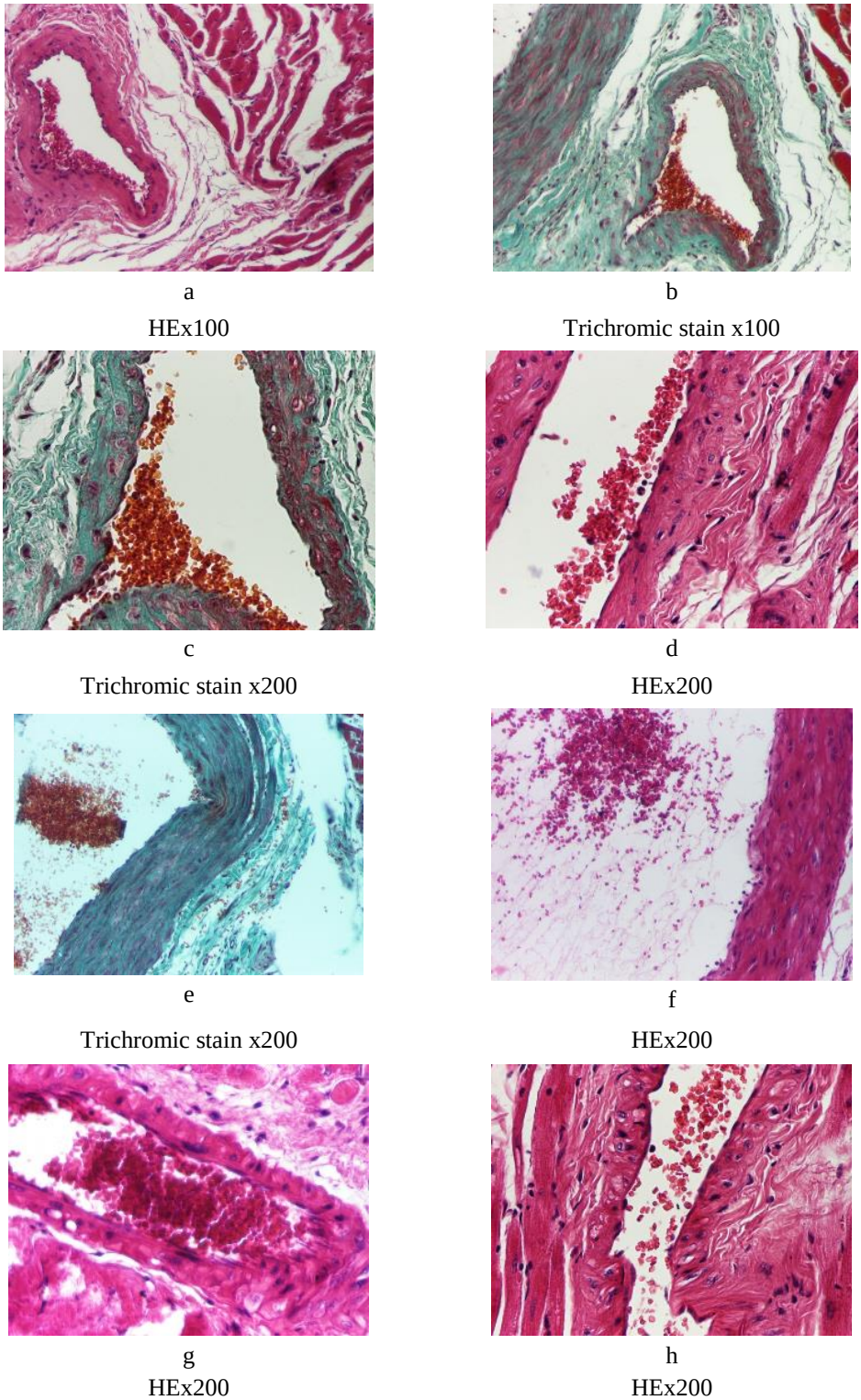
c HEx200 d HEx200

e HEx200 f HEx200

g Trichromic stain x100 h Trichromic stain x200

Figure 11. *Vascular wall with early atherosclerotic changes – group III (C+V): reduced erythrocytes and platelets adhesion to the arterial walls; internal elastic lamina and elastic layers of the coronary wall within normal histological limits (HE stain - a, b, c, d, e, f x200; Goldner-Szekely trichrome stain – g x100, h x200)*

As summarized in Table XII, the third group showed reduced erythrocyte and platelet adherence to both types of artery walls (aorta and coronaries). The progression of alterations in the fourth group of rats demonstrated a similar pattern to that seen in the third group. Reduced erythrocytes, leukocytes, and platelet adherence at the endothelium level was seen, keeping the arrangement of the elastic layers within the structure of medium layer (Table XII, Figure 12).



a HEx100 b Trichromic stain x100

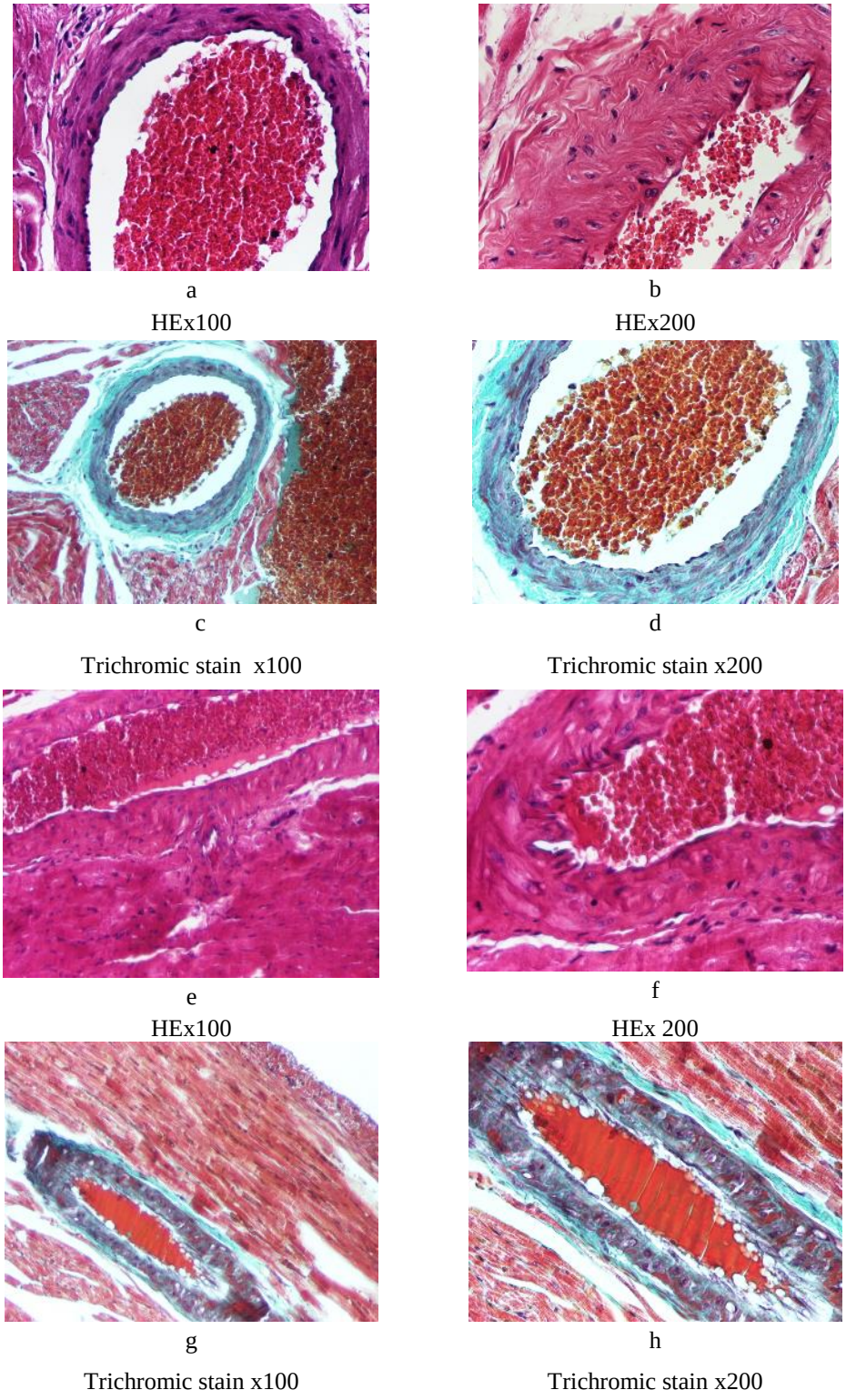
c Trichromic stain x200 d HEx200

e Trichromic stain x200 f HEx200

g HEx200 h HEx200

Figure 12. *Vascular wall with early atherosclerotic changes – group IV (C+L): reduced erythrocytes, leukocytes, and platelets adhesion at the endothelium level; preserved disposition of the elastic layers within the structure of coronaries tunica media (HE stain - a x100; d, f, g, h x200; Goldner-Szekely trichrome stain – b x100; c, e x200)*

When compared to groups III and IV, the fifth group showed arterial lumens, sometimes with irregular endothelium, and with a minor adhesion of erythrocytes, and platelets due to intimal discontinuity (Table XII, Figure 13).



a HEx100 | b HEx200

c Trichromic stain x100 | d Trichromic stain x200

e HEx100 | f HEx 200

g Trichromic stain x100 | h Trichromic stain x200

Figure 13. *Vascular wall with early atherosclerotic changes – group V (C+i-L): some arterial lumens with irregular endothelium and with a slight adhesion of erythrocytes, and platelets (HE stain - a, e x100; b, f x200; Goldner-Szekely trichrome stain - c, g x100; d, h x200)*

I.2.2.5. Discussion

Hypercholesterolemia represents one of the major risk factor of atherosclerosis, involving many genetic and environmental factors which are difficult to control [Bennett et al., 2015]. Oxidative stress also has a role in the development of hypercholesterolemic atherosclerosis. Anandhi et al. used male Wistar rats fed an atherogenic diet for 45 days in order to conduct an experimental investigation on atherosclerosis. The results of the study show considerably higher average levels of blood lipid profile parameters (total cholesterol, triglycerides, and low-density lipoprotein cholesterol) [Anandhi et al., 2014], a finding that was also documented in our investigation.

Leucine, isoleucine, and valine are considered branched-chain essential amino acids. Previous studies showed that a diet with amino acids deficiency used for a period of 7 days quickly reduces abdominal fat mass, in mice [Xiao et al., 2016]. Other scientific evidences have reported a relationship between levels of branched- chain essential amino acids and obesity [Kitsy et al., 2014]. Several studies proved that leucine is involved in the systemic cholesterol metabolism. For instance, examined the effect of leucine supplementation on the progression of atherosclerosis in mice. For 8 weeks, the mice were fed a diet enriched with leucine (1.5% w/v), in drinking water. The results of leucine supplementation were a 57.6% reduction in aortic atherosclerotic lesions in mice, a 41.2% decrease in serum levels of LDL cholesterol, and an increase by 40.2% in serum levels of HDL-cholesterol [Zhao et al., 2016]. Total cholesterol levels were found to be considerably lower in rats fed a diet supplemented with leucine, according to our findings. Similarly, the study conducted by our research team found that the group receiving valine had a lower total cholesterol level.

In previous investigations, Cojocaru et al. found that combining amino acids like valine and leucine in diets can result in the regression of atherosclerosis [Cojocaru et al., 2012]. In 2013, the research team of Miasoedov et al. conducted an experimental study in which they administered essential amino acids leucine, glycine, and proline to experimental animals and showed that this diet may reduce the risk of vascular blood clots, and thus may prevent the occurrence of atherosclerotic plaques in the arterial wall [Miasoedov et al., 2013].

The fact that atherosclerosis plaques in mice have similar characteristics to human atherosclerotic plaques [Matoba et al., 2013] makes it easier to apply the findings to humans.

Whereas the significance of hypercholesterolemia in the atherosclerotic process is well understood, there are only a few studies on the role of hypercholesterolemia in the absence of atherosclerotic lesions in the literature. In 2011, Garjani et al. 2011 investigated the effects of hyperlipidemia generated by a high-cholesterol diet on the rat aorta isolated in the absence of an atherosclerotic lesion. According to the findings, a high-cholesterol diet significantly increased total cholesterol and LDL-cholesterol levels in the blood ($p<0.001$). Despite the absence of aortic atherosclerotic plaques, the study indicated that elevated cholesterol levels were linked to endothelial dysfunction [Garjani et al. 2011].

The scenario is similar to that reported in our study, in which atherosclerotic plaques did not occur because the investigation was only 60 days long, not long enough to cause significant injuries. We did, however, discover biochemical alterations in lipid metabolism and morphological alterations that indicated prelesional phases of atherosclerosis. Despite the fact

that LDL and HDL values are more specific targets in the management of hyper/dislipidemias, we did not analyse these markers, which may be a limitation of our current study.

The biochemical research showed that the nonpolar type of essential amino acids such as valine, leucine, and isoleucine have a direct influence on lowering total cholesterol plasma levels by assessing different sets of lipid metabolism parameters according to the study design. As a result, the endothelium is protected, lowering the risk of endothelial dysfunction. In terms of biochemical parameter improvement, valine generates a faster reaction than leucine and isoleucine, however there are no significant differences between the three amino acids in terms of their protective abilities as shown by histopathological lesions assessment.

Our research found that the onset of early changes at the level of the vascular intima that involves the endothelium, the subendothelial tissue, and the tunica media (the occurrence of some superficial endothelial erosions, of a discrete, medium, or severe adhesion of erythrocytes and platelets) was linked with the degree of the arterial wall damage, of the leukocytes adhesion at the arterial intima level, and of the discontinuities of the internal elastic lamina. These microscopic aspects of endothelial dysfunction can be explained by oxidative theory of atherogenesis according to which the origin of this process is located in the endothelial cells. As a consequence, any type of aggression exerted on the endothelium has a major significance, contributing to the initiation of the lesion.

The excess of reactive oxygen species affects endothelial cells and subendothelial extracellular matrix which leads to adhesion of formed elements of blood. The oxidative theory of atherogenesis, according to which the beginning of this process is located in endothelial cells, can explain these microscopic characteristics of endothelial dysfunction. As a result, any form of injury directed towards the endothelium plays an important role in the genesis of the lesion. Endothelial cells and the subendothelial extracellular matrix are affected by an excess of reactive oxygen species, resulting in the adherence of blood components [De Brito et al., 2016].

I.2.2.6. Conclusions

Our findings support the view that essential amino acids valine, leucine, and isoleucine cause a considerable reduction in biochemical alterations caused by a high cholesterol diet. By comparing the vascular fragments collected from the experimental animals, the histopathological examination demonstrates that the essential amino acids have a beneficial effect on preserving the vascular wall architecture. The experimental study provides substantial evidence for the protective effects of a diet supplemented with amino acids, paving the way for the development of new products that could be used to prevent atherosclerosis.

I.2.3. Valine and Leucine's Hypolipemiant Actions in an experimentally induced model of atherosclerosis

I.2.3.1. Introduction

Emerging epidemiological evidence suggests that hypertriglyceridemia is a common risk factor for cardiovascular disease and plays a key role in atherosclerosis development. As a result, current ideas on how dyslipidemia is handled should be updated to include triglyceride normalization [Goldberg, 2018].

There is still a risk of atherosclerosis once serum LDL levels are reduced, either through medication or a hygienic-dietetic regimen [Peng et al., 2017]. This is owing to lipoproteins that are known to carry triglycerides. The reduction of apoB (apolipoprotein B) appears to be the mechanism by which lowering plasma triglycerides reduces total cardiovascular risk [Ference et al., 2019]. Another evidence of triglycerides' involvement in the atherosclerotic process is that they increase the synthesis of inflammatory cytokines, fibrinogen, and coagulation factors, influencing fibrinolysis [Tenenbaum et al., 2014]. Although the role of triglycerides in atherosclerosis and increasing total cardiovascular risk is clear, more research is needed to determine whether elevated serum triglycerides cause ischemic heart disease per se or in conjunction with other known cardio-vascular risk factors such as diabetes mellitus or obesity [Simha, 2020; Packard, Boren, Taskinen, 2020].

I.2.3.2. Aim

Our previous research analysed the anti-atherogenic potential of valine and leucine, two non-polar amino acids, in the context of hypercholesterolemia induced by a cholesterol-rich diet, and we followed their effects on lipid metabolism (total cholesterol, HDL (high-density lipoprotein)-cholesterol, LDL-cholesterol) and oxidative stress parameters [Cojocaru et al., 2020; Cojocaru et al., 2012a; Cojocaru et al., 2012b; Cojocaru et al., 2014]. The present study focuses on the results regarding the study of triglycerides levels in the same context, agreeing that hypertriglyceridemia could become an important therapeutic target in the management of atherosclerosis.

I.2.3.3. Material and methods

Study design

Over a period of 60 days, an experimental investigation on male Wistar rats weighing 250-280g was conducted. All animal protocols were carried out in strict conformity with the Guide's recommendations for animal care and scientific use, as well as international ethical norms [NACLAR, 2004; AVMA, 2013].

The animals were divided into the following 4 groups: control group I (n=8): fed a regular diet composed of agricultural by-products; group II– C (n=8): received regular diet supplemented with 0.4g/kg/day cholesterol; group III –C+V (n=8): received regular diet supplemented with 0.4g/kg/day cholesterol and 62.5mg/kg/day valine powder for animal nutrition; group IV–C+L (n=8): received regular diet supplemented with 0.4g/kg/day cholesterol and 69.985mg/kg/day leucine powder for animal nutrition.

We collected blood samples from the retro-orbital plexus, under anaesthesia of animals with 75 mg/kg of intraperitoneal ketamine, in three moments of the experiment as follows: R0 – 1st day, R1 – 30th day R2 – 60th day. As in prior research], we used a Diagnosticum Zrt kit acquired in Budapest, Hungary to assess triglyceride levels [Fossati, Prencipe, 1982].

Statistical analysis

All information was centralized in EXCEL and SPSS databases and processed with the statistical functions that they are suitable for. Statistical confidence intervals with a 95%

significance level were used. We utilized the ANOVA test to determine the statistical significance of our groupings (including repeated measures ANOVA). The statistical significance threshold is the highest level of probability that allows for an error. In practice, a significance level of 0.05 percent implies enough precision, while 95 percent probability indicates reliability.

I.2.3.4. Results

Triglycerides were measured in R0, R1, and R2 for each group. The levels of triglycerides throughout the experiment, as well as the variation of the observed values series, are shown in Table XIII and Figure 14. The variance in the control group was mild, ranging from 3.27 to 3.77 CV (coefficient of variation) %, with R2 results being the most homogenous (3.27 CV%).

The coefficients of variation in groups II, III, and IV ranged from 2.45 to 14.82 CV%. The most consistent findings were found in R2 in group II – C (8.04 CV%) and group IV – C+L (4.36 CV%), and in R1 in group III – C+V (2.45 CV%). Individual triglyceride levels were highest in group II, which corresponded to rats who solely received cholesterol (Figure 14).

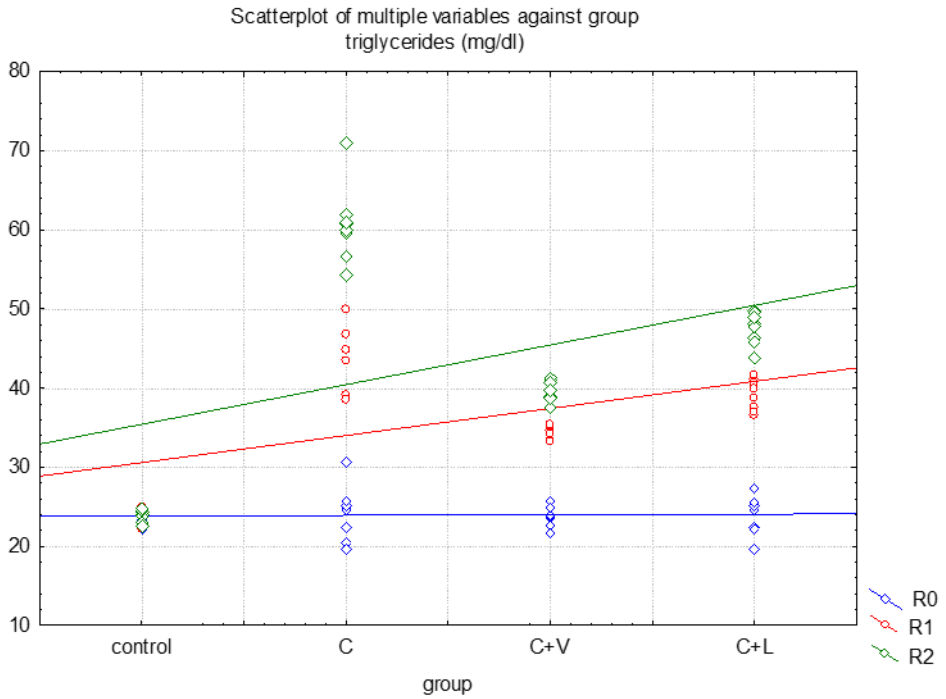


Figure 14. *Individual levels of triglycerides in all groups.*

The null hypothesis that there is no change in triglyceride values in distinct subgroups evaluated in R0, R1 and R2 (N=32) was tested using a one-way repeated measure analysis of variance (ANOVA) (Table XIV).

The results of the ANOVA indicated a significant time effect, Wilks' Lambda = 0.015, $F(2, 27) = 879.33$, $p < 0.01$, $\eta 2 = 0.98$. As a result, there was enough evidence to rule out the null hypothesis.

Table XIII. Levels of triglycerides in all groups

Group	R0	R1	R2
Group I: control			
Average mg/dl	23.70	23.88	23.90
SD	0.89	0.90	0.78
CV%	3.76	3.77	3.27
Group II: C			
Average mg/dl	24.19	45.73	60.61
SD	3.44	6.78	4.87
CV%	14.22	14.82	8.04
Group III: C+V			
Average mg/dl	23.91	34.15	39.73
SD	1.31	0.84	1.30
CV%	5.48	2.45	3.27
Group IV: C+L			
Average mg/dl	24.04	39.15	47.56
SD	2.43	2.01	2.07
CV%	10.13	5.14	4.36

Table XIV. Multivariate tests using one-way repeated measure analysis of variance

Multivariate Tests[d]

Effect		Value	F	Hypothesis df	Error df	Sig.	Partial Eta Squared	Noncent. Parameter	Observed Power[b]
Time	Pillai's Trace	0.985	879.337[a]	2.000	27.000	0.000	0.985	1758.674	1.000
	Wilks' Lambda	0.015	879.337[a]	2.000	27.000	0.000	0.985	1758.674	1.000
	Hotelling's Trace	65.136	879.337[a]	2.000	27.000	0.000	0.985	1758.674	1.000
	Roy's Largest Root	65.136	879.337[a]	2.000	27.000	0.000	0.985	1758.674	1.000
Time * Type	Pillai's Trace	1.058	10.487	6.000	56.000	0.000	0.529	62.920	1.000
	Wilks' Lambda	0.028	44.831[a]	6.000	54.000	0.000	0.833	268.989	1.000
	Hotelling's Trace	31.694	137.340	6.000	52.000	0.000	0.941	824.037	1.000
	Roy's Largest Root	31.596	294.898[c]	3.000	28.000	0.000	0.969	884.694	1.000

[a] Exact statistic. [b] Computed using alpha = 0.05. [c] The statistic is an upper bound on F that yields a lower bound on the significance level.

[d] Design: Intercept + Type. Within Subjects Design: Time. * Statistical difference between time and type (groups).

Each pairwise difference was significant, according to follow-up comparisons, $p<0.01$. There was a significant increase of values over time (Table XV). There were no significant variations in average triglyceride levels between groups II, III, and IV, or between these and the control group, in R0 ($p<0.05$). Triglyceride levels were considerably higher in all groups in R1 and R2 when compared to the control group ($p<0.001$).

Table XV. Pairwise comparisons between the studied groups

Pairwise Comparisons						
(I) Time	**(J) Time**	**Mean Difference (I-J)**	**Std. Error**	**Sig.[a]**	**95% Confidence Interval for Difference[a]**	
					Lower Bound	**Upper Bound**
R0	R1	-11.766*	0.459	0.000	-12.934	-10.597
	R2	-18.986*	0.454	0.000	-20.143	-17.829
R1	R0	11.766*	0.459	0.000	10.597	12.934
	R2	-7.220*	0.321	0.000	-8.038	-6.401
R2	R0	18.986*	0.454	0.000	17.829	20.143
	R1	7.220*	0.321	0.000	6.401	8.038

Based on estimated marginal means. *. The mean difference is significant at the 0.05 level.
a Adjustment for multiple comparisons: Bonferroni.

In addition, in R1 and R2 measurements, the average triglycerides in group II receiving cholesterol only (C) were considerably greater than those in group III receiving valine (C+V) as well as in group IV receiving leucine (C+L) ($p<0.001$; $p<0.05$). At the end of the experiment (R2), the average triglycerides in group III were significantly lower than in the case of rats who received leucine ($p<0.001$) (Table XVI, Figure 15).

In previous studies, we evaluated the antiatherogenic potential of valine and leucine in the context of hypercholesterolemia induced by a cholesterol-rich diet, following their effects on lipid metabolism (total cholesterol, HDL -cholesterol, LDL-cholesterol) and oxidative stress parameters. The mean values can be seen in Table XVII [Cojocaru et al., 2020; Cojocaru et al., 2012a; Cojocaru et al., 2012b; Cojocaru et al., 2014].

Table XVI. Statistical differences between the average levels of triglycerides in all groups.

Time	**Group**	**Control Group (n=8)**	**C (n=8)**	**C+V (n=8)**
R0	C (n=8)	p = 0.737	-	
	C+V (n=8)	p = 0.884	p = 0.849	-
	C+L (n=8)	p =0.814	p = 0.919	p = 0.928
R1	C (n=8)	p < 0.001*	-	
	C+V (n=8)	p < 0.001*	p < 0.001*	-
	C+L (n=8)	p < 0.002*	p = 0.001*	p =0.0422*
R2	C (n=8)	p < 0.001*	-	
	C+V (n=8)	p < 0.001*	p < 0.001*	-
	C+L (n=8)	p < 0.001*	p < 0.001*	p < 0.001*

Post-hoc analysis: Newman-Keuls test; (*) Marked differences are significant at p < 0.05

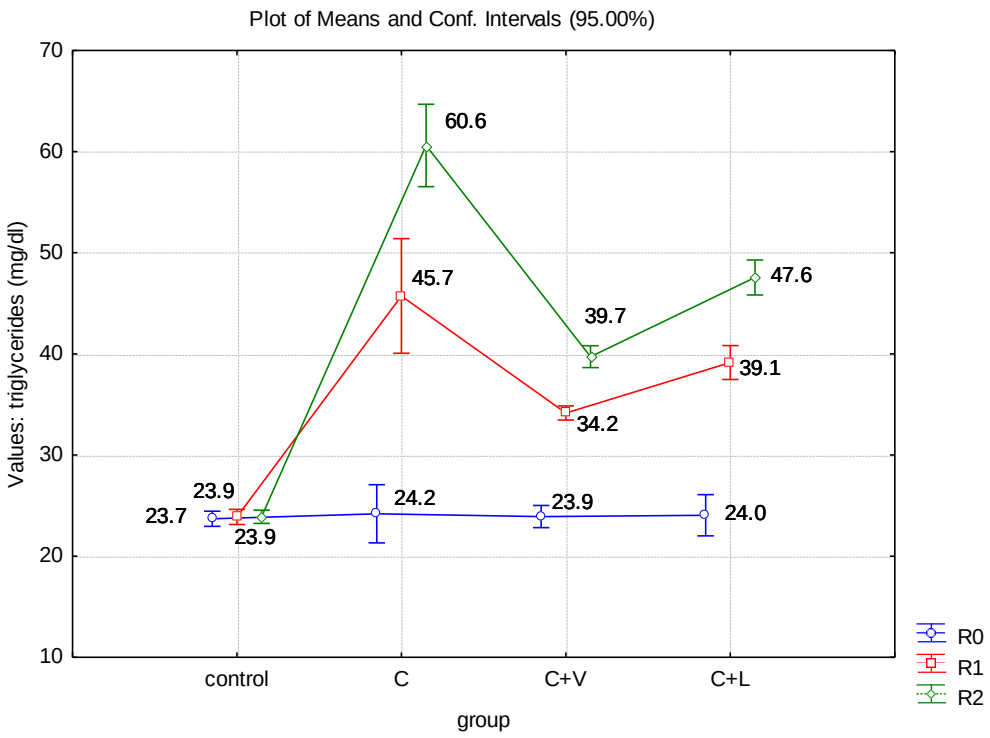


Figure 15. *Average levels of triglycerides in all groups.*

According to our findings, valine and leucine increased serum HDL- cholesterol levels. After one month and at the end of the study, HDL-cholesterol levels in animals given only cholesterol (C) were considerably lower than those in animals given cholesterol and valine (C + V) or cholesterol and leucine (C + L) ($p < 0.001$) [Cojocaru et al., 2012a]. We also found that valine and leucine reduced LDL-cholesterol levels in the blood, indicating that they have lipid-lowering actions [Cojocaru et al., 2012b].

In our study, we also investigated at glucose levels and discovered that when cholesterol is added to the diet, the glycemic levels growth significantly, but when amino acids (valine and leucine) are added, the glycemic levels reduce. As a result, the two amino acids demonstrated antioxidant properties, which may help to reduce endothelium damage associated with atherosclerosis [Cojocaru et al., 2014].

The formation of atheroma plaque in the vessel's intima in rats fed a hypercholesterolemic diet takes about 8 months, according to the literature. We were unable to obtain atherosclerotic plaques in our investigation since the duration of the study was just 60 days, which was insufficient to produce significant lesions. However, we discovered changes in lipid metabolism and oxidative stress indicators that define atherosclerosis in the prelesional phases.

Furthermore, according to our findings, adding valine and leucine to this diet has a direct impact on lipid metabolism parameters by reducing triglycerides, total cholesterol, and LDL-cholesterol levels. When comparing the two necessary amino acids, we discovered that valine reacts more efficiently than leucine. As a result, we assume a possible hypolipemiant action and a consequently anti-aterogenic action of the two compounds.

Table XVII. Mean values of HDL, LDL and glucose at R0, R1 and R2

	Group	R0	R1	R2	p–value
Cholesterol I mean ± SD	Control group	37.14±2.56	37.50±2.09	37.61±1.45	0.0783
	C (n=8)	36.41±4.15	49.89±3.99	76.61±3.46	<0.001*
	C+V (n=8)	36.67±1.28	41.12±1.27	44.87±1.22	0.001*
	C+L (n=8)	36.50±2.70	46.04±2.71	49.53±2.12	<0.001*
	p–value	**0.577**	**0.006***	**<0.001***	
HDL mean ± SD	Control group	23±1.48	22.88±1.22	22.89±1.68	0.911
	C (n=8)	22.43±3.29	19.44±1.45	15.93±1.20	0.004
	C+V (n=8)	22.98±1.48	24.64±2.79	26.85±2.95	0.114
	C+L (n=8)	22.51±2.15	22.97±1.90	23.17±1.81	0.523
	p–value	**0.637**	**0.001***	**<0.001***	
LDL mean ± SD	Control group	9.39±3.39	9.83±2.52	9.93±2.71	0.749
	C (n=8)	7.73±4.54	21.3±3.64	47.94±5.47	<0.001*
	C+V (n=8)	8.9±2.01	9.64±2.79	10.07±2.75	0.486
	C+L (n=8)	9.17±3.43	15.23±2.73	16.84±2.28	0.0004*
	p–value	**0.319**	**0.001***	**<0.001***	
Glucose mean ± SD	Control group	122.75±5.87	122.61±5.84	122.77±6.08	0.962
	C (n=8)	122.78±8.77	149.26±7.73	162.82±5.83	<0.001*
	C+V (n=8)	121.98±4.71	140.93±4.84	142.37±4.70	<0.001*
	C+L (n=8)	121.93±5.13	143.79±6.32	148.08±4.78	<0.001*
	p-value	**0.994**	**<0.001***	**<0.001***	

Post-hoc analysis: Newman-Keuls test; (*) Marked differences are significant at $p < 0.05$.
SD –standard deviation

I.2.3.5. Discussion

The study of the antiatherogenic potential of various compound is understandable as atherosclerotic cardiovascular disease represents nowadays a leading cause of death worldwide [Laslett et al., 2012; De Backer et al., 2018]. Triglycerides are found in chylomicrons, which contain the most lipids absorbed in the intestines, as well as very-low-density lipoproteins (VLDL), which contain triglycerides generated in the liver. Therefore, any increase of these two causes an increase in serum triglycerides. The result is hypertriglyceridemia as a clinical syndrome with a heterogeneous combination of symptoms, each of which contributes to a degree in the increase of total cardiovascular risk [Navar, 2019].

Based on the physiopathological principles of atherosclerosis, we wanted to investigate the antiatherogenic potential of two amino acids (valine and leucine) in settings of induced hypercholesterolemia via a cholesterol-rich diet. We looked at a number of animal models

addressed in the literature [Leong, Ng, Jaarin, 2015] and decided on Anitschkow's 1913 atherosclerosis experimental model [Anitschkow, Chalatow, 1913].

It is known that amino acids are required for the synthesis of a wide range of chemicals, all of which play an important part in maintaining homeostasis [Harris, Joshi, Jeoung, 2004; Wu, 2009]. The branched-chain essential amino acids such as leucine, valine, and isoleucine are involved in protein synthesis and cell division cycle regulation. Leucine, in particular, participates in growth and development of cells in a mTOR-dependent manner [Chotechuang et al., 2009].

Several hypotheses have been proposed in recent years regarding the involvement of essential amino acids in atherosclerotic pathogenesis modulation. Unfortunately, because research has so far produced inconsistent results, the precise impact and timing of these amino acid interventions are not entirely understood [Rom, Aviram, 2017]. At the moment, scientists are debating whether branched-chain amino acids have a potential proatherogenic effect as well as a potential antiatherogenic role [Grajeda-Iglesias, Aviram, 2018].

In a previous study we evaluated the role of valine, leucine and isoleucine on the onset and progression of atherosclerosis in rats fad a hypercholesterolic diet. The study of the three essential amino acids revealed that valine induced a faster response than leucine and isoleucine on the improvement of biochemical parameters, but no significant differences between the three amino acids in terms of their protective ability, according to the histopathological lesion assessment [Ifrim et al., 2018]. However, more research is needed to determine the particular biochemical pathway by which these amino acids modify triglyceride levels.

In contrast to our findings, Bhattacharya et al. (2013) proposed that branched-chain amino acids are responsible for an increase in cardiovascular-related mortality, and that they are linked to severe forms of ischemic heart disease. They were able to show that the link exists even when established risk factors like diabetes mellitus or insulin resistance have been eliminated [Bhattacharya et al., 2014].

Ruiz-Canela et al. postulated that elevated serum levels of branched-chain amino acids such as valine, leucine, and isoleucine are associated with increased global cardiovascular risk, which may not be affected by dieting [Ruiz-Canela et al., 2016]. Furthermore, Sun et al. established that cardiac depression symptoms are caused by a deficiency in branched-chain amino acid catabolism mediated by Kruppel-like factor 15 (KLF 15) in a mouse model [Sun et al., 2016].

Tobias et al. concluded that the causal association between branched-chain amino acids and cardiovascular disease is similar to the causal relationship between plasma levels of LDL cholesterol and cardiac mortality based on the findings of a prospective cohort study that lasted 18.6 years [Tobias et al., 2018].

Numerous published research, on the other hand, indicate branched-chain amino acids' positive benefits in regulating lipid metabolism and functional cardiac parameters. Comparable to our findings, Noguchi et al. emphasized the importance of valine and leucine in reducing the impact of aberrant lipid concentrations, which are directly involved in the development of atherosclerotic lesions. [Noguchi et al., 2006].

In the study of Terakura et al., the authors found that adding branched-chain amino acids to the diet reduces hepatic triglyceride uptake and the chronic inflammatory process linked to obesity, most likely by inhibiting the expression of interleukin-6 (IL-6), tumor necrosis factor-

alpha (TNF-alpha), and monocyte chemoattractant protein-1 (MCP-1). In addition, mice fed branched-chain amino acids had lower average adiposity, which was possibly mediated by PPAR-gamma (peroxisome proliferator-activated receptor gamma) [Terakura et al., 2012].

Chen *et al*. published their results on leucine's impact on body weight and blood lipids in 2012. Regardless of how the amino acid was delivered (orally or intracerebroventricularly), they observed a modulation of glucose and lipid metabolism [Chen et al., 2012].

In 2014, Pedroso et al. have shown that in rats with metabolic syndrome, restricting their caloric intake concurrent with adding leucine to their diet facilitates an improved protein anabolism, as well as an increase in the levels of leptin and IL-6. The study drew attention to the fact that supplementing the diet with branched-chain amino acids modifies the hepatic metabolism by influencing the metabolism of fatty acids and cholesterol [Pedroso et al., 2014].

The role of leucine in lowering the levels of triglycerides and LDL and in raising HDL-cholesterol in diabetic rats to which food was supplemented with this amino acid was highlighted in a study of Sadri *et al*. in 2017 [Sadri et al., 2017]. In the literature, a potential increase in leptin levels as a result of a leucine-rich diet has been studied. Yet, the observed leptin levels were not statistically significant [Lynch et al., 2006].

As a result, research findings are incongruent, and various viewpoints are expressed. Some studies link branched-chain amino acids to an increase in worldwide cardiovascular risk, while others point to the benefits of adding leucine, valine, and isoleucine to the diet in order to improve lipid metabolism parameters. Our findings suggest that leucine and valine reduce plasma triglycerides, improving lipid balance and, as a result, vascular wall integrity.

I.2.3.6. Conclusions

Our biochemical research demonstrated that essential amino acids such as valine and leucine lower the amount of triglyceride by comparing the triglyceride values across different groups according to the design of the experiment. As a result, the endothelium of the blood vessels is protected, and the danger of endothelial dysfunction is reduced.

When the two necessary amino acids were compared, it was shown that valine acts more quickly than leucine. The findings of this study back up the theory that valine and leucine play separate and independent roles in the progression of induced atherosclerosis. The way these two amino acids interact opens up plenty of new research possibilities in terms of therapeutic goals, and our study is an attempt to highlight one of them.

I.2.4. The evolutive dynamic of aortic wall parameters in newborn and child

I.2.4.1. Introduction

The management of aortic disturbances such as such as atherosclerosis, aneurysms and dissections, requires an accurate understanding of its structure as it relates to age. The biomechanical properties and vascular morphology of the aortic walls define an exceptionally large and extensible artery, with a special dynamic in terms of compliance. As a result, the aortic wall maintains a high systemic blood pressure due to its near proximity to the heart and to its large diameter [Sherifova, Holzapfel, 2019].

Three layers, or tunics, make up the aortic wall. A flat endothelium is supported by a subendothelial layer in the tunica intima, the innermost layer surrounding the aortic lumen

(which ensures a variable degree of local mobility). The tunica media, which is responsible for the aortic wall's distensibility and is also the most representative component in every large artery, is separated from the tunica intima by an internal elastic lamina. Tunica media typically consists of a variable number of concentric fenestrated lamellae of elastic fibers, as well as dispersed collagen fibers and smooth muscle cells. The elastic structure of the middle aortic layer is necessary for its expansion during continuous blood flow [Cocciolone et al., 2018]. The adventitial layer connects the aorta to the neighbouring connective tissue, being separated from the tunica media by the external elastic lamina [Kim et al., 2017; Kau et al., 2007; Sherifova, Holzapfel, 2019].

The aortic wall faces a new circulatory status after birth, and it must adapt at this situation. The connective tissue elements together with vascular smooth muscle cells (VSMC) from the medium layer must define an appropriate aortic architecture [Wagenseil, Mecham, 2009]. New studies reveal that VSMC can differentiate into the tunica adventitia of stressed aorta, as well as the fact that they have a highly reversible potential. The VSMC phenotypic switching which is now widely used, refers to the ability of VSMC to modify their phenotype in response to a variety of circumstances without losing their secretory function, making them into an ideal candidate for therapeutic intervention [Majesky et al., 2011; Chen et al., 2020].

Collagen fibers, which are commonly arranged in dispersed bundles between medial elastic lamellae, have no effect on the function of elastic fibers, unless they're overextended, in which case collagens prevent vascular rupture. When collagen synthesis is disrupted, aortic integrity and tensile strength suffer significantly [Wolinsky, Glagov, 1964; Cheng, Wagenseil, 2012]. Each vascular component, whether directly or indirectly involved in aortic distensibility, has its place in the aortic edifice [Hungerford et al., 1996; Huang et al., 2001; Tsamis et al., 2013].

Aorta pathology is quite diverse and varies greatly by age, opening a wide debate field in which the most important problems originate from the still unknown parts of aortic structural features. These constituents are extremely responsive to vascular mechanical characteristics and aortic injury, according to convincing evidence. Analytical examination of aortic wall components in normal status and disease at various stages of life can help to better understand the aorta wall's evolutive dynamics [Huang et al., 2006; Kassab, 2006; Zhu et al., 2018].

I.2.4.2. Aim

The purpose of the study was to reveal that the development of elastic lamellae should be regarded not only as an indispensable step for the aortic wall configuration, but also like a process in a firm connection with the rest of the aortic wall components. In order to maintain a continuous pulsatile blood flow, the transition from intrauterine life to a new life stage, childhood, necessitates an appropriate adaptation of nearly all components of the aorta wall.

I.2.4.3. Material and methods

Study design

The morphometric analysis was performed on samples of thoracic aorta prelevated from 12 neonates and 12 children (who did not die of any cardiovascular disease). The children

ranged in age from two to twelve years. The study was approved by the Ethics Committee of "Sfânta Maria" Emergency Children Hospital, Iassy, Romania.

Histological assessment

Thoracic aorta fragments were specifically processed by paraffin embedding technique and stained with trichromic Szekely and Verhoeff's iron Hematoxylin. The histological examination was done with a Nikon Eclipse 50i microscope and digital images were obtained with DS Camera Control Unit DS – L2. Using PRODIT 5.2, an interactive digital software, we were able to perform a morphometric assay of the structural elements of the aortic wall. The validity of these procedures was reliant on an initial standardization procedure, which was carried out to eliminate any interference caused by particular tissue processing or technical variability.

Stereological analysis

To quantify percentage volumes of the significant aortic components, a standard grill with Weibel parallels was utilized; a combined test-line and test-point in a standard surface will allow the assessment of the percentage volume of the component structures. The exam was realized at 40×, on a test surface corresponding to 540 sampling points on superimposed Weibel standard grill, the distance between two points totalling 15.07 μm.

The sequence of stereological events is formed by the following stages: identification of investigated structure (collagen fibers, elastic fibers, muscular cells, interstitium); overlapping of Weibel grill on the microscopic acquisition image; determination of total point number which has to be count; adjustment of lines position in their random intercrossing of randomized structures; automatically assay of stereological account; statistical assessment of investigated parameters.

Statistical analysis

The application calculates the statistics for all of the measured fields in each group automatically. All comparisons between the two explored groups were realized with $\chi 2$-test and Student's t-test (for differences between investigated groups). Differences were considered statistically significant if $p<0.05$.

I.2.4.4. Results

The aortic samples prelevated from newborns (group 1) and from children (group 2) were analysed and the quantification of volume percentage of connective tissue, muscular cells and interstitium was done on trichromic Szekely stain (Figures 16 and 17). We analysed the tunica media from each aortic sample for a better understanding of the structures under investigation.

After morphometric assessment, the stereological assay for newborn medial aortic structures revealed the following specific percentage volumetric values: connective tissue 79.07%, smooth muscle cells 1.11%, interstitium 19.81% (Table XVIII).

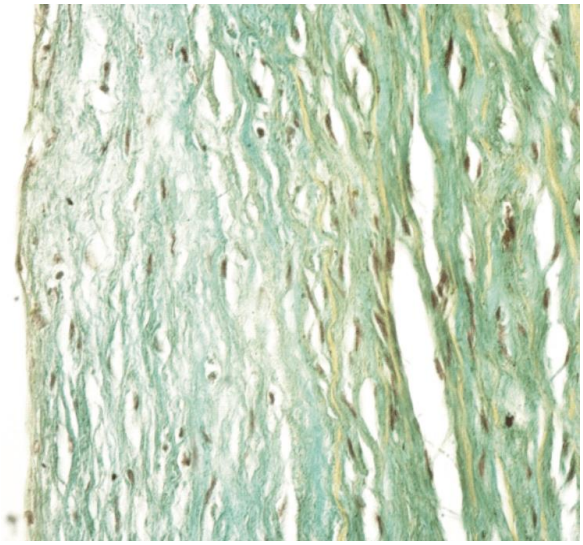


Figure 16. ***Group 1: Aortic medial layer, trichromic Szekely stain (ob. ×40).***

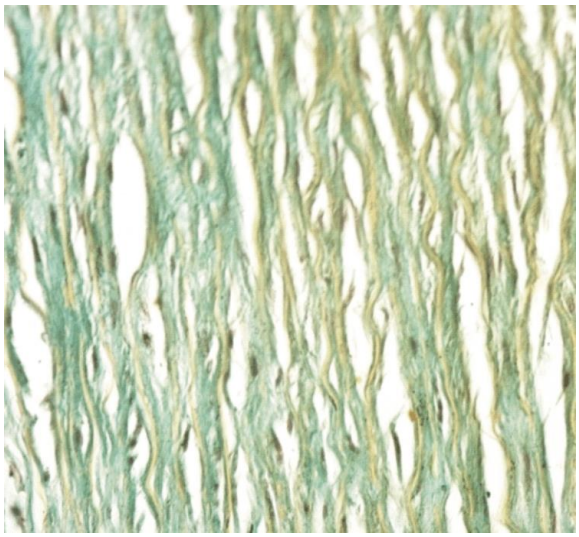


Figure 17. ***Group 2: Aortic medial layer, trichromicSzekely stain (ob. ×40).***

After morphological evaluation, the stereological assay for children's medial aortic structures revealed the exact % volumetric values: connective tissue 67.59%, smooth muscle cells 16.11%, interstitium 16.3% (Table XVIII).

Table XVIII. Quantification of aortic parameters

Quantified aortic parameters [%]			
Group	***Connective tissue***	***Muscle cells***	***Interstitium***
1	79.07	1.11	19.81
2	67.59	16.11	16.3

The distribution of aortic media components revealed that infants and children have distinctive features (Figures 18 and 19). To highlight the variations between the analyzed groups, the dynamic changes in the aortic structure identified throughout this investigation are summarized in figure 20.

On Verhoeff's stained aortic samples, the collagen and elastic fibers, as well as other structures dispersed in the aorta media, were stereologically studied (Figures 21 and 22). The stereological assay for newborn aortic fibrillar content indicated specific percentage volumetric values: collagen fibers 16.26%, elastic fibers 39.97%, interstitium 16.18% and other structures 27.6% (Table XIX).

The stereological assay for child aortic fibrillar content indicated the specific percentage volumetric values: collagen fibers 25%, elastic fibers 34.26%, interstitium 14.63% and other structures 26.11% (Table XIX).

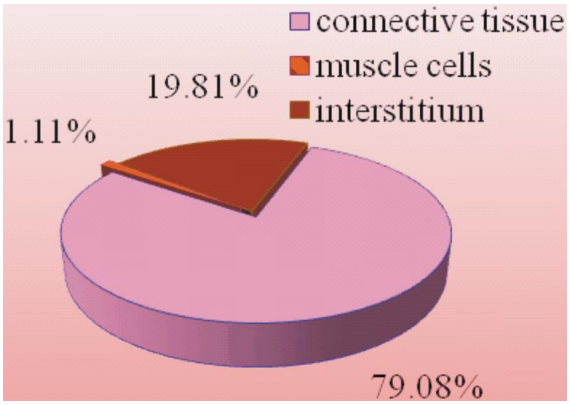


Figure 18. *Group 1: Stereological estimation of structural components of the aortic media.*

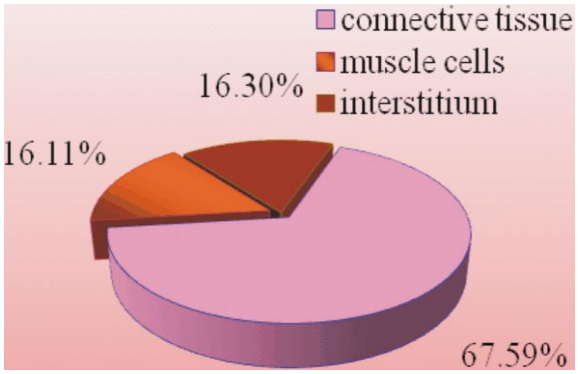


Figure 19. *Group 2: Stereological estimation of structural components of the aortic media.*

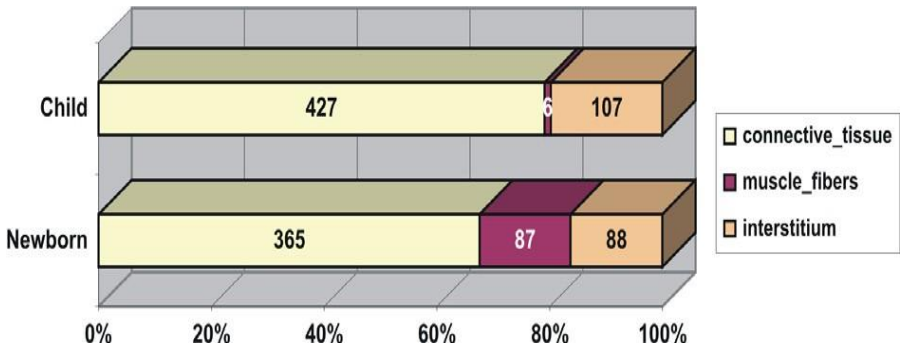


Figure 20. ***Quantification of percentage volumes of main aortic structures.***

Figure 21. *Group 1: Aortic medial layer, Verhoeff'sstain (ob.×40)*

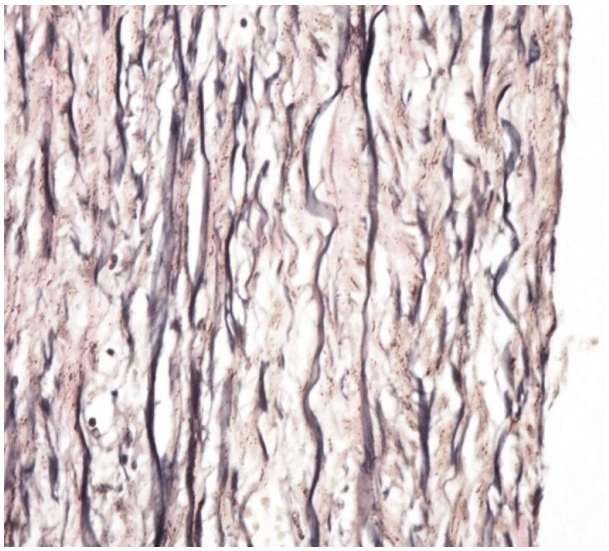


Figure 22. *Group 2: Aortic medial layer, Verhoeff's stain (ob×40)*

Table XIX. Quantification of aortic parietal elements

	Quantified structural parietal elements [%]			
Group	***Collagen fibers***	***Elastic fibers***	***Interstitium***	***Other structures***
1	16.26	39.97	16.18	27.6
2	25	34.26	14.63	26.11

The differences between the two groups were revealed by the median distribution of aortic fibrillar content (Figures 23 and 24). The evolution of aortic fibrillar medial content was summarized in order to allow a correct evaluation for the two investigated groups (Figure 25).

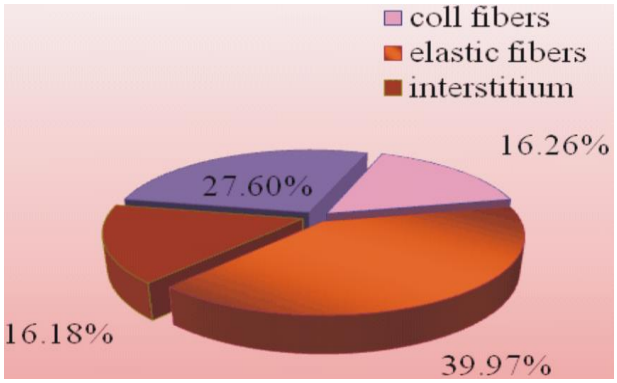


Figure 23. *Group 1: Stereological estimation of the aorticfibers*

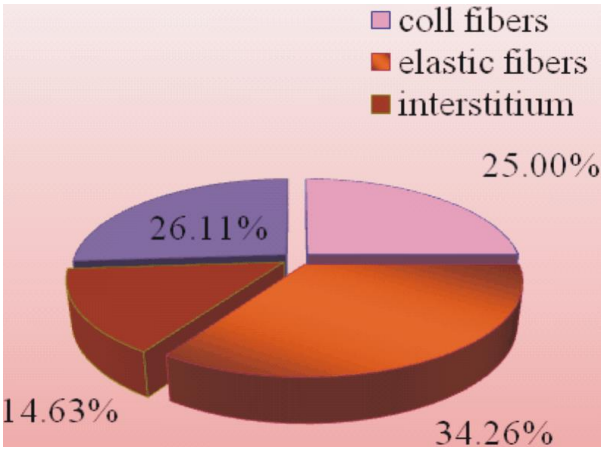


Figure 24. *Group 2: Stereological estimation of the aorticfibers*

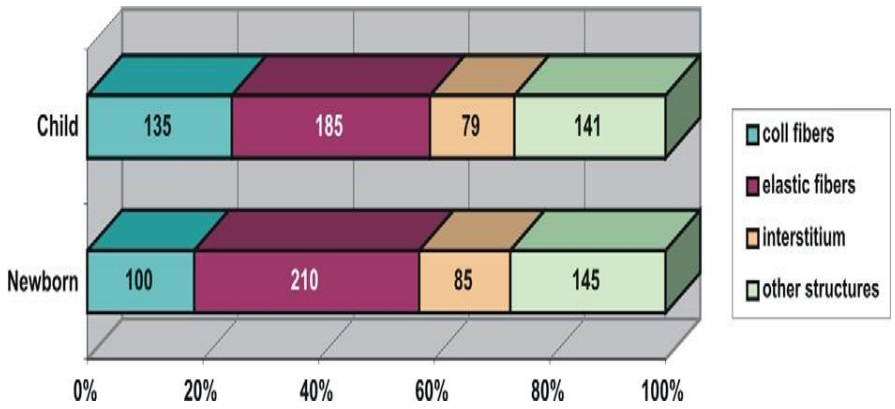


Figure 25. *Quantification of percentage volumes ofmain aortic structures*

I.2.4.5. Discussion

The aorta hemodynamic mechanisms differ significantly depending on the time of birth, infancy, and youth, with substantial implications for the structure and diameter of the arterial wall as previously shown [Wolinsky, Glagov, 1969; Machii, Becker, 1997]. The aorta wall's ability to adapt to progressive variations in blood pressure has been connected to the development of its structural components under normal circumstances. As the medial layer of the aorta is thought to be the determining factor in controlling the functions of the human body's largest elastic artery, the constituents that compose this tunic are thought to be responsible for

the vessel's overall integrity as well as its responsiveness to tensional pressures [Van Meurs-Van Woezik, Krediet, 1982; Cocciolone et al., 2018].

Medial thickness rises with age, from birth to adulthood. Disturbances in aortic responsiveness to various vascular circumstances, particularly those caused by longitudinal stretch, will be reflected in any change in aortic wall composition. The major component that guarantees aortic stability is made of elastin, VSMC, and collagen fibers [Shadwick, 1999; Pratt, Curci, 2010; van den Munckhof et al., 2018].

Elastin has to be regarded as the essential component of the aortic wall, responsible for its large viscoelasticity [Cheng, Wagenseil, 2012; Tsamis et al., 2013]. The lamellar unit is still the most important structural and functional component of the aortic wall. The presence of elastin pores and their specific distribution provide for a better understanding of elastic design [O'Connell et al., 2008]. Elastin and collagen, on the other hand, are key components in maintaining aortic microarchitecture, as the two interrelated types of fibers have a similar manner of assembling in the tunica media [McEniery et al., 2005; Cocciolone et al., 2018].

Individual or bundles of collagen fibers are placed between elastic lamellae, along with VSMC. The most appropriate perception of the proper distribution of all these elements in the aortic media, referring to their prevailing orientation, close relationship, and interplaying relations [Orlandi et al., 1994; Halloran et al., 1995; Feng et al., 1999] is still being debated. Because the entire extracellular matrix is dependent on the secretory profile of VSMC, it remains a hotly debated topic. VSMC ensures the synthesis and degradation of this matrix, as well as the essential stability and force of the vascular wall; there is growing evidence that VSMC are mechanically dependent, age-related, and that their secretion may interact with gender determinants [Jackson et al., 2002; Chen et al., 2020; Chakraborty et al., 2021].

The purpose of this study was to use a quantitative approach to demonstrate how structural features of the aorta change from newborn to child. There are two independent stages of life, and it appears to be important to determine which of the constitutive aortic components has the major implication in defining vascular unique design. We have to take into account the fact that various hemodynamic circumstances are linked to appropriate aortic remodelling.

With this regard, we had to consider that various hemodynamic circumstances are intricately linked to appropriate aortic structural remodelling. We further believe that VSMC, through their distinct secretory pattern of elastic and collagen fibers, are responsible for the changing age-related shape of the aorta. The findings support the notion that VSMC dynamic engagement is critical for each stage of development, as previously discussed [Fujiwara, Uehara, 1992; Karnik et al., 2003; Chen et al., 2020].

Connective tissue predominates in both groups studied, however it is more abundant in newborns, according to stereological assessment. We can assume that the reduced hemodynamic demand during the prenatal stage is solely responsible for this result. In addition, the volume percentage of VSMC appears as a significant factor in order to demonstrate a reduced implication in direct synthesis of elastic and collagen fibers immediately after birth, while in children, the volume percentage of VSMC becomes more prominent. In both studied groups, the interstitium structure remained comparable.

The analysis of fibers from the aorta medial layer shows that elastic fibers have a decreased degree of variability in both groups; as vascular adaptation requires an increase in elastic fibers percentage volume, a noticeable increase in aortic samples from children is

expected. The result can be attributed to the wide age dispersion for the second group. Regarding the collagen fibers, they are moderately increased in second group.

I.2.4.6. Conclusions

The findings of the present research highlight the importance of gaining a better understanding of age-related aortic morphology, which has a direct and significant impact on vascular pathology. Collagen and elastic fibers as the main components of the aorta wall, have their own sequential pattern of development, and together with VSMC, have a complex interaction which results in maintaining aortic architecture.

I.2.5. An immunohistochemical study of atherosclerotic plaque macrophages

I.2.5.1. Introduction

Previous studies showed that endothelial dysfunction is an early sign of atherosclerosis, which leads to increased vascular permeability, leukocyte and platelet adhesion, vascular smooth muscle cell proliferation, and eventually a vasoconstrictor and pro-inflammatory state. This endothelial status can be thought of as the sum of a person's risk factors, which can be associated with a variety of disorders that raise cardio-vascular risk, such as coronary disease, hypertension, diabetes mellitus, and dyslipidemia., etc [Barquera et al., 2015; Boudoulas et al., 2016; Geovanini, Libby, 2018].

Changes in the state of leukocytes in the arterial wall are related with a substantially vascular wall inflammation. The most prevalent type of vascular inflammation is generated by the influx of monocytes early in the course of atherosclerosis [Ley, Miller, Hedrick, 2011] followed by the recruitment of other macrophages in response to inflammatory signals [Murray, Wynn, 2011; Chawla, Nguyen, Goh, 2011; Aziz, Yadav, 2016].

Endothelial damage results in peroxide degradation of the intima's lipids. The release of proinflammatory mediators and growth factors, and an increased proliferation of smooth muscle cells, with subsequent collagen production, are the steps that follow endothelial injury [Kumar, Abbas, Aster, 2018; Geovanini, Libby, 2018].

I.2.5.2. Aim

CD68 antibodies are used in current practice as markers for monocytes/macrophages present in normal or pathological tissue. The purpose of this research was to study the immunohistochemical expression of the anti-CD68 antibody at the level of the atherosclerotic plaque in order to assess the inflammatory reaction at this level.

I.2.5.3. Material and methods

Study design

Our retrospective study included 213 patients, aged between 33 and 78 years, hospitalized in the Cardiovascular Surgery Department of the Institute of Cardiovascular Diseases “Prof. Dr. George IM Georgescu”, during 2005–2009. According to the severity of

the symptoms and degree of lumen stenosis, endarterectomies with the subsequent removal of plaques stenosis were done.

The tissue samples were processed by the classic histopathological technique of paraffin embedding, microtome sectioned in 5 μm thick slices and stained with the classical Hematoxylin and Eosin method. On representative blocks, immunohistochemical stains were performed for the study of various factors involved in the pathogenesis of atherosclerosis such as neoangiogenesis, smooth muscle cells proliferation or inflammatory reaction.

The present research refers to the immunohistochemical study of the inflammatory response of atherosclerotic plaque at different stages of development, using the anti-CD68 antibody, a marker strongly expressed by blood monocytes and tissue macrophages present in normal or pathological tissue. As primary antibody, we used a CD68 monoclonal antibody, clone KP1 AM416-5M. The immunohistochemical staining technique was performed using the Super Sensitive Detection System kit (Biogenex). Positive control for the CD68 was performed on slides prepared from stomach. Negative control was used to confirm that the tested positive reaction is the result of a specific antigen-antibody link. The primary antibody, clone KP1, required a Citra Plus antigen retrieval pre-treatment by boiling in a microwave.

I.2.5.4. Results

Endarterectomy fragments were divided into six different categories of lesions based on the AHA categorization (American Heart Association). The categories V and VI had the highest rate (Figure 26).

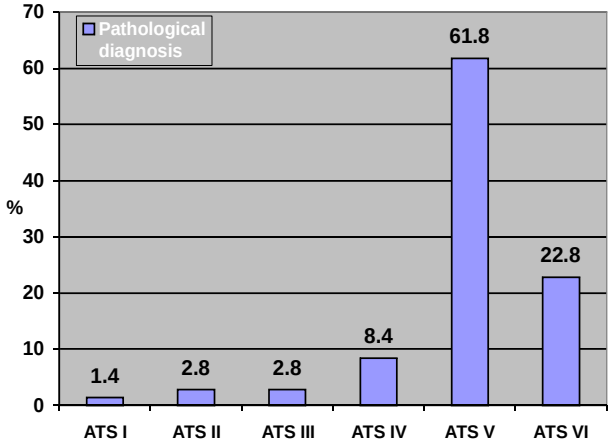


Figure 26. *Distribution of cases according to pathological diagnosis.*

Lesions appeared mostly as a single plaque with a single lipid core or as stratified plaques with several lipid cores and fibrotic layers, with calcium deposits or primarily fibrotic alterations.

Inflammatory activity in atherosclerotic lesions, observed in routine stain (Figures 27 and 28), was also immunohistochemically demonstrated. As plaque progresses, macrophages migrate into the plaque area. Plaque macrophages make up the majority of leukocytes in atherosclerotic lesions, and their secretory activity may be linked to the plaque's fragilization and subsequent rupture.

The analysed samples showed a variable degree of infiltration with foamy macrophages. Some areas showed a low density of CD68 positive immuno-reactivity (Figures 29 and 30). The fibrous collagen cap revealed the presence of macrophages (CD68 positive), although other inflammatory cells were also present.

CD68 was positive in a varying number of macrophages in the core of atherosclerotic plaques, depending on the severity of the inflammatory response. Near the necrotic lipid core was a large number of foamy macrophages; in contrast, the fibrous cap itself had only a few macrophages.

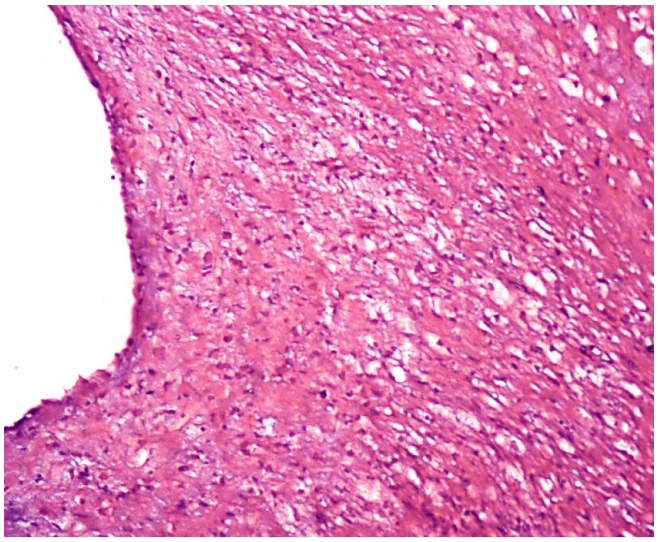


Figure 27. *Atherosclerotic plaque: discontinuous endothelium, foamy cells, extracellular lipid deposits (HE stain, ob. 20×)*

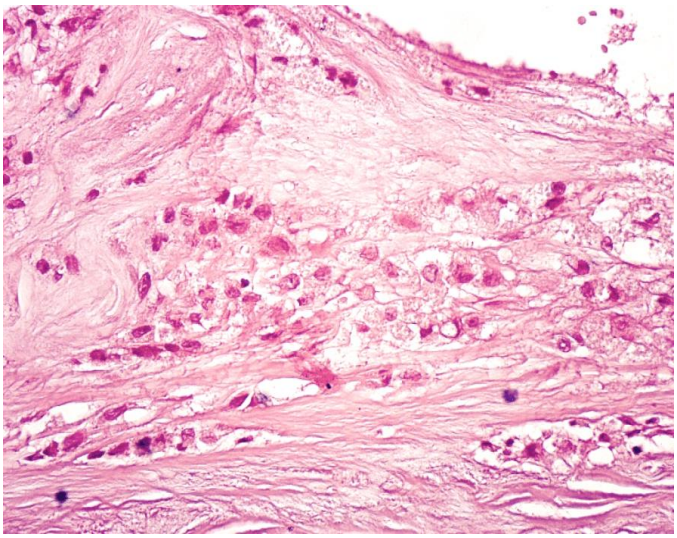


Figure 28. *Atherosclerotic plaque: abundant extra-cellular matrix, foamy cells (HE stain, ob. 40×)*

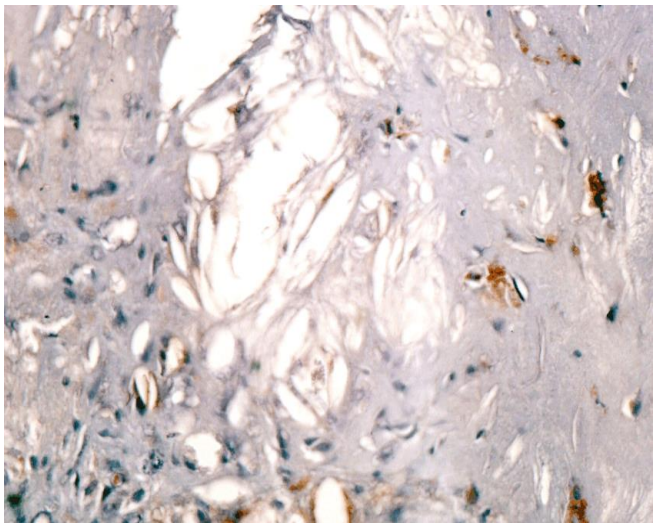


Figure 29. *Atherosclerotic plaque: positive immuno-reactivity for CD68, low density (ob. 40×)*

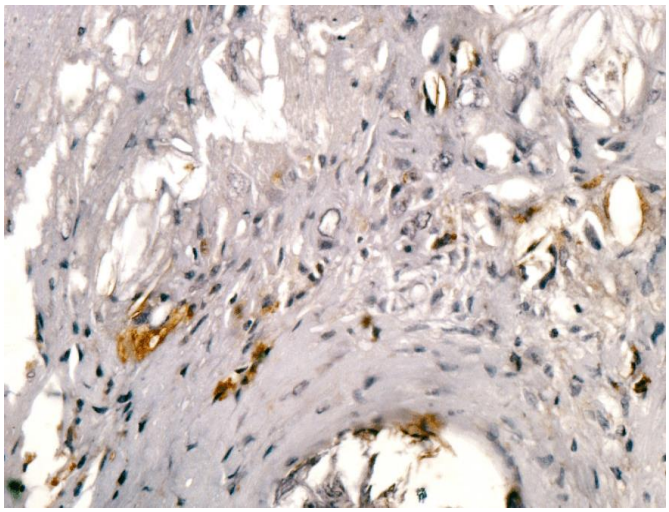


Figure 30. Atherosclerotic plaque: positive immuno-reactivity for CD68, low density (ob. 40×)

I.2.5.5. Discussion

According to the American Heart Association, there are six different forms of atherosclerotic lesions. Foamy cells occur as solitary cells in the first type, followed by fatty streaks and fibroatheromatous plaques, which progress to more complicated lesions [Kumar, Abbas, Aster, 2020]. Types I and II lesions are more common in the first decade of life, but they can also be detected in adulthood. Preatheroma, also known as type III, is a form of lesion that appears in adolescence and has a structure that is halfway between a lipid streak and an atheroma. Types IV, V, and VI are considered advanced lesions. Vascular stenosis, thrombosis, and/or bleeding can occur in the V and VI types as a result of accidents, and can be clinically silent or obvious. The evolution of a lesion from stage I to stage VI takes several decades, with distinct growth mechanisms [Kumar, Abbas, Aster, 2020].

Several studies suggest that atherosclerosis is a chronic immune-inflammatory disease in which interactions between blood monocytes and activated endothelium play a crucial role in causing artery intima impairment [Bobryshev, 2006; Webb, Moore, 2007].

Macrophages are now regarded as major mediators of inflammatory and metabolic signals in all stages of atherosclerosis, from the genesis and progression of lesions to necrosis leading to rupture and clinical symptoms of atherosclerosis. In addition, they are involved in the resolution and regression of atherosclerotic lesions [Moore et al., 2013]. Monocytes migrate into the subendothelial layer, where they develop into macrophages or dendritic cells, in the early stages of atherosclerosis. Most cells in the subendothelial tissue become foamy macrophages when atherogenic lipoproteins are present. The atheroma core can form when foamy cells clump together; as the process progresses, the lesion's center becomes necrotic, containing fat, cholesterol crystals, and cell debris [Webb, Moore, 2007; Spacek et al., 2018].

The inflammatory response in the plaque was assessed in our study using the immunohistochemistry marker CD68. The CD68 was positive in the atherosclerotic plaques, depending on the extent of the inflammatory reaction. In stable plaques, either a moderate zonal or diffuse positive immunoreactivity or a high zonal or diffuse immunoreactivity near the lipidonecrotic core with a large number of foamy macrophages were revealed. In contrast, in the fibrous cap itself, only a few macrophages were present. In the cap of complicated plaque, numerous CD68 positive macrophages were also observed.

It is known that white line cells do not adhere to normal endothelium in a physiological manner, but early in atherosclerosis, arterial endothelial cells begin to express on their surfaces selective adhesion molecules that link various types of leukocytes: ICAM-1, VCAM, ELAM [Boyle, 2005; Ulbrich, Eriksson, Lindbom, 2005; Zhu et al., 2018]. VCAM-1 (vascular cell adhesion molecule-1) specifically binds the two types of leukocytes present in early atheromas in humans and animals: monocytes and T-lymphocytes. Monocytes, significantly stimulated by local production of chemokines and receptors for chemokines, move through endothelial cells to the intima, where they become macrophages and begin to accumulate lipoproteins, after their entry by the endothelium layer. Thus, they become foamy cells which are a rich source of inflammatory mediators, encouraging inflammation and the growth of the lesion, in addition to serving as a reservoir for excess lipid [Spacek et al., 2018].

Typically, monocyte recruitment and macrophage differentiation protects against damage by absorbing modified lipids, but as fat load develops, damage progression follows [McLaren et al., 2011; Moore, Tabas, 2011; Bui, Prempeh, Wilensky, 2009; Loppnow, Werdan, Buerke, 2008]. Macrophages express numerous metalloproteinases and serinproteases that rupture. They release a variety of different substances, including reactive oxygen species, eicosanoids, TNF- and IL-1, and MCP-1, all of which contribute to leukocyte adhesion [Loppnow, Werdan, Buerke, 2008; Holvoet, 2008; Zhu et al., 2018].

MCP-1 (monocyte chemoattractant protein-1) is the most potent and powerful activator of monocyte migration into atherosclerotic lesions, despite the fact that other stimuli are known to stimulate monocyte chemotactic migration. In these lesions, colony-stimulating factor is required for macrophage differentiation, proliferation, and survival. A minor population of macrophages can proliferate even inside the atherosclerotic lesions, especially in the early stage [Matoba, Egashira, 2011; Shi, Pamer, 2011; Wolfs, Donners, de Winther, 2011].

It is known that macrophages have a number of receptors, including scavenger receptors, that allow them to take in various modified lipoproteins. Foamy cells occur as a result of the accumulation of cholesterol esters in the cytoplasm in the developing lesions. Different scavenger receptors are expressed by macrophages, however the MSR-A I and II (MSR-A I, II)

play the most critical role in the uptake of oxidized low density lipoprotein. Furthermore, macrophages and foamy cells produced from macrophages generate ceroid and advanced glycation end products (AGEs), which they collect in their cytoplasm. Other macrophages take up the produced extracellular AGEs via specialized receptors, such as MSR-A I and II. The majority of cells die by apoptosis inside the plaques, although others escape into the bloodstream.

In addition, macrophages also generate enzymes, activators, inhibitors, and bioactive mediators that help in plaque rupture, blood coagulation, and fibrinolysis. During the progression of atherosclerosis, macrophages continuously interact with vascular endothelial cells, medial smooth muscle cells, and other inflammatory cells, primarily T-cells and dendritic cells [El Khatib et al., 2011; Takahashi, Takeya, Sakashita, 2002].

CD4+ T-cells and macrophages, which release proinflammatory cytokines in atherosclerotic plaque, are the subject of several investigations on immunological effectors in atherosclerosis [Ikonomidis et al., 2008; Ingersoll et al., 2011; Moriya, 2019]. CD4+ cells have been observed to interact with oxidized LDL, and this interaction is thought to turn them into autoantigens. T helper 1 (T_H1) cells play a pro-atherogenic role, while regulatory T (Treg) cells play an anti-atherogenic role, according to significant research. Treg cells, on the other hand, can become pro-atherogenic. Other T_H cell subsets, such as T_H2, T_H9, T_H17, T_H22, follicular helper T cells, and CD28null T cells, as well as other T cell subsets, such as CD8+ T cells and T cells, play less well-known roles in atherosclerosis [Saigusa et al., 2020].

Moreover, it was suggested the possible role of antibodies and autoantigens in early atherosclerosis [Palinski, Witztum, 2000; Hansson GK, Hermansson, 2011]. In this context the participation of immunological factors in early human hypertension it has been reported. Anti-HSP-65 and endothelial cell antibodies (AECA) increase in patients with borderline hypertension [Noble, Shen, 2012]. Interaction with scavenger receptors, modulation of inflammation-related molecular pathways, such as miRNA regulation, and activation of the NLRP3 inflammasome are just a few of the ways OxLDL works [Poznyak et al, 2021]. However, anti-oxLDL and anti-lysophosphatidylcholine are low in patients with hypertension, implying that alternative autoantigens may have an anti-atherogenic role

Different phenotypes of macrophages are found in atherosclerotic lesions in experimental animals and humans, each of which plays a different role in mediating inflammation, removing dead cells, and possibly tissue healing. The accumulation of lipids in plaques enables macrophages to modify their phenotypic and biological functions, activating certain sets of genes [Wilson, 2010]. The M1 inflammatory phenotype of macrophages is caused by the activation of inflammasome by cholesterol crystals and the interaction of particular receptors with oxidized lipids. When oxidized phospholipids stimulate the Nrf2 response to stress genes, a novel MOX phenotype appears. Additional lipid mediators, such as nitrosilated fatty acids and omega-3 fatty acids, polarize macrophages in atherosclerotic plaques, causing them to change into anti-inflammatory phenotypes [Adamson, Leitinger, 2011; Taghizadeh et al., 2019].

To conclude, monocytes and macrophages play a role in the formation, progression, and destabilization of atherosclerotic plaques through a variety of mechanisms. Interaction to the plaque's complex microenvironment, which contains a variety of stimuli, causes macrophages to polarize and modify their phenotypic and activities [Nagenborg et al., 2017]. As a result,

targeting monocyte/macrophage therapy to achieve anti-inflammatory effects could be a potential choice for atherosclerosis prevention.

I.2.5.6. Conclusions

The examination of endarterectomies samples revealed varying degrees of foamy macrophage infiltration. The qualitative investigation of the inflammatory component, as indicated by immunohistochemical research, revealed differences in the location and in number of inflammatory cells in the vascular wall, which can be linked to plaque formation and progression. Deciphering how lipids accumulated in atherosclerotic plaques alter macrophage phenotype and functions, and hence the progression of such lesions, will aid in the development of innovative therapeutic techniques in the future. The heterogeneity of atherosclerotic disease, with aspects that are still unknown and a negative consequence on population health, keeps academics and physicians focused on the subject.

I.3. Consideration regarding the therapeutic approach and patient education in coronary heart disease

I.3.1. Scientific context

Coronary artery disease (CAD) is the leading cause of death, followed by cerebrovascular diseases. Stroke is the leading cause of long-term disability and the third highest cause of death in Europe, so it has a huge impact on public health. INTERHEART and INTERSTROKE, two case-control studies with a majority of patients from developing countries [Teo, Dokainish, 2017], were essential in identifying shared risk factors for acute myocardial infarction and stroke, respectively. Stroke can be driven by a range of factors. Ischemic stroke can occur as a result of the rupture of an atherosclerotic plaque (atherosclerotic thrombotic stroke) or as a result of the atherosclerotic process stimulating the start of an embolus (atherosclerotic embolus) (cerebral embolism). The elderly is the most impacted since the atherosclerosis process influences the emergence of the disease. Stroke can also be hemorrhagic, caused by a vascular rupture, however in this situation, atherosclerotic process intervention is low, with young people being the most commonly affected group [Barquera et al., 2015; Kobiyama, Ley, 2018].

Surveys data of coronary patients prove that the implementation of guidelines regarding cardiovascular diseases prevention in clinical practice needs improvement [De Backer et al., 2018; Kotseva et al., 2016]. Scientific studies related to atherosclerosis are being constantly published, in time with an increase in morbidity and mortality due to ischemic heart disease. The medical community is justifiably focused on cardiovascular diseases prevention, following the control of risk factors involved in the etiology of these disease (total cholesterol, LDL (low-density lipoprotein)-cholesterol, glycemia, uric acid, smoking, hypertension, hyperhomocysteinemia, etc) [Filip et al., 2010; Yuan et al., 2014; Tuñón et al., 2018].

Lately, the view of who is at risk for a heart attack has changed radically. A heart attack used to bring up images of a white man middle-aged smoker, with high cholesterol levels and high blood pressure, but these traditional notions of what adds to risk have shifted in recent years. Accordingly, new thinking on the following topics is included in these updated

perspectives: the main cause of death worldwide nowadays is atherosclerotic cardiovascular disease (ASCVD); in addition, women, younger people, and patients from all categories of life are increasingly affected by atherosclerosis; the importance of high-density lipoprotein (HDL) cholesterol in heart disease prevention has been highlighted, while triglycerides have risen as a prospective target for lowering cardiovascular risk. Finally, inflammation may have a role in the relationship between recognized risk factors such as abnormal lipids, smoking, and diabetes, as well as atherosclerotic complications such as heart attack and stroke [Meha et al., 2020].

Recently, some placebo-controlled clinical studies proved that the lower the achieved LDL-C values, the lower the risk of future CV events, with no lower limit for LDL-C values, or 'J'-curve effect. Furthermore, investigations of the clinical safety of these extremely low LDL-C values have been encouraging, although longer-term monitoring is required. Recent studies have found that currently available medications for increasing high-density lipoprotein (HDL) cholesterol (HDL-C) do not diminish the incidence of ASCVD. Additionally, human Mendelian randomization experiments have shown that LDL-C and other cholesterol-rich ApoB-containing lipoproteins play a critical role in the development of atherosclerotic plaques and subsequent CV events. As a result, there is no longer a "LDL-C hypothesis," but rather proven facts that higher LDL-C levels are directly connected to ASCVD, and that decreasing LDL particles and other ApoB-containing lipoproteins as much as feasible lowers CV events [Mach et al., 2020]. Consequently, new LDL-C objectives, as well as a revised CV risk classification, which are especially relevant to high- and very-high-risk patients, have been proposed by the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) Task Force members. These new ESC/EAS Lipid Guidelines give critical new patient treatment guidance, allowing more doctors to effectively and safely lower CV risk through lipid modulation [Raygor, Khera, 2020].

Taking into account these aspects, numerous cardiovascular risk assessment charts have been developed, the newest and recommended by the European Society of Cardiology being the SCORE diagram, according to which patients with cholesterol values above 310 mg / dl and LDL-cholesterol values over 190 mg / dl have an increased risk (> 15%) of developing a fatal cardiovascular disease in the next 10 years [Adamkiewicz et al., 2017].

The National Cholesterol Education Program Expert Panel III defines metabolic syndrome as a combination of risk factors for cardiovascular disease. Abdominal obesity, high serum cholesterol, high blood pressure, insulin resistance with or without impaired glucose tolerance, a pro-inflammatory status with a high level of C-reactive protein, and a pro-thrombotic status with a high level of plasma fibrinogen and coagulation factors are just a few of them [Grundy et al., 2004; Khoshdel et al., 2012; Moore et al., 2020].

Lowering cholesterol levels is critical, and it can be accomplished through a combination of lifestyle changes and pharmacological agents such as statins or newer drugs such protein convertase inhibitor subtilisin / kexin type 9 (PCSK9) [Agabiti Rosei, Salvetti, 2016]. Furthermore, multiple research suggests that lowering triglyceride and lipoprotein (a) levels would reduce the risk of developing ischemic heart disease in addition to lowering LDL cholesterol [Sanin, Pfetsch, Koenig, et al., 2017].

Statin drug treatment is widely used around the world, but side effects such as rhabdomyolysis, liver damage, decreased glucose tolerance that can lead to type 2 diabetes, and cognitive dysfunction are common. These side effects are more common in women over 65,

patients with hepatic or renal impairment, and those who take multiple hypocholesterolemic medications [Thompson et al., 2016; Mach et al., 2018]. Furthermore, new research has shown that side effects can arise in people who have defective genes implicated in drug metabolism, indicating that such patients should undergo genetic testing [Sirtori et al., 2012].

Personal contribution related to atherosclerosis management was synthesized in the following papers:

ISI ARTICLES

1. Grosu C, Mastaleru A, Nita O, Cobzaru RG, Rapa CV, Leon-Constantin MM, **Cojocaru E**. Effects of Statin Therapy in Patients with Stroke and Atheromatosis. *Rev Chim(Bucharest)* 2018; 69(12): 3698 - 3701. **IF : 1.605**
   https://revistadechimie.ro/Articles.asp?ID=6822
2. Bostan MM, Stătescu C, Anghel L, Șerban IL, **Cojocaru E**, Sascău R. Post-Myocardial Infarction Ventricular Remodeling Biomarkers—The Key Link between Pathophysiology and Clinic. *Biomolecules* 2020; 10: 1587. **IF: 4.879**
   biomolecules-10-01587 (1).pdf
3. Cojocariu SA, Maștaleru A, Sascău RA, Stătescu C, Mitu F, **Cojocaru E**, Trandafir LM, Leon-Constantin M-M. Relationships between Psychoeducational Rehabilitation and Health Outcomes—A Systematic Review Focused on Acute Coronary Syndrome. *Journal of Personalized Medicine* 2021; 11(6):440. **IF: 4.945**
   https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8223782/
4. Manta A, **Cojocaru E**, Leon-Constantin MM, Maștaleru A, Roca M, Rusu C, Cojocariu SA, Mitu F. IPAQ-L and CPET Usefulness in a North-Eastern Romanian Population Undergoing Cardiac Rehabilitation. *Applied Sciences*. 2021; 11(12):5483. **IF: 2.679**
   https://www.mdpi.com/2076-3417/11/12/5483/htm
5. Zota IM, Leon Constantin MM, Statescu C, Sascau RA, Roca M, Gavril RS, Vasilcu TF, Boisteanu D, **Cojocaru E**, Mastaleru A, Mitu O, Mitu F. Clinical and biological impact of CPAP therapy in patients with obstructive sleep apnea and cardio-metabolic comorbidities. *Acta Medica Mediterranea*. 2020; 36:1975. **IF: 0.219**
   https://www.actamedicamediterranea.com/archive/2020/medica-3/clinical-and-biological-impact-of-cpap-therapy-in-patients-with-obstructive-sleep-apnea-and-cardio-metabolic-comorbidities

BDI ARTICLES

1. Leon MM, **Stefanachi (Cojocaru) E**, Cobzaru R, Mitu F. The effects of hydroxymethylglutaryl coenzime A reductase inhibitor in elderly patients. *Revista Română de Anatomie funcţională şi clinică, macro- şi microscopică şi de Antropolgie* 2013; 12 (1): 35-40.
   http://revanatomie.ro/ro/abstract.php?an_rev=2013&nr_rev=1&nr_art=13
2. Mitu F, **Stefanachi (Cojocaru) E**, Leon MM. The incidence of essential hypertension in elderly patients with metabolic syndrome. *Rev Med Chir Soc Med Nat Iasi* 2013; 117 (3): 630-634.
   http://www.ncbi.nlm.nih.gov/pubmed/24502027
3. Leon MM, **Stefanachi (Cojocaru) E**, Cobzaru R, Mitu F. Impact of metabolic syndrome on the development of cardiovascular disease. *Rev Med Chir Soc Med Nat Iasi* 2013; 117 (3): 635-640.
   http://www.ncbi.nlm.nih.gov/pubmed/24502028

4. Mitu O, Mitu F, Constantin S, **Cojocaru E**, Leon MM. Therapeutical considerations in associated atrial fibrillation and heart failure. *Rev Med Chir Soc Med Nat Iasi* 2014; 118 (3): 624-630.
http://www.ncbi.nlm.nih.gov/pubmed/25341275
5. Manta A, Leon-Constantin MM, Maștaleru Alexandra, Cojocariu SA, **Cojocaru E**, Roca M, Mitu F. Utility of the international physical activity questionnaire in patients with heart failure - the experience of a cardiovascular rehabilitation clinic. *Rev Med Chir Soc Med Nat Iasi* 2021; 125(3):352-358.
https://www.revmedchir.ro/index.php/revmedchir/article/view/2448/1766

I.3.2. The influence of statin therapy in stroke and atheromatosis outcome

I.3.2.1. Introduction

Following ischemic heart disease and oncologic pathology, stroke is the third leading cause of morbidity and mortality in both Europe and the United States [Lopez et al., 2006; Rothwell et al., 2005; Wafa et al., 2018]. It is a condition caused by a combination of genetic and environmental factors, as is the case with several other diseases. Age, familial antecedents, ethnicity, sex, and prior vascular events are all unchangeable stroke risk factors, while blood pressure, atrial fibrillation, diabetes, cardiac disease, asymptomatic carotid atheromatosis, hypercholesterolemia, sedentarism, obesity, smoking, poor socioeconomic status, drug use, and alcohol abuse are among the modifying risk factors [Sun et al., 2017].

Depending on the aetiology and concomitant disorders, secondary prevention usually includes the use of preventive medication such as anti-aggregation, hypolipidemic, antihypertensive [Mortensen et al., 2017; Kernan, 2014; Amarenco et al., 2009]. Atherosclerosis and dyslipidemia are two major risk factors in the pathogenesis of stroke [Polak et al., 2013; Osawa et al., 2018; Kim et al., 2017]. As a result, according to current international guidelines, statins should be used in patients with a history of stroke because they lower cholesterol and triglyceride levels while also alleviating carotid or cerebral atheroma plaques [Amarenco et al., 2009; Zhong et al., 2017; Sirtori, 2014].

The use of statins in secondary prevention, according to the 2016 ACC/AHA blood cholesterol management recommendations, demonstrated a reduction of 16% for stroke, of 27% for nonfatal myocardial infarction and 20% for mortality from cardiac events [Piepoli et al., 2016; Miller, and Martin, 2016; Amarenco et al., 2007].

Statins can cause cytotoxicity, hepatic injury or necrosis, renal damage, and myopathy if used in excess or for a long time [Mancini et al., 2016; Reiss et al., 2011]. To avoid any negative consequences, hepatic and kidney function, as well as muscle enzymes, must be monitored on a regular basis [Abd and Jacobson, 2011; Karahalil et al., 2017]. In addition, many studies have linked statins to the onset of diabetes, cognitive impairment, and haemorrhagic stroke [Mach et al., 2018; Betteridge et al., 2016].

Nevertheless, current research and guidelines indicate that the benefits of statin medication outweigh any potential risks or side effects [Bellaosta et al., 2012].

I.3.2.2. Aim

The objective of this research is to show how statins impact other cardiovascular risk factors such as diabetes, atheromatosis, and uric acid level, in stroke patients who are

undergoing hypolipidemic therapy with statins as secondary prophylaxis. We also investigated the liver enzymes to see if statins had any negative impacts.

I.3.2.3. Material and methods

We achieved a retrospective study on 58 patients with a history of ischemic stroke, admitted in the Neurology Department inside the Clinical Rehabilitation Hospital in Iasi, Romania during 01.01-30.09.2018. Anthropometric measurements (age, weight, height, BMI) and biochemical analysis (hepatic enzymes, uric acid, glycemia, glycosylated hemoglobin, and lipid profile) were performed on all patients. Doppler cervical ultrasound was also performed at the level of the bilateral common carotid artery for the identification of atheromatosis and its degree, with a Siemens Accuson X300 system using a 7.5 MHz linear probe through a standardized method [Stein et al., 2008].

Patients who had an embolic stroke or had an arterial obstruction were excluded from the study. Before being tested and included in the study, all subjects were informed of the research technique and completed an informed consent form. SPSS v.18 was used for statistical analysis. We used p=0.005 as a reference value for significance in interpreting statistical results, which equates to a 95% confidence range. Continuous type variables were presented as mean ± standard deviation.

I.3.2.4. Results and discussion

We had 23 females and 35 males in our study, with a mean age of 65.9±13.11 years. A history of high blood pressure was common among the patients (82.8% vs.17.2%) and also presented with atheroma plaques in the carotid artery at the Doppler examination (53.4% vs.46.5%). 36.2% of the patients were overweight and 25.8% were obese. 53 of the patients included associated type 2 diabetes mellitus insulin dependent.

The mean value for AST was 25.5±17.7 mg/dl, with a maximum of 106 mg/dl, for ALT it was 36.7±36.7 mg/dl, with a maximum of 191.6 mg/dl, for uric acid the mean value was 4.5±1.7 mg/dl, with a maximum of 8.16 mg/dl. As for the lipid profile, mean cholesterol was 145.7±40.4 mg/dl and for triglycerides was 116.7±56.2 mg/dl (Table XX).

Table XX. Biological analysis for the study group

	AST	ALT	Uric acid	Cholesterol	Triglycerides
Mean	25.5500	36.7741	4.5569	145.7848	116.7788
Std. Deviation	17.78476	36.76194	1.73366	40.45919	56.29924
Minimum	11.00	10.60	1.26	85.80	43.40
Maximum	106.60	191.60	8.16	256.00	302.00

Most of the study subjects had statins (87.9%) in the treatment regimen, but there were also patients who did not receive statin therapy at home, even after the cerebrovascular event. Among the statin type, atorvastatin was the most used (69%), then rosuvastatin (17.2%) and simvastatin (1.7%) last (Table XXI).

Table XXI. Types of statins used in the study

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Without	7	12.1	12.1	12.1
	Atorvastatin	40	69.0	69.0	81.0
	Simvastatin	1	1.7	1.7	82.8
	Rosuvastatin	10	17.2	17.2	100.0

When it came to statin doses, the most common was 10 mg (37.9%), followed by 20 mg (31.0%), then 40 mg (17.2%) and only 1.7% of patients were treated with the 80 mg dose (Table XXII).

Table XXII. Dosage of statins used in the study

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Without	7	12.1	12.1	12.1
	10 mg	22	37.9	37.9	50.0
	20 mg	18	31.0	31.0	81.0
	40 mg	10	17.2	17.2	98.3
	80 mg	1	1.7	1.7	100.0

The use of statins as a secondary preventative strategy is mentioned in international guidelines for ischemic stroke [Piepoli et al., 2016].

In our study, 12% of patients did not have chronic statin treatment, 37.9% had statin at the 10 mg dose, 31% had a statin dose of 20 mg, 17.2% had 40 mg of statin and 1.7% had the statin dose of 80 mg. The recommended doses in the European Cardiology Guide are 40 mg, respecting 80 mg in patients with a history of stroke [Piepoli et al., 2016].

When liver enzymes increase by 3-5 times their normal value 3 weeks after starting a statin-lipid-reducing therapy, the same guidelines suggest lowering the statin dose. In our investigation, patients taking statins of 10 mg, 20 mg, 40 mg, or 80 mg had an abnormal aspartate aminotransferase (AST) value in a similar percentage of 1.7 %. It's worth noting that in the absence of hypolipidemic medication, a statistically significant number of patients had higher liver enzymes by 3.4 % (figure 31).

We discovered a statistically significant correlation between the AST value and the statin dose ($p = 0.039$) among liver enzymes.

Uric acid is another risk factor for vascular disease. The difference between the uric acid value and the statin concentration was statistically significant in our investigation. As a result, increased uric acid was discovered in 2% of patients taking 10 mg statin, in the same proportion as those taking a 20 mg or 40 mg dose. When the dyslipidaemia therapy is given at the maximal dose of 80 mg, the uric acid level is unaffected. In 6% of patients, uric acid levels were higher, 65.3 % were lower, and 28.7% were normal.

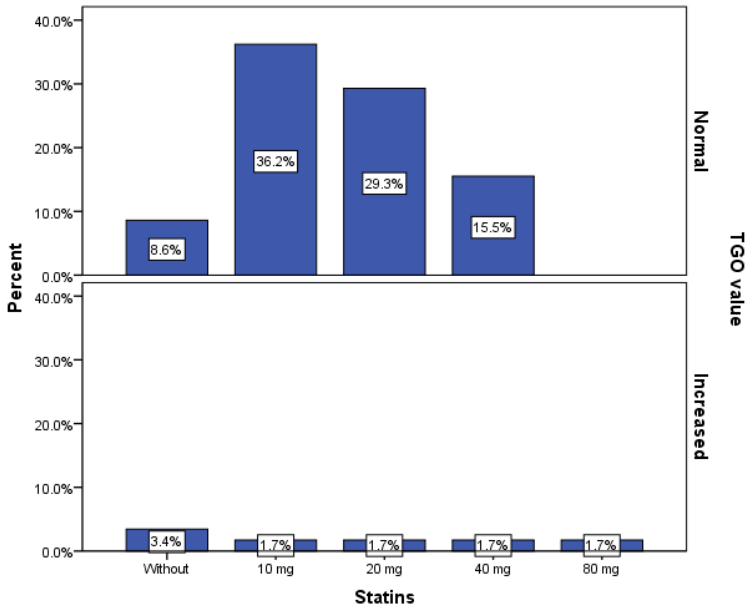


Figure 31. *Correlations between dosage of statins and ALT value*

The level of uric acid was considered in all patients, whether or not they were on urate-reducing medication, suggesting that statins, in addition to their hypolipemiant action, may also have a role in lowering uric acid, a known cardiovascular risk factor. The statin dose compared to the normal uric acid value (p = 0.046) and the high uric acid value (p = 0.047) showed a statistically significant difference (figure 32).

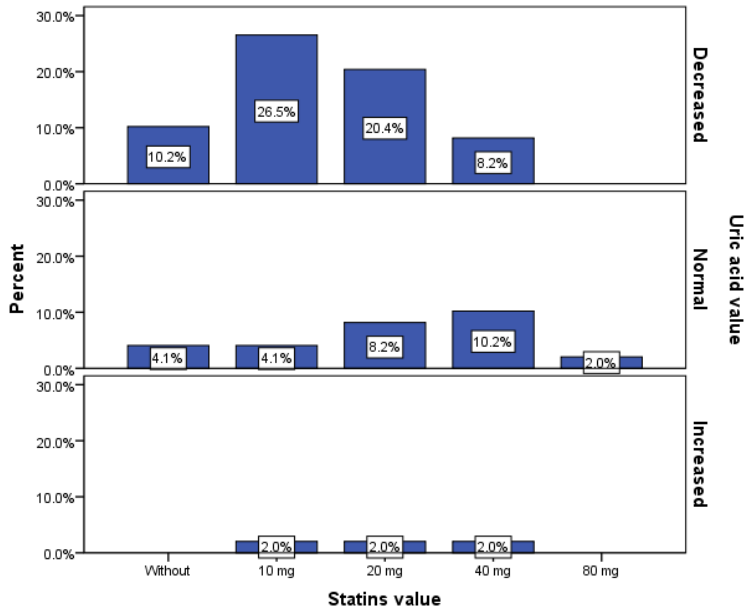


Figure 32. *Correlations between statin dose and uric acid value*

In individuals with a history of stroke, statin medication is extremely important, especially if they also have diabetes. However, only 8.5 % of diabetic patients receiving insulin were also prescribed a statin, and the difference in statin dose between diabetic and non-diabetic individuals was statistically significant (p = 0.003).

There was no statistically significant correlation between statin doses in patients with oral diabetes mellitus. Despite the modest number of diabetic patients getting dyslipidemic medication, we found that the majority (3.4%) took the 40 mg dose, which was statistically significant compared to the other doses utilized in our patients. The percentages for the 10 mg and 20 mg doses are comparable. It's worth noting that the 80 mg dose is only found in patients with stroke and diabetes, but in a low percentage of 1.7% (figure 33).

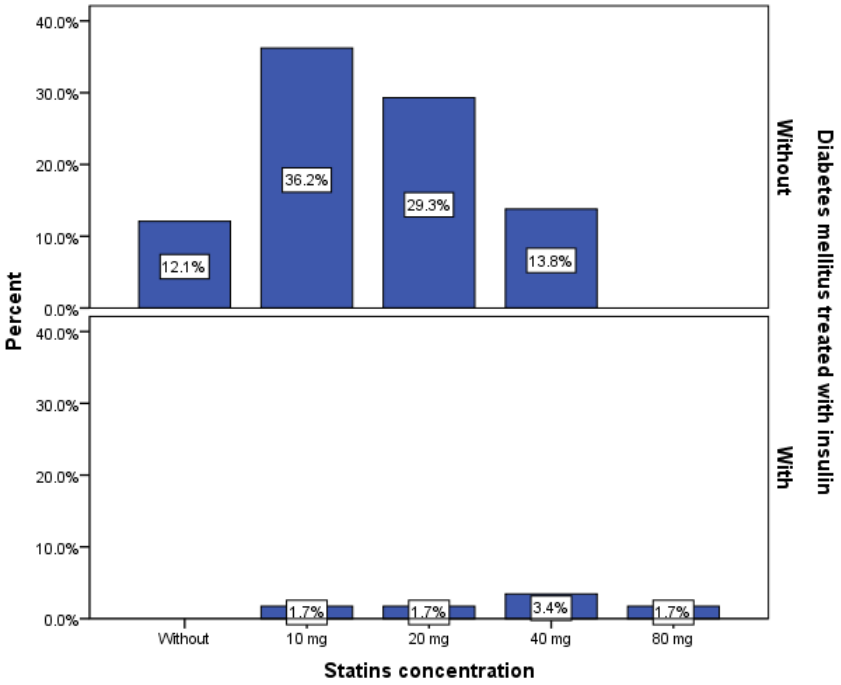


Figure 33. *Correlations between statins concentration and presence of diabetes mellitus*

The use of cervical Doppler ultrasound to measure carotid atheromatosis is suggested by worldwide protocols for the secondary preventive therapy of ischemic stroke patients. Atheromatosis was observed in 53.4 % of patients with ischemic stroke in our study. Only 8.6% of people were not taking statins. Between the percentage of patients with atheromatosis and the percentage of patients without atheromatosis, there was a statistically significant difference. In comparison to atherosclerotic patients, the percentage of patients treated with statin but without atheromatosis was statistically significant at 10 mg and 20 mg, respectively (22.4 % vs 15.5 %at the 10 mg dose, respectively 17.2 % vs. 13.8 % at the 20 mg dose).

The percentages are reversed with a statistically significant difference in patients with stroke, atheromatosis and 40 mg statin and patients without atheromatosis. The statin dose of 80 mg, as recommended by the guidelines, was identified in 1.7% of stroke patients with well-known atheromatosis (figure 34).

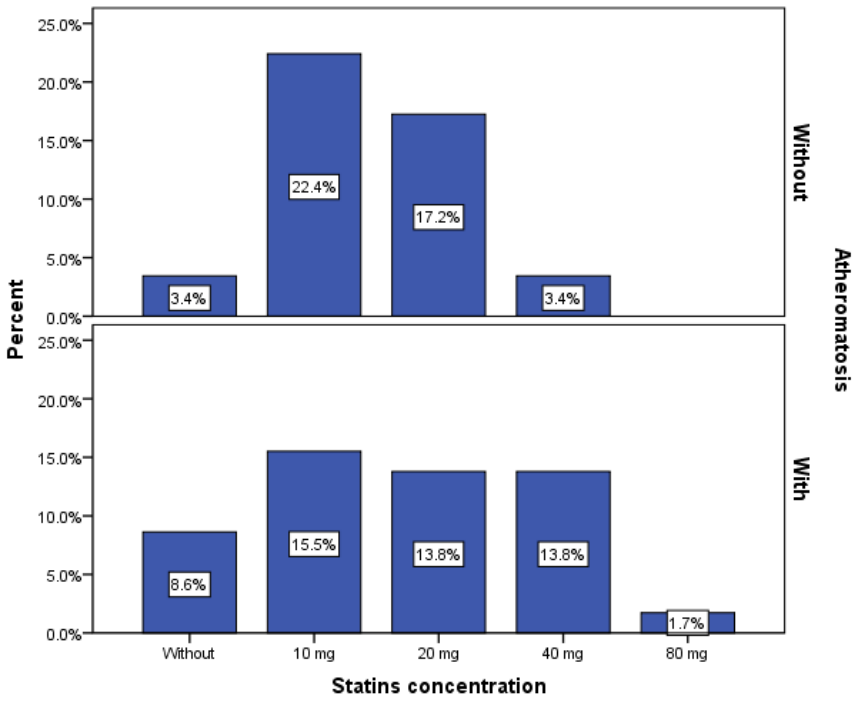


Figure 34. *Correlations between statins concentration and atheromatosis*

As a result, 15.5 % of patients with atheromatosis and stroke receive appropriate treatment. There was no statistically significant relationship between cholesterol and triglycerides and statin dosage.

I.3.2.5. Conclusions

We noticed a strong relation between atheromatosis and statin treatment in our research, which was more significant in patients taking higher doses. We also identified a link between statin dosage and uric acid level, which is another cardiovascular risk factor. Our result, in line with other literature data, highlights the necessity of statins administration in order to reduce cholesterol levels and to consolidate atheroma plaque in stroke patients.

I.3.3. Psychoeducational rehabilitation and health outcomes in acute coronary syndrome

I.3.3.1. Introduction

The most prevalent disease requiring emergency cardiac treatment is acute coronary syndrome (ACS), which is associated with a high risk of morbidity and mortality, as well as a significant burden on patients and healthcare systems [Ibanez et al., 2021]. Myocardial infarction (MI) with ST-segment elevation (STEMI) and without ST-segment elevation (NSTEMI) as well as unstable angina (UA) are all part of the ACS spectrum [Collet et al., 2021]. Coronary atherosclerotic plaque disruption coupled by thrombosis is the most common pathophysiological mechanism involved [Crea and Libby, 2017]. Hypertension, smoking, diabetes, hyperlipidemia, and obesity are frequently controllable risk factors for coronary thrombosis, while age, male sex, family history, and ethnicity are unmodifiable [Kumar and Cannon, 2009]. The histological basis is cardio-myocyte necrosis in MI, and myocardial ischemia without cell damage in UA [Chapman, Adamson, and Mills, 2017].

Cardiac rehabilitation (CR) is a multidisciplinary approach that includes social and emotional support and patient education, as well as physical activity, cardiovascular risk factor management, and dietary counselling [Ambrosetti et al., 2021]. The customization of the program for specific cardiac manifestation started in 2010 when the key steps to deliver CR were established [Piepoli et al., 2010]. Patients with ACS should continue a rehabilitation program, according to the newest guidelines from the European Society of Cardiology (ESC) [Ibanez et al., 2018]. The most significant confirmed advantages in the modern age are a 26% reduction in cardiac mortality and an 18% reduction in recurrent hospitalization.

Previous research stated that depression and anxiety after a MI develop in 30-40% of the patients [Reid et al., 2013], both being associated with substantial increases in the risk of adverse cardiovascular outcomes [Nicholson, Kuper, Hemingway, 2006; Roest et al., 2010]. It is proved that psychological interventions can reduce the prevalence of emotional disorders, relaxation training [Price et al., 2016], stress management [Blumenthal et al., 2016], and low-level cognitive-behavioral therapy (CBT) techniques [Kira et al., 2015] being recommended in the CR program. Furthermore, metacognitive treatment for distressed CR patients [McPhillips et al., 2019], as well as problem-solving therapy for depressed patients, may be beneficial [Lichtman et al., 2008]. Motivational interviewing is another psychological strategy that has been shown to be effective in inducing behavioural change (starting an exercise program and modifying eating habits) [Pietrabissa et al., 2015] and enhancing physical activity [Reid et al., 2015]. Patient education, which is linked to psychotherapy, should be incorporated into the CR program to make it effective [Piepoli et al., 2014]. Despite the fact that current data show that education-based strategies have no effect on overall mortality, total revascularizations, or hospitalizations, the main benefit received is a decrease in fatal MI and/or non-fatal cardiovascular events [Anderson et al., 2017].

Several systematic reviews in the field offer significant information on the positive effects of psychological and educational interventions in patients with cardiovascular disease, the most narrowed group investigated being the one that included patients with coronary artery disease (CAD) [Linden et al., 1996; Dusseldorp et al., 1999; Rees et al., 2004; Whalley et al., 2011; Reid et al., 2013; Anderson et al., 2017; Richards et al., 2017; 21-26]. Because the negative psychological effect of acute myocardial infarction is higher than that of stable ischemic heart disease [Norlund et al., 2018], we thought it was important to review studies that only included patients with acute coronary syndrome.

I.3.3.2. Aim

The purpose of the study was to focus on the the available and updated evidence on the effectiveness of psychological and educational interventions (as a stand-alone measure or as part of a cardiac rehabilitation program) in comparison to the usual medical care for patients who have suffered an acute coronary syndrome. We aimed to make an inclusive summary of the dose and types of interventions currently administered and their benefits on rehospitalisation and quality of life. We also focused on the control of the cardiovascular risk factors, exercise capacity, and adherence to cardiac rehabilitation. Moreover, we also considered important to include patients' understanding and attitude towards the disease, as well as its effects on the psychological and medical symptoms.

I.3.3.3. Material and methods

The protocol was registered on PROSPERO (CRD42021239578). The systematic review was carried out according to Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist [Liberati et al., 2009]. Additionally, we have taken into consideration the recommendations from the latest Prisma statement 2020 [Page et al., 2020].

Our systematic review was based on the following research question: "Is psychological rehabilitation effective in preventing major adverse cardiovascular events in patients with ACS?"

Adults with ACS made up the population investigated in this study. Psychoeducational rehabilitation was used for individuals with ACS, either alone or in addition with standard cardiac rehabilitation. The control group involved standard cardiac rehabilitation. The outcomes were: rehospitalisation, the quality-of-life evaluation, the control of the cardiovascular risk factors, exercise capacity and adherence to cardiac rehabilitation, the understanding and the attitude towards the disease, but also the effects regarding the psychological and medical symptoms.

A systematic search in the following electronic databases was used to index relevant papers: MEDLINE (Ovid), Cochrane Central Register of Controlled Trials (CENTRAL), PsycINFO (Ovid), and Embase (Ovid). We searched the WHO International Clinical Trials Registry Platform and the US ClinicalTrials.gov Registry for on-going clinical trials on February 15, 2021. Furthermore, we performed a manual search of the reference lists for the selected articles. We applied several selection criteria such as: study type (randomized controlled trials); language (English); types of participants (adults of all ages who have been diagnosed with an acute coronary syndrome); types of interventions (psychotherapy, mental fitness, education during hospitalization for the acute event, interpersonal counselling, short-term psychological intervention, motivational interviewing, and positive psychology); outcome (all-cause rehospitalisation, quality of life evaluation, the control of the cardiovascular risk factors, the exercise capacity, and the adherence to the cardiac rehabilitation program, but also the understanding and the attitude towards the disease, as well as the effects on psychological and medical symptoms); follow-up duration (without restrictions; if a study was reported in several publications, all follow-up results were taken into account).

A number of exclusion criteria have been established: (i) interventions or conditions within a study that were not fully randomized, (ii) studies available only in abstracts, (iii) studies that included patients with stable ischemic heart disease without a history of an acute ischemic event, (iv) studies that evaluated the physical exercise or other components of the cardiac rehabilitation, (v) studies with an intervention arm less than 30 participants (to avoid unreliable findings) and (vi) dissertations, conference abstracts and studies with a sample size $\leq$100 patients.

Three reviewers independently assessed the risk of bias for the included randomized controlled clinical trials using AUB KQ1, a modified version of the Cochrane technique [Higgins et al., 2011]. As a result, we valued random sequence generation, allocation concealment, selective reporting, other sources of bias, blinding, and incomplete outcome data. Each of these domains has been graded as having a high risk of bias, a low risk of bias, or

unclear. The contradictions of the results between the three reviewers were solved by a fourth one.

I.3.3.4. Results

The screening and selection process for the papers included in this systematic review is depicted in figure 35. Our search in databases has identified 6248 studies in databases during our search. We read 149 full-text publications after eliminating duplicates and scanning titles and abstracts. Only 11 of them matched the requirements for inclusion in our study. Around 35 % of all studies were at a high risk of bias due to other sources of bias, such as groups being unbalanced at baseline and the intention to treat analysis. Less than 10% of studies did not provide sufficient methodological detail to allow assessment of possible bias in outcome assessment and other sources. The methods of allocation concealment and attrition bias were unclear for no more than 20 % of studies.

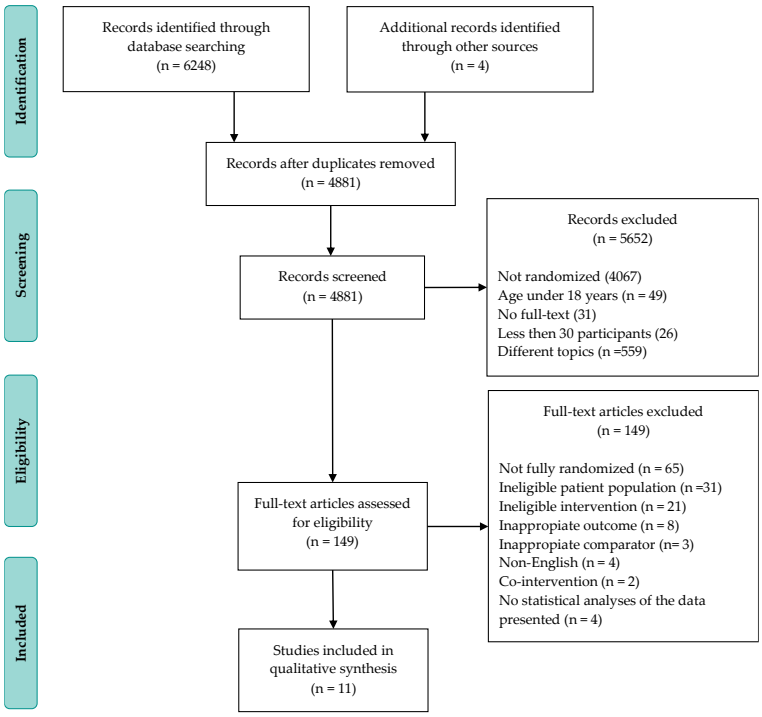


Figure 35. *Flow diagram showing selection process*

The characteristics and demographic data for each study are presented in Table XXIII. The analysis showed that out of the 11 studies, six were single-sited: Italy [Roncella et al., 2013; Chiavarino et al., 2016], Portugal [Fernandes et al., 2017], USA [Huffman et al., 2019], Iran [Nasiri et al.,2020], Finland [Oranta et al., 2010] and five took place in multiple centers [Davidson et al., 2010; O'Brien et al., 2014; O'Neil et al., 2015; Sunamura et al., 2017; Norlund et al., 2018]. A total of 3090 patients with AMI were included in the selected randomized controlled trials. Among patients, 2237 (72.39%) were men and 853 (27.61%) were women, with a sex ratio of 2.62. Only one study enrolled mainly women, 68.78% [Davidson et al., 2010]. The mean age for all the people in the included studies was over 50 years old, with one exception that classified the age as less than 60 years and 60-75 years old, without including

the mean value [Oranta et al., 2010]; no study specified how many patients were young, how many middle-aged and how many elderly individuals at the time of diagnosis of ACS.

In terms of the intervention, seven studies applied standard methods [Chiavarino et al., 2016; Davidson et al., 2010; Fernandes et al., 2017; Nasiri et al., 2020; O'Brien et al., 2014; Roncella et al., 2013, Sunamura et al., 2018], one study used the tele-phone [Huffman et al., 2019], one used an online portal [Norlund et al., 2018] and two studies used the hybrid method telephone-based and in-hospital [O'Neil et al., 2015; Oranta et al., 2010] as a means of delivering psychotherapy. Only one study of those who had set up in the hospital was conducted on an outpatient basis [Sunamura et al., 2018], the other six being in a continuous hospitalization. The longest follow-up time was five years [Pristipino et al., 2019], but the initial design of the study was sized to evaluate one-year outcomes. The shortest follow-up time was 1 month [Nasiri et al.,2020].

The effect of problem-solving therapy on depressive symptoms can be extended across gender, according to Davidson et al., with just a minor difference between the intervention and control groups: mean −3.6, 95% CI −7.5 to 0.3, p = 0.07 for male patients versus mean −4.0, 95% CI −7.6 to −0.3, p = 0.03 for female patients. In addition, the effect proved to be generalizable across the ethnic background, without a significant difference between the intervention group and the control group: mean −3.5, 95% CI −7.6 to 0.5, p = 0.09 for Hispanic patients versus mean −3.5, 95% CI −7.6 to 0.5, p = 0.04 for African American patients [Davidson et al., 2010].

Norlund et al. found that internet-based cognitive behavioural treatment had no effect on the total score on the Hospital Anxiety and Depression Scale (HADS) 14 weeks after baseline for the main analysis. Separate exploratory analyses at follow-up revealed that men had a lower HADS - total score compared to women (β = −2.04, 95% CI −3.60 to −0.47, p = 0.01), and there was a borderline significant reduction in HADS - total score per unit increase in age (β = −0.08, 95% CI −0.16 to 0.01, p = 0.09). In contrast, both the main analysis and separate exploratory analyses showed no effect of treatment on either HADS - anxiety or HADS - depression subscales [Norlund et al., 2018].

Table XXIII. General characteristics of studies included in present systematic review

Authors and Year of Publication	Enrollment Place (No. of Centers) and Time	No. of Patients F/M (no.)	Mean Age years (SD)	Education (mean, no.)	Timing and Setting
Chiavarino et al., 2016	Italy (1) 2 year period	118 17/101	56.5 (8.70)	10.3 ± 4.0 years	8 months Hospital
Davidson et al., 2010	USA (5) Between 1 January 2005 and 29 February 2008	157 108/49	61.2 (10.6)	13.1 ± 3.8 years	15 months Hospital
Fernandes et al., 2017, 2018	Portugal (1) 6 months period	121 37/84	61.77 (12.11) versus 66.11 (12.61)	< 4 years: 25 4 years: 50 4-12 years: 31 >12 years: 15	2 months Hospital
Huffman et al., 2019	USA (1)	47 11/36	60.80 (10.7)	Not specified	6 months Telephone

	Between May 2017 and April 2018				
Nasiri et al., 2020	Iran (1) Between September 2018 and July 2019	64 26/38	52.7 (10.94)	Elementary: 12 Cycle degree: 18 Diploma: 14 Associate degree: 2 Bachelor's degree: 10	1 month Hospital
Norlund et al., 2018	Sweden (25) Between September 2013 and December 2016	239 80/159	58.4 (9.0) versus 60.8 (7.8)	Elementary: 48 High school: 91 University: 100	3.5 months Internet-based portal
O'Brien et al., 2014	Dublin (5) Between October 2007 and October 2009	1,136 316/820	62.65 (12.3)	Little formal/ primary: 404 Second level: 509 Third level: 222	12 months Hospital
O'Neil et al., 2015	Australia (6) Between December 2009 and February 2011	121 30/91	61.0 (10.2) versus 58.9 (10.7)	High School: 67 Diploma/trade: 23 Bachelor/Master: 19	12 months Hospital Telephone
Oranta et al., 2010-2012	Finland (1) Between September 2004 and January 2007	103 30/73	< 60 years: 45 60-75 years: 58	Professional education: 41 II grade education: 39 College-level education: 18 University education: 5 Profession Worker: 62 Official: 25 Businessman: 16	18 months Hospital Telephone
Pristipino et al., 2019	Italy (1) Between June 2005 and January 2011	45 10/35	55 (9) versus 55 (8)	Not specified	5 years Hospital
Roncella et al., 2013					12 months Hospital
Sunamura et al., 2017	Netherlands (10) Between November 2011 and August 2014	615 124/491	57.5 (9.2) versus 57.4 (9.3)	Low = 19 Intermediate = 319 High = 139	18 months Hospital Outpatient
Ter Hoeve et al., 2018		324 64/260	58.8 (9) versus 59.1 (9)	Low = 16 Intermediate = 198 High = 78	

The study published by O'Brien et al., indicated that the individualized education session delivered using motivational interviewing techniques was more effective in increasing belief scores in patients with a lower level of education at enrolment compared to those who had a second or over level of education ($p = 0.014$). Concerning knowledge and attitude endpoints of the study, the intervention did not have a significant effect for any of the measured covariates: employment, education, insurance, diabetes, and age [O'Brien et al., 2014].

Oranta et al. examined the benefits of interpersonal counselling for depression, distress, and quality of life during the 18-month follow-up. For distress, the intervention tested was more effective in patients under 60 years of age compared to patients over 60 years of age ($p = 0.033$). For the other endpoints, there were no significant differences in effect size between the two age categories [Oranta et al., 2010; Oranta et al., 2011; Oranta et al., 2012].

The primary objective of Roncella et al. was the determination of the combined incidence of new cardiovascular events (including myocardial re-infarction, death, stroke, any revascularization procedure, life-threatening ventricular arrhythmias, and recurrence of typical angina pectoris) during one year of follow-up. The authors reported that short-term psychotherapy had a significantly higher primary endpoint effect in patients with a life-event score > 10 calculated by Systematic Coronary Risk Evaluation compared to those who had a life-event score < 10 at enrolment (OR = 5.78, 95% CI 1.28 to 26.18) [Roncella et al., 2013].

The effectiveness of psychoeducational therapy may be influenced by the patients' previous medical history, familial history, environmental factors, and support. In this regard, no clinical trial included in this systematic review reported effect sizes on these covariates at follow-up, with only descriptive data of baseline participant characteristics available.

Chi-square, t-test, paired t-tests, and Fisher's test were used to assess differences in patient characteristic variables between the intervention and control groups. The Mann–Whitney U-test was performed to evaluate the differences in the changes between the groups using repeated measures analysis of variance with heterogeneous compound symmetry covariance structure. Binary logistic regression was used to examine the differences in changes between the groups as well as changes within the intervention and control groups.

The intervention details of each trial are summarized in Table XXIV.

Table XXIV. The intervention details for each study

RCT	Type	Description	Delivered by	Dose	Comparator
				Minutes (no. of sessions)	
Chiavarino et al., 2016	Mental fitness	The sessions were conducted in small groups and lasted 90 minutes. The intervention was focused on emotions and thoughts. The protocol was set on cognitive theory being designed for patients with ACS and adapted to the individual power of control of perceptions. The program contained cognitive strategies so that patients were trained to understand and confront the event they were experiencing.	Two specifically trained clinical psychologists	360 (4)	Usual care
Davidson et al., 2010	Problem solving therapy	The meetings were weekly, in person or on the phone, each visit lasting 30-45 minutes. The intervention focused on solving the problem. The protocol was set on increasing the patient's skills. Participants were taught to assess and expose each psychosocial problem. Pleasant regular activities tailored to each patient were encouraged.	Clinical nurse specialist, psychologist, social worker, and/or psychiatrist	120-160 (6-8)	Usual care
Fernandes et al., 2017, 2018	Brief psychological intervention in phase I of	The program was made of three sessions: education on ACS and cardiac rehabilitation, promotion of psychosocial adjustment in post-ACS rehabilitation (cognitive-behavior strategies for reducing stress and anxiety, education for disease awareness and confidence, promoting adaptive coping, self-	Session 1: psychologist, cardiologist	200 (3)	Usual care

	cardiac rehabilitation	monitoring planning and family involvement in coping after discharge) and follow-up after hospital discharge.	Session 2 and 3: Psychologist		
Huffman et al., 2019	Positive psychology exercises combined with motivational interviewing	The sessions were weekly, delivered by phone, with a duration of 30-45 minutes each, for a period of 12 weeks. The intervention was composed of two components: a positive psychology component (focused on completing activities set on positive psychology and their application in everyday life) and a motivational interviewing component (used for goal-setting to specifically promote physical activity).	Study interventionist	360-540 (12)	Positive psychology exercises alone
Nasiri et al., 2020	Mindfulness training program	The meetings were weekly and lasted 2 hours each. The intervention focused on the stress perceived after the acute coronary event and on understanding the disease.	NS	1080 (9)	Usual care
Norlund et al., 2018	Internet based cognitive behavioral therapy	The intervention included 10 modules with different themes adapted to patients with MI: managing worry, fear and avoidance, behavioral activation, problem solving, communication skills, applied relaxation training, managing negative thoughts, coping with insomnia, values in life and relapse prevention. Each module consisted of 2-4 treatment steps. Each treatment stage provided psychoeducation in the form of an electronic text (PDF) along with 1-2 homework assignments. Patients also benefited from additional material and videos that exemplified coping strategies. In addition, patients had access to a discussion board where they could communicate with other patients.	Licensed psychologists	NM (20-40)	Treatment as usual
O'Brien et al., 2014	Individualized education session delivered using motivational interviewing techniques	The meetings were monthly, each visit lasting 40 minutes. The first session was delivered within 2-4 days of hospital admission at the bedside or in a room off the ward. The intervention consisted of face-to-face education sessions, tailored to the needs and impact of the disease on the patient's cognition and emotions. Through motivational training, patients were encouraged to act promptly and appropriately to seek medical attention if the symptoms required.	NS	80-160 (2-4)	Usual care
O'Neil et al., 2015	Telephone based psychotherapy	The sessions took place over the phone for 6 months, with an average duration of 48.4 minutes per session. Intervention sessions were delivered most intensively over the first 3 months. The goal of the program was the depression management and cardiovascular risk reduction. The components of the psychological intervention were: motivational interviewing, goal	Master's level qualified psychologists	384 (8)	Usual medical care

		setting, behavioral activation and cognitive restructuring.			
Oranta et al., 2010-2012	Interpersonal counseling	The content of the intervention was modified for MI patients to take from 1 to 6 sessions (mean 4.6, SD 1.24, mode 5), consisting of: • starting (sessions 1–2): linking the depressive symptoms to the patient's interpersonal situation and choosing the problem area; • encouragement (sessions 3–4): working in the problem area, encouragement, processing life changes, finding resources and coping strategies; • ending phase (sessions 5–6): encouragement to seek help, encouraging and consolidating the gains, developing ways of identifying and countering depressive symptoms in the future.	Psychiatric nurse trained for one day in the practice of interpersonal counseling	130 (1-6)	Standard care after MI
Pristipino et al., 2019 **Roncella et al., 2013**	Short-term psychotherapy	Individual psychotherapy: 3 to 10 sessions of 1 hour each with included personal history elaboration, body language insights, relaxation techniques and dreams analysis. Group psychotherapy: 5 sessions, 2 hours each included the same items of individual sessions plus couple analysis, medical/psychological education and music-therapy.	Single psychotherapist	540-960 (6-13)	Usual care
Sunamura et al., 2017 **Ter Hoeve et al., 2018**	Group counseling sessions delivered using motivational interviewing technique	The intervention was structured in 3 group counseling sessions, face to face, regarding the physical activity performed. Each session lasted 75 minutes. In addition, patients participated in 2 more face-to-face group sessions at 3 and 9 months. Each of these sessions consisted of behavioral counseling on heart-healthy lifestyle lasting 1 hour per session.	Physiotherapist trained in motivational interviewing	345 (5)	Standard cardiac rehabilitation

RCT: randomized controlled trial, ACS: acute coronary syndrome, MI: myocardial infarction, NM: not measurable: NS: not specified

Some psychotherapies were built around the approach of controlling the perception of an event such as mental fitness [Chiavarino et al., 2016] and mindfulness training program [Nasiri et al., 2020], others around solving problems [Davidson et al., 2010], while most psychological interventions consisted of multiple components: education, promoting adaptive coping and cognitive-behavioral strategies [Roncella et al., 2013; Fernandes et al. 2017; Norlund et al., 2018]. Interventions based on motivational interviewing approaches in the presence of positive psychology [Huffman et al., 2019] or in the lack of positive psychology [O'Brien et al., 2014; O'Neil et al., 2015; Sunamura et al., 2018] were among the studies considered. Only one study looked at interpersonal counselling in patients with MI [Oranta et al., 2010]. Only two trials studied group psychological sessions [Chiavarino et al., 2016; Sunamura et al., 2018], most followed the effects of the individual sessions [Oranta et al., 2010;

Davidson et al., 2010; Fernandes et al. 2017; Huffman et al., 2019; Nasiri et al., 2020; Norlund et al., 2018; O'Brien et al., 2014; O'Neil et al., 2015]. A single trial administered both individual and group sessions [Roncella et al., 2013]. The total dose of psychotherapy was expressed as total minutes. Among the included studies, the highest dose was 1080 minutes [Nasiri et al., 2020], the lowest dose being 80-160 minutes [O'Brien et al., 2014].

The findings of the included studies are visualized in Figure 36, being systematically described as follows:

The impact on morbidity

Regarding this aspect, we analysed major adverse cardiovascular events, New non-cardiovascular events and rehospitalisation.

In the COPES trial, Davidson et al. [Davidson et al., 2010] followed *major adverse cardiovascular events* (MACE), defined as MI or hospitalization for UA. At 9 months, patients receiving problem-solving therapy had fewer MACE events (3 events) compared to those in the control group (10 events), with a $p = 0.047$. In the STEP-IN-AMI trial, Roncella, Pristipino et al. [Roncella et al., 2013; Pristipino et al., 2019] had as a primary outcome a composite index consisting of re-infarction, death, stroke, revascularization, major adverse cardiac and cerebrovascular events, life-threatening ventricular arrhythmia, and recurrence of typical angina. Roncella et al. followed the group of patients for one year: patients who received short-term psychotherapy had a significantly reduced incidence of the primary composite endpoint (21 events) compared to the group who received the usual care (35 events), with a $p = 0.0006$ and with a 35% reduction of the absolute risk [Roncella et al., 2013].

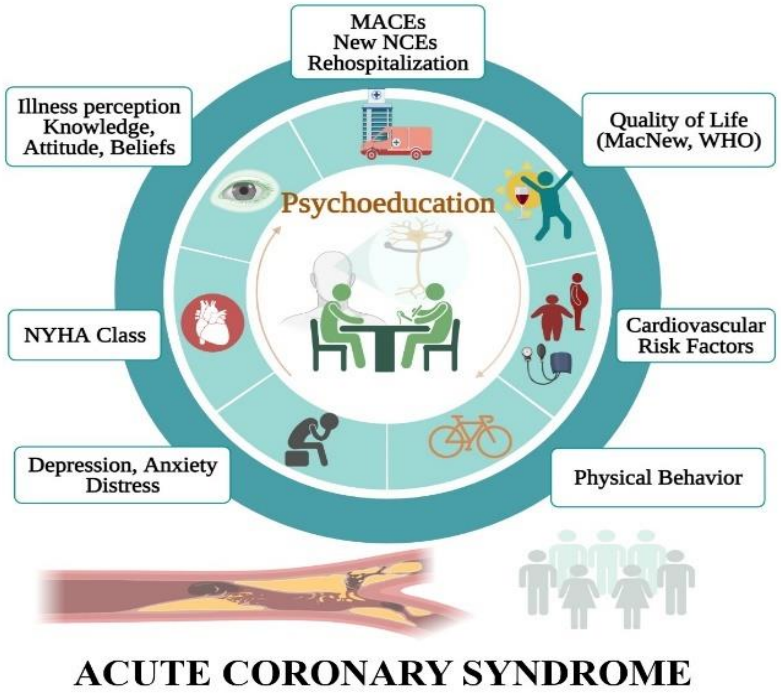


Figure 36. Benefits of psychoeducation rehabilitation focused on acute coronary syndrome

Pristipino et al. followed the group of patients up to 5 years: both for the combined incidence of new cardiovascular events and for the cardiovascular events monitored

individually, there was no statistically significant reduction in the intervention group compared to the control group [Pristipino et al., 2019].

The STEP-IN-AMI trial showed a marked reduction of the *newly diagnosed non-cardiac events* in the group of patients who received short-term psychotherapy at one year ($p < 0.0001$) according to the results published by Roncella et al. [Roncella et al., 2013], as well as at 5 years ($p < 0.0001$) according to the results published by Pristipino et al. [Pristipino et al., 2019]. In addition, the authors highlight the fact that such a significant effect has an important impact in improving the long-term prognosis of a post-ACS patient.

With regard to rehospitalisation, Oranta et al. proved that patients who received interpersonal counselling had a significant reduction in the use of any specialized healthcare service compared to usual care ($p = 0.007$) [Oranta et al., 2012]. Though, the characteristics of the patients proved to be important for the patient's initiative to seek medical attention. The STEP-IN-AMI trial aimed to assess the total number of hospitalizations, the number of cardiovascular and non-cardiovascular hospitalizations in patients who received short-term psychotherapy compared to usual care. Roncella et al. demonstrated that patients in the intervention group had a significant one-year reduction in the total number of hospitalizations ($p = 0.02$) [Roncella et al., 2013]. Pristipino et al. revealed the absence of significant improvement at 5 years for any component of the outcome [Pristipino et al., 2019].

Health related quality of life

Health-related quality of life (HRQoL) was measured by the MacNew Questionnaire in 2 of the studies included in the analysis, both with evidence of its benefits through psychological interventions. The study of Sunamura et al. identified an improvement in the emotional component ($p = 0.004$) and also in the physical one ($p = 0.015$) [Sunamura et al., 2018]. Roncella et al. demonstrated positive effects on the physical component ($p = 0.03$), lack of significant enhancement for the other two components (emotional and social), and absence of the overall score improvement [Roncella et al., 2013]. The SF-12 questionnaire was used to quantify the quality of life in 2 of the included studies. O'Neil et al. [O'Neil et al., 2015], respectively, Huffman et al. [Huffman et al., 2019] demonstrated the absence of significant improvement for physical and mental component building HRQoL score. Chivarino et al. evaluated the quality of life through the World Health Organization Quality of Life – Brief questionnaire (WHOQOL- Brief) [Chiavarino et al., 2016]. At the 8-month evaluation, significant time*group interactions were noted for total score ($p < 0.001$), physical health ($p < 0.001$), psychological health ($p < 0.001$), social relationships ($p < 0.001$) and environment ($p = 0.026$). Oranta et al. measured the quality of life using the EuroQol-5D (EQ-5D) questionnaire. Compared to that standard care, interpersonal counselling did not improve quality of life after myocardial infarction, but the intervention provided positive effects on quality of life in patients over 60 years old [Oranta et al., 2011].

Cardiovascular risk factors

According to Chivarino et al., there is a strong link between mental fitness and the following medical parameters: systolic blood pressure ($p = 0.019$), heart rate ($p = 0.023$), ventricular ejection fraction ($p = 0.021$), low-density lipoprotein cholesterol ($p <0.001$), high-density lipoprotein cholesterol ($p < 0.001$), triglycerides ($p = 0.047$) and serum creatinine ($p =$

0.002) [Chiavarino et al., 2016]. The study's findings revealed no statistically significant data on diastolic blood pressure, blood glucose, and BMI. Additionally, the OPTICARE trial [Roncella et al., 2013] strengthened the favourable results of a psychoeducational intervention on total cholesterol ($p < 0.001$) and smoking cessation ($p < 0.001$), without obtaining statistically significant data on SCORE Risk Score, WC (cm), and systolic BP. Consequently, the two studies obtained contradictory results for systolic blood pressure.

Physical behavior

The study of Chivarino et al. found that mental fitness had a substantial impact on the number of patients who continued *physical activity* from enrolment to follow-up (p 0.001) when compared to usual care [Chiavarino et al., 2016]. Psychoeducational interventions delivered using motivational interviewing techniques have proven their effectiveness in promoting physical activity. Huffman et al. evaluated the impact of positive psychology exercises combined with motivational interviewing on physical activity measured with the accelerometer, the results obtained being promising: higher moderate-to-vigorous physical activity (MVPA) at 24 weeks ($p = 0.026$) by completing 9–15 more minutes per day and taking 1600–1800 more steps per day in the intervention group compared to the control group [Huffman et al.,2019]. In 2018, Ter Hoeve et al. described the statistically significant impact of group counselling sessions delivered using a motivational inter-viewing technique on the volume of physical behaviour: higher daily step count ($p = 0.035$, additional 513 steps per 14.5 h of day-time waking hour) and increased time in prolonged MVPA ($p = 0.002$) in the intervention group compared to the control group [Ter Hoeve et al., 2018].

Psychological variables

We focused on depression and anxiety, distress, positive affect and coping strategies, self-esteem, and health locus of control. Multiple research has examined the impact of psychoeducation on emotional states, with a set of validated questionnaires used to determine the results. Therefore, Beck Depression Inventory (BDI) was used in 3 of the analysed studies, all with substantial evidence regarding the association between intervention and depression relief [Davidson et al., 2010; Oranta et al., 2010; Roncella et al., 2013]. The results of studies that measured *depression and anxiety* using the HADS were inconsistent. Specifically, Fernandes et al. demonstrated that psychological intervention enhanced statistically significant both the total score and the 2 components: anxiety and depression (all $p < 0.0001$) [Fernandes et al., 2017]. On the opposite, Nourlund et al. found that patients in both groups (intervention and control) reported a reduction in the class of depressive symptoms, with no difference between the two groups during follow-up [Norlund et al., 2018]. Besides the two depression assessment scales, O'Brien et al. noted the absence of depressive symptoms improvement when measured with Cardiac Depression Scale (CDS), but reported statistically significant effect when evaluated with Patient Health Questionnaire 9 (PHQ 9), with a $p = 0.025$ [O'Brien et al., 2014].

Anxiety was provided as an independent outcome of depression in 2 of the studies, the results being contradictory. More specifically, for anxiety measured using the Cardiac Anxiety Questionnaire [Norlund et al., 2018] psychotherapy didn't prove a significant outcome, while

for anxiety assessed using Anxiety Score [Sunamura et al., 2018] the intervention was associated with favorable results (p = 0.036).

Nasiri et al. demonstrated that the mindfulness-based training program was associated with *decreased stress* levels measured with the Perceived Stress Scale - 14 (p <0.001) two months after the intervention [Nasiri et al., 2020]. In contrast, studies that assessed stress through Symptom Checklist-25 did not obtain statistically significant results when evaluated 12 months after surgery [Roncella et al., 2013], respectively, at the 18 months evaluation [Oranta et al., 2010].

In their study, Huffmann et al. evaluated the outcome regarding the positive psychology exercises combined with motivational interviewing on the *positive affect*, a variable measured using the Positive and Negative Affect Schedule (PANAS) [Huffman et al., 2019]. In essence, the authors demonstrated a statistically significant association between intervention and improvement in positive affect (p <0.001). The authors propose that increasing positive affect in post-ACS patients may have important implications, proving the association between positive affect and lower risk of overall mortality in healthy persons and chronic illnesses such as HIV and diabetes [Kubzansky et al., 2018; Massey et al., 2017].

A study of Chivarino et al. aimed to evaluate *coping strategies, self-esteem, and health locus of control* that they measured using Brief Coping Orientations to Experienced Problems (Brief-COPE), General Self-Efficacy Scale (GSES), respectively Multidimensional Health Locus of Control Scale - form C (MHLC-C). The results were statistically significant regarding the association between mental fitness and coping strategies, both for the total score (p = 0.027) and for two of the three components: emotion-focused (p = 0.001) and problem-focused subscale (p = 0.002). A substantial out-come was also observed in the relationship between intervention and health locus of control (p = 0.002). There hasn't been observed a significant effect regarding self-esteem (all p> 0.652) [Chiavarino et al., 2016].

Illness variables

We followed cardiac symptomatology, illness perception and knowledge, attitude, and beliefs about illness. At both a one-year follow-up (p = 0.01) [Roncella et al., 2013] and a five-year follow-up (p = 0.01) [Pristipino et al., 2019], the NYHA class improved significantly in patients who received short-term psychotherapy compared to usual care. Despite having a higher NYHA class, the echocardiographic parameters (ejection fraction and wall motion score index) in the intervention group were identical to those in the control group at follow-up. Furthermore, the authors hypothesized that psychotherapy affects the severity of symptoms rather than the degree of dyspnea itself.

According to Nasiri et al., the mean score of *illness perception* assessed using the Brief Illness Perception Questionnaire (BIPQ) was substantially higher in patients in the intervention group (mindfulness-based training program) than in the control group (p <0.001) [Nasiri et al., 2020]. Fernandes et al. evaluated illness cognition through Portuguese versions of BIPQ [Fernandes et al., 2017].

The term "illness representations" refers to a patient's perception of the illness's outcomes, timeline, symptom experience, emotions, concern, personal control, and comprehensibility. For all parameters of illness representations, the study revealed significant time/group interaction effects (all p <0.001). Precisely, patients in the intervention group (short

psychological intervention in phase I of cardiac rehabilitation) felt less unpleasant experiences related to their condition and more positive events related to their maintenance during the follow-up period. Patients in the control group, on the other hand, showed an increase in perception of the unfavourable repercussions of their disease following discharge.

Knowledge was evaluated in two of the trials included in our analysis, both with significant results regarding the beneficial outcome of the studied intervention. Accordingly, O'Brien et al. demonstrated a substantial effect of individualized educational intervention on *knowledge* ($p < 0.001$), *attitude* ($p = 0.003$) and *belief* ($p < 0.001$) about ACS [O'Brien et al., 2014]. Furthermore, Fernandes et al. proved a notable impact of psychoeducational intervention in improving knowledge about the disease and maintaining it throughout the follow-up ($p = 0.000$) [Fernandes et al., 2019].

I.3.3.5. Discussion

As far as we know, this systematic review is the first that aimed to summarize the evidence regarding the impact of psychoeducational rehabilitation in patients with ACS. From our point of view, the provided data is clearly encouraging in terms of the utility of these interventions to improve hard endpoints as well as the quality of life including alleviation of symptoms of depression and anxiety. Still, considering the heterogeneity of the included studies, we emphasize the need for large RCTs with structured integrated multi-modality psychological interventions with a detailed methodology of implementation. More specifically, it provides conclusive data for the effectiveness of psychological intervention compared with usual care.

We propose a personalized medicine approach in the psychoeducational rehabilitation of ACS by providing in detail the interventions used in the included randomized controlled trials (type of psychotherapy, number of sessions, and total dose performed) and therefore exposing their heterogeneity.

Figure 37 depicts the effects of psychoeducational therapies on various components of cardiac rehabilitation.

The majority of research in the area included subjects without a determination of various mental health comorbidities among ACS before the intervention. Only a few trials have separated the intervention group into two subgroups: with and without this comorbidity, according to the literature. We did not find any RCT that studied the benefits of a psychoeducational intervention applied only to patients without a mental disorder diagnosed with ACS. This is an important area for future research, taking into account a substantial increase in the prevalence of mental health disorders among patients with acute MI, according to Sreenivasan et al. [Sreenivasan et al., 2021].

Particularly for depression, is described as a multifaceted and bidirectional relationship with cardiovascular disease, especially with ACS [Amadio et al., 2020]. Therefore, depression by itself may be the cause of MI, but it is not known whether psychoeducation in this category of patients has similar benefits to the same intervention in patients without depressive symptoms.

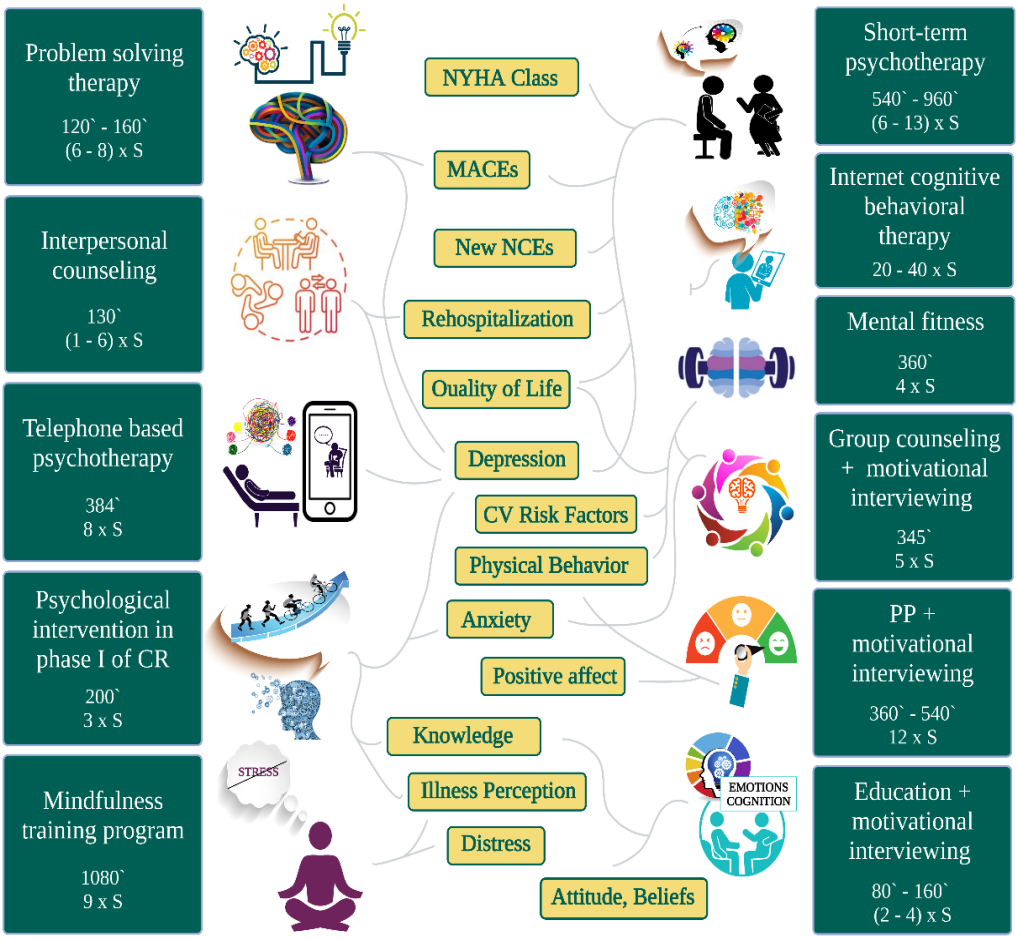


Figure 37. The benefits of psychoeducational interventions specific to the type of cardiac re-habilitation programs and their total dose expressed in number of sessions (S) and minutes (`). CR = cardiac rehabilitation, PP = positive psychology, NCEs = non-cardiovascular events.

The benefit of psychological counseling on mortality and morbidity in CAD has been proven in meta-analyses in this field [Linden et al., 1996; Rees et al., 2004; Nicholson et al., 2006; Whalley et al., 2011; Richards et al. 2017; Norlund et al., 2018]. The latest update of the most rigorous reviews (by Cochrane Collabo-ration) [Rees et al., 2004] showed the benefit in the current era of optimal psychoeducational intervention. Regarding prognostic outcomes, the positive clinical outcome that resulted from the analysis was cardiac mortality (RR 0.79, 95% CI 0.63 to 0.98). on the other hand, no obvious effect was demonstrated in terms of risk reduction for total mortality (RR 0.90, 95% CI 0.77 to 1.05), rates of revascularization (RR 0.94, 95% CI 0.81 to 1.11), and rates of non-fatal MI (RR 0.82, 95% CI 0.64 to 1.05). The meta-analysis has revealed a reduction in depressive symptoms (SMD -0.27, 95% CI -0.39 to -0.15), anxiety (SMD -0.24, 95% CI -0.38 to -0.09), and stress (SMD -0.56, 95% CI -0.88 to -0.24) in the intervention group compared to the comparator group. Furthermore, by direct comparison of the studies, the authors demonstrated positive effects on health-related quality of life, type A behaviour, and vital exhaustion. Additionally, the systematic review of Reid et al. completes the data from the literature and shows the benefits of psychological intervention on blood pressure for patients [Reid et al., 2013]. Also, the authors describe a positive effect on knowledge and satisfaction for both patients and their partners. As demonstrated in the

Cochrane review [Dusseldorp et al., 1999], education-based intervention in CAD reduced fatal and/or non-fatal cardiovascular events (other than MI) compared to control groups receiving no education (RR 0.36, 95% CI 0.23 to 0.56). Concerning the health-related quality of life, the heterogeneity of measures applied in the studies included in this me-ta-analysis made it impossible to have consistent evidence. Nevertheless, there is limited information regarding the improvement of some domain scores. There was no difference in the outcomes for total mortality, fatal and/or non-fatal MI, total revascularizations, and hospitalizations.

This paper has some *limitations*. First, the group of patients analysed has a defective distribution between the 2 genders (male / female sex ratio = 2.62), which limits the generalization to the general population. This is due to the fact that sex is a risk factor for CAD, including ACS [Byars et al., 2018] which will lead to a preponderance of male patients in our study [Haider et al., 2020]. Secondly, we searched in the databases only articles published in English. Thirdly, the comparison with placebo does not apply to psychological and educational interventions, in all trials the control group being the usual care one. Thus, the nonspecific effects of psychotherapy and education were not accounted for. Fourth, we did not investigate intervention for other emotional disorders such as bipolar disorder. As in any systematic review, there may be a publication bias and the overall picture may be underlined based on positive results, being known that unsuccessful studies do not end up being published. Finally, there are some important gaps in the literature. It is important to emphasize that the findings are limited by the paucity of randomized controlled trials that have studied psychoeducational intervention exclusively in patients with ACS. Psychoeducational interventions were miscellaneous in terms of the type of intervention, the number of sessions and the total duration, the enrolled population, and the setting (phone or in-person or both). Therefore, there is no possibility of a meta-analysis, mainly due to the heterogeneity of outcomes and their measurement tools.

I.3.3.6. Conclusions

Psychoeducational rehabilitation appears valuable in ACS, being associated with improvement in new non-cardiovascular events, quality of life, most cardiovascular risk factors, physical behaviour, mental health outcomes such as depression, anxiety, and distress, along with illness perception and cognitions. Most therapies, on the other hand, failed to improve diastolic blood pressure, blood glucose, BMI, abdominal circumference, or self-esteem. MACEs and rehospitalisation were dramatically reduced one year following psychotherapies, however this benefit did not last five years.

In the era of personalized medicine, patients with ACS should benefit from specific psychoeducational strategies and the choice of type of intervention should be chosen in accordance with the evidence-based guidelines. Unfortunately, there are currently a limited number of clinical trials that study the effect of psychoeducation focused on MI. Taking into account the heterogeneity issue of these studies available in the literature at the moment, we highlight the need for large RCTs with structured integrated multi-modality psychological interventions with detailed methodology of implementation. Additionally, there is a critical need to establish a number of sessions and a total dose standardized by experts in the field, but this requires further studies. Given the possible health consequences and significant costs of untreated emotional disorders (especially depression) in patients with heart disease, there is a necessity for RCT to evaluate the impact of psychotherapy on cardiac morbidity and mortality.

I.3.4. IPAQ-L and CPET usefulness in cardiac rehabilitation

I.3.4.1. Introduction

Sedentarism is one of the most important modifiable cardiovascular risk factors [AACPR, 2013] and a vital component of any cardiovascular rehabilitation (CR) program. According to current European Guidelines [Piepoli et al., 2016], adults should engage in at least 150 minutes of moderate-intensity aerobic physical activity per week, or 75 minutes of vigorous-intensity aerobic physical activity per week, or an equal combination of moderate and vigorous-intensity activity. In line with the guidelines, effort should be divided into at least 10 minute sessions. Significant exercise intolerance is a hallmark of heart failure with reduced ejection fraction (HFrEF) [Piepoli et al., 2011]. For the first time, the efficacy and safety of exercised-based cardiac rehabilitation were documented by Coats et al. [Coats et al., 1990]. A recent meta-analysis assessed the effectiveness of the program in patients with heart failure. The results showed an increase in quality of life and exercise capacity, but without finding a significant advantage in terms of mortality and hospitalization [Bjarnason-Wehrens et al., 2020].

Obesity has risen dramatically in the absence of physical exercise, particularly in the present COVID-19 pandemic, imposing a multidisciplinary approach. Numerous efforts must be made to enhance physical activity and to reduce obesity - promoting lifestyles in order to achieve long-term morbidity and mortality reduction [Zupo et al., 2020]. Variations in moderate to vigorous physical activity and sedentary activity assessed by the international physical activity questionnaire (IPAQ) or accelerometry can contribute to define a population's health profile, according to a study published in 2020. Moreover, a 16-week supervised aerobic exercise program was successful in improving self-reported physical activity, lowering sedentarism, and optimizing sleep quality in overweight/obese adults with hypertension [Martinez Aguirre-Betolaza et al., 2020].

The design of methods to reliably quantify physical activity (PA) is critical for both research and follow-up of CR patients, who often deal with poor compliance to lifestyle changes. In a thorough assessment, the duration, frequency, and intensity of PA should all be included, making such a measurement complex and challenging [AACPR, 2013]. Direct observation, PA questionnaires, patient diaries, and direct measurement have all been presented as approaches to evaluate PA [Hills et al., 2014]. The most common methods utilized in the CR program are self-report surveys and physical activity monitors.

In the face of recent significant advancements in portable direct physical activity monitors, clinical evidence on their reliability in cardiovascular disease patients is still scarce [Maiorana et al., 2017]. The monitor must be worn 12 h / day for at least 4 days to produce an acceptable PA assessment. Even though it has the benefit of quantifying inactivity period, such a system is still unavailable for the majority of Romanian middle-aged and elderly adults, who constitute the majority of patients admitted to Cardiac Rehabilitation Clinics, making self-report questionnaires a better method for such facilities [AACPR, 2013; Maiorana et al., 2017]. Even if these devices are available on the Romanian market at inaccessible prices for patients, the Clinical Rehabilitation Hospitals in Romania do not have the economic potential to offer these devices to patients. In Romania, CR programs are not funded separately and there are also no

policies through which cardiovascular patients are sent to this service, so that addressability is minimal.

The ability of the patient to describe and categorize their recent PA into easy, moderate, and vigorous is a fundamental limitation of self-report surveys. The IPAQ questionnaire was validated in 14 locations across 12 nations and yields consistent PA estimates. IPAQ is available in two versions. While the short form is recommended for large prevalence studies, the IPAQ long form (IPAQ - L) provides the advantage of a more detailed analysis of PA on 4 different domains (occupation, transportation, home, leisure time) [Craig et al., 2003]. The IPAQ - L addresses PA performed in the past 7 days, assessing the frequency and average time of walking, moderate and vigorous effort, re-ported for each of the 4 domains listed above.

I.3.4.2. Aim

Considering its accessibility and minimal cost, we hypothesized that the IPAQ-L questionnaire would be useful for Romanian patients. The study's primary objective was to see how beneficial the IPAQ-L questionnaire was in patients with HFrEF who were enrolled in a CR program. The secondary objectives of the study were: (i) the evaluation of the relationship between the items of the IPAQ-L questionnaire and the clinical, biological and paraclinical characteristics of the patients; (ii) the influence of cardiovascular comorbidities including, obesity, hypertension and type 2 diabetes on IPAQ-L results, and (iii) the relationship between the PA parameters evaluated by the IPAQ-L questionnaire and the effort capacity determined by cardiopulmonary exercise testing (CPET) for a suitable subgroup. The usefulness of IPAQ-L evaluation compared to CPET was to assess the criterion validity for a PA questionnaire using a direct method. The validation by using a direct method is needed to estimate the absolute amount of PA and is most relevant when monitoring adherence to health-enhancing PA recommendations.

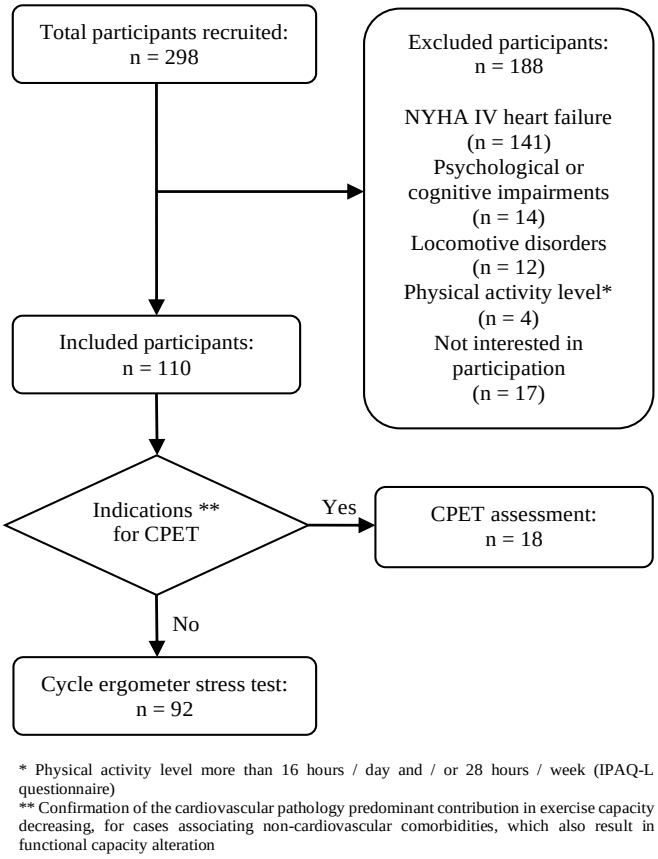
I.3.4.3. Material and methods

Study design and setting

A single-center cross-sectional study including 110 patients aged 18-69 years admitted between 01 January 2017 and 31 December 2017 in the Cardiovascular Clinic of the Rehabilitation Clinic Hospital from Iasi, Romania was conducted. The aim of the research was to assess PA patterns in patients with HFrEF which adhered to a CR program. The diagnostic criteria of HFrEF during the admission was left ventricular ejection fraction less than or equal to 40%, a hemodynamic parameter measured with trans-thoracic echocardiography. We obtained the approvals of the local Ethics Committee for Scientific Research of the University of Medicine and Pharmacy of Iasi and the Ethics Committee for Scientific Research of the Rehabilitation Clinic Hospital. All patients signed an informed consent and underwent clinical examination, blood tests, cycle ergometer stress test, and self-evaluation of PA level using the IPAQ - L questionnaire.

Initially 298 hospitalized patients with the HFrEF diagnosis were screened for their eligibility to participate in the study. One hundred and forty-one of them were diagnosed with NYHA IV heart failure, 14 of them were diagnosed with psychological or cognitive impairment that limited the CR, 12 patients had locomotive disorders that excludes the participation in an

exercise training program, 4 individuals performed PA for more than 7 hours / day and / or 28 hours / week, and 17 were not interested in participating in the study. As a final point, 110 patients with HFrEF were eligible as being included the study. All of the above can be seen in Figure 38.



* Physical activity level more than 16 hours / day and / or 28 hours / week (IPAQ-L questionnaire)
** Confirmation of the cardiovascular pathology predominant contribution in exercise capacity decreasing, for cases associating non-cardiovascular comorbidities, which also result in functional capacity alteration

Figure 38. *Study flow chart*

Study procedures and outcome assessment

Patients' assessment included medical history such as comorbidities, clinical information, anthropometrics; in addition, blood tests such as lipid profile, cardiovascular and pulmonary evaluation were done. PA assessment was conducted using the IPAQ - L, which was present within the first 24 hours of admission. The English version of the questionnaire was translated into Romanian, according to current guidelines and recommendations [IPAQ, 2010]. The interview was conducted by a single physician trained to deal with patient inter-rogation. The total time spent performing different types of PA was converted into minutes. As short trainings are known to have insignificant metabolic effects [Ambrosetti et al., 2021], the final analysis of PA level included only activities with a minimum interval of 10 minutes. Physical activity levels are expressed by total metabolic equivalent of task (MET) - min / week, obtained by multiplying predefined MET scores with the duration of a specific PA (in minutes) [Wu et al., 2016]. We calculated the total MET - min / week, as well as the value for each activity (walking, moderate and vigorous effort) and for each do-main (work, transportation, leisure and domestic and garden), respectively. Further-more, according to IPAQ scoring guidelines [Bauman et al., 2009], we divided our study group in 3 categories, based on their PA levels:

low, moderate and high. Sitting time / week is re-ported as total (minutes). Sedentary time is quantified as minutes / week.

According to body mass index (BMI), our study group was categorized as normal weight (18.5 - 24.9 kg/m²), overweight (25 - 29.9 kg/m²), first degree obesity (30 - 34.9 kg/m²), second degree obesity (35 - 39.9 kg/m²) and third-degree obesity (> 40 kg/m²). The abdominal circumference (AC) was measured at the midpoint of the line be-tween the rib or costal margin and the iliac crest in the midaxillary line. Abdominal obesity was defined as waist circumference > 88 cm for female and > 102 cm for male. Fitness was quantified by the percentage of age-predicted maximal heart rate (%HR), exercise resistance (W) and workload (METS), on a symptom-limited, cycle ergometer stress test using a standard protocol [McDonough et al., 1969].

Patients in whom the etiology of exercise capacity limitation could not be clearly established were suitable for cardiopulmonary exercise testing (CPET). A subgroup of 18 patients was thus formed, in which this investigation was carried out on the same day with PA assessment with IPAQ - L, a few hours after the patients completed it. The most important CPET parameters were: absolute value of maximal oxygen uptake (VO2 max) and percentage of his predicted value (VO2 max%), absolute value of the maximal work rate (WR) and percentage of his predicted value (PR%), oxygen uptake at the anaerobic threshold (AT), maximal value of the respiratory exchange ratio (RER), maximal heart rate (HR), heart rate reserve (HRR). The HRR is determined by difference between maximal HR and resting HR.

Statistical analysis

We used SPSS v 20.0 for all statistical analyses (SPSS Inc., Chicago, Illinois, USA). For group comparisons, chi-square and student's t tests were done. The Mann - Whitney U test has been used as an alternative to the student's t test when the data were not normally distributed. Descriptive data were displayed as means ± SD (standard deviation), medians with interquartile range or percentages, as appropriate. A p value < 0.05 was considered statistically significant. The Spearman correlation coefficients were used to analyse the relationships between variables.

I.3.4.4. Results

There were 110 patients in our study, with an average age of 57.2 years. Table 1 shows the descriptive data of our research population. On admission, the group had a balanced gender ratio (47.27 % men and 52.72 % females), as well as similar BMI and blood pressure levels. Despite the fact that males had a higher average glycemia than females, females had a lower lipid profile than males. Regarding cycle ergometer stress test results, females achieved a slightly higher %HR, but male subjects presented both better exercise resistance (105.84 W versus 75.84 W) and a higher workload (5.21 METS versus 4.54 METS), as are described in Table XXV.

Overweight, first, second and morbidly obese patients accounted for respectively 47.27 %, 27.27 %, 13.63 %, and 2.72 % of our study group. Among the study participants, 80 % of them were hypertensive and more than 50 % were diagnosed with third degree hypertension (Table XXV). The presence of hypertension and / or diabetes did not influence IPAQ - L results.

Table XXV. Descriptive statistics of the study population

Variables	Total	Males	Females	*p* Value*
Number, n (%)	110 (100)	52 (47.27)	58 (52.72)	
Age (years), mean (SD)	57.20 (6.45)	57.11 (7.05)	57.27 (5.92)	0.897
Weight (kg), mean (SD)	83.48 (12.40)	88.15 (12.41)	79.29 (10.89)	< 0.001
BMI (kg/m^2), mean (SD)	30.28 (4.80)	29.66 (5.22)	30.84 (4.36)	0.200
AC (cm), mean (SD)	97.81 (12.41)	101.09 (13.18)	94.87 (10.96)	0.008
SBP (mmHg), mean (SD)	134.44 (15.31)	132.96 (17.57)	135.77 (12.97)	0.346
DBP (mmHg), mean (SD)	83.65 (10.52)	83.88 (10.20)	83.44 (10.89)	0.829
Glycemia (mg/dl), mean (SD)	114.73 (38.90)	121.61 (35.79)	108.45 (32.49)	0.048
TC (mg/dl), mean (SD)	200.00 (42.97)	190.15 (45.68)	208.82 (38.68)	0.022
HDL - C (mg/dl), mean (SD)	48.22 (13.15)	45.17 (11.87)	51.05 (13.75)	0.019
LDL - C (mg/dl), mean (SD)	121.88 (37.99)	115.98 (39.76)	127.45 (35.73)	0.127
Non - HDL cholesterol (mg/dl), mean (SD)	151.39 (41.00)	144.98 (44.14)	157.35 (37.26)	0.118
TG (mg/dl), mean (SD)	158.72 (99.80)	161.09 (111.53)	156.60 (88.91)	0.815
EGFR (ml/min/1.73 m^2), mean (SD)	84.02 (16.58)	84.02 (17.28)	81.34 (15.60)	0.075
LVEDD (mm), mean (SD)	48.28 (6.29)	51.63 (5.14)	45.08 (5.61)	< 0.001
LVMI (g/m^2), mean (SD)	120.96 (35.58)	134.11 (33.44)	107.19 (32.74)	< 0.001
%HR (%), mean (SD)	75.34 (12.54)	72.82 (11.89)	77.60 (12.77)	0.045
Exercise resistance (W), mean (SD)	90.02 (30.17)	105.84 (30.01)	75.84 (22.38)	< 0.001
Workload (METS), mean (SD)	4.85 (1.33)	5.21 (1.39)	4.54 (1.20)	0.008
Weight status, n (%): Normal weight	10 (9.09)	6 (11.54)	4 (6.9)	0.512
Overweight	52 (47.27)	25 (48.08)	27 (46.50)	1.000
First degree obesity	30 (27.27)	15 (28.85)	15 (25.86)	0.831
Second degree obesity	15 (13.63)	5 (9.62)	10 (17.24)	0.278
Third degree obesity	3 (2.72)	1 (1.92)	2 (3.45)	1.000
Hypertension, n (%): Normotensive	22 (20)	15 (28.85)	7 (12.07)	0.033
First degree HTN	10 (9.09)	2 (3.85)	8 (13.79)	0.099
Second degree HTN	20 (18.18)	5 (9.62)	15 (25.86)	0.046
Third degree HTN	58 (52.72)	30 (57.69)	28 (48.28)	0.345
Type 2 Diabetes, n (%)	31 (28.18)	21 (40.38)	10 (17.24)	0.010

** Mann - Whitney U test; BMI: body mass index; AC: abdominal circumference; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; non-HDL: non–high-density lipoprotein cholesterol; TG: triglycerides; EGFR: estimated glomerular filtration rate; LVEDD: left ventricular end-diastolic diameter, LVMI: left ventricular mass index; %HR: percentage of age-predicted maximal heart rate; HTN: hypertension*

Obese and overweight patients had a longer time to accomplish the walking parameter compared to the subjects with a body mass index less than 25 kg/m2 (Δ= 2306.56 MET – minutes / week, p< 0.001and Δ= 1765.81, p< 0.001, respectively), but no significant difference was reported regarding total weekly PA among these subgroups.

Moderate PA is the most common activity level for both male and female, ac-counting for over 60 % of total PA. Although females were more likely to be involved in physical activities, 31.79 % of males had a low PA rate (Table XXVI).

There were no statistically significant differences between genders in terms of weekly PA, also per each of the 4 analysed domains, and per difficulty (walking, moderate and respectively vigorous exercises), as are described in Table XXVII. The domestic and garden domain was the chosen type of PA for both males and females, accounting for approximately 60 % of total MET - minutes per week.

Table XXVI. Distribution of total physical activity among subgroups

	Total	**Males**	**Females**
Low level of PA (%)	27.07	31.79	22.40
Moderate level of PA (%)	65.35	62.62	68.04
High level of PA (%)	7.57	5.58	9.54

PA: physical activity

Table XXVII. IPAQ questionnaire results

	Age		**MTCF**	
	r Value	*p* Value*	*r* Value	*p* Value*
Total MET – minutes / week at work	- 0.28	0.002	0.28	0.002
Total MET – minutes / week for transportation	- 0.01	0.850	0.01	0.850
Total MET – minutes / week domestic and garden	- 0.01	0.852	0.01	0.852
Total MET – minutes / week in leisure time	0.04	0.631	- 0.04	0.631
Vigurous PA (leisure time)	- 0.12	0.183	0.12	0.183
Total physical activity MET – minutes / week	- 0.18	0.053	0.18	0.053
BMI (kg / m²)	0.15	0.107	- 0.15	0.107
AC (cm)	0.10	0.295	- 0.10	0.295
LVEF (%)	0.10	0.338	- 0.10	0.338

* Spearman's rho; MET: total metabolic equivalent of task; MTCF: maximum tolerated cardiac frequency; MET: metabolic equivalent of task; PA: physical activity; BMI: body mass index; AC: abdominal circumference; LVEF: left ventricular ejection fraction

Table XXVIII. Correlations between CPET parameters and IPAQ - L questionnaire

CPET parameters	**IPAQ - L and Physical activity (METS – minutes / week)**							
	Vigorous activity		Moderate activity		Walking		Total PA	
	r Value	*p* Value	*r* Value	*p* Value	*r* Value	*p* Value	*r* Value	*p* Value
VO2max%	0.52	0.025	0.19	0.447	0.32	0.195	0.34	0.168
AT	0.53	0.026	0.12	0.632	0.16	0.522	0.30	0.228
RER	0.02	0.923	0.18	0.468	0.16	0.502	0.07	0.781
WR%	0.52	0.027	0.45	0.061	0.48	0.040	0.48	0.040
HRR	0.61	0.007	0.63	0.005	0.59	0.009	0.65	0.003

VO2max% - percentage of the predicted maximal oxygen uptake; AT - oxygen uptake at the anaerobic threshold; RER - maximal value of the respiratory exchange ratio; HRR - heart rate reserve

Outcomes

Vigorous activity was statistically significant correlated with VO2 max% (r= 0.52, p= 0.025), and with AT (r= 0.53, p= 0.026). However, the correlation between VO2 max% and total physical activity did not reach statistical significance (r= 0.34, p= 0.168). Total physical activity presented significant correlations with WR% (r= 0.48, p= 0.04) and HRR (r= 0.65, p= 0.003), determined by CPET. HRR also correlated with vigorous activity (r= 0.61, p= 0.007), moderate activity (r= 0.63, p= 0.005), and walking (r= 0.59, p= 0.009) (Table XXVIII).

Age was significantly correlated with total MET - minutes / week at work (r= - 0.28, p= 0.002), but not with total activity in the other 3 analysed domains or with sit-ting time (p> 0.05). Furthermore, age presented a weak borderline correlation with total physical activity MET – minutes / week (r= - 0.18, p= 0.053), but not with BMI, AC or EF. Maximum tolerated cardiac frequency Fitness (MTCF) exhibited a significant positive correlation with total MET- minutes / week at work (r= 0.28, p= 0.002), and a borderline correlation with total physical activity MET – minutes / week (r= 0.18, p= 0.053), but not with BMI or AC (Table XXIX).

Table XXIX. Correlations between age and MTCF and physical activity, BMI, AC and LVEF

	Total		**Males**		**Females**		***p* Value***
	Mdn (IQR)	(%)	Mdn (IQR)	(%)	Mdn (IQR)	(%)	
Total MET – minutes / week at work	6132 (1936-11370)	21.1	15210 (6194-25524)	22.6	2866 (2087-6936)	19.7	0.384
Total MET – minutes / week for transportation	594 (198-1386)	18.1	988 (181-8752)	20.3	877 (330-4788)	15.9	0.517
Total MET – minutes / week domestic and garden activities	2520 (240-5790)	54.5	12675 (4147-17085)	50.8	1350 (180-6720)	58.1	0.019
Total MET – minutes / week in leisure time	198 (33-594)	6.1	123 (49-198)	6.1	579 (99-1482)	6.1	0.983
Total sitting (minutes)	540 (420-660)		545 (300-692)		540 (360-600)		0.899
Total walking (MET – minutes / week)	1072 (346-2227)	27.1	5131 (1249-10065)	31.7	2095 (280-5841)	22.4	0.799
Total moderate activity (MET – minutes / week)	2840 (415-8115)	65.3	13575 (11797-17085)	62.6	5340 (1620-7920)	68.0	0.210
Total vigorous activity (MET – minutes / week)	2880 (780-7440)	7.5	7440 (4800-19800)	5.5	2400 (600-4320)	9.5	0.057
Total physical activity (MET – minutes / week)	4735 (1614-12515)		29382 (23833-37727)		14061 (3080-20091)		0.964

* Mann - Whitney U test; MET: total metabolic equivalent of task; Mdn: medians; IQR: interquartile ranges

I.3.4.5. Discussion

The IPAQ questionnaires have been investigated in both developed and developing countries' urban populations. Nevertheless, when interpreting IPAQ findings from rural or low-literacy communities in developing countries, caution should be taken [Hills et al., 2014]. Although the IPAQ - L was validated for PA monitoring among adults [Hills et al., 2014], like most self-reported methods of evaluating PA levels, it lacks accuracy, as patients frequently over-estimate their level of PA [van der Ploeg et al., 2010]. The IPAQ - L, in particular, yields an overestimation of moderate-vigorous PA and an underestimation of sitting time [Van Dyck et al., 2015; Clemeset al., 2012]. Yet, since a 2015 study demonstrated that patients interviewed by a trained professional reported PA levels much closer to the real ones than those who filled in the IPAQ questionnaire by themselves [Van Dyck et al., 2015], we decide to use this approach in our evaluation.

While it was reported that the IPAQ - L is associated with a superior overestimation of total PA compared to the IPAQ - short version [Craig et al., 2003], its benefit consists in the ability to differentiate domain-related PA, offering a detailed picture of the study group’s

activity patterns. Such data may be useful in the creation of effective intervention programs to combat sedentary behavior [Van Holle et al., 2015]. Despite the fact that other authors have reported a negative correlation between physical activity levels and BMI [Lee et al., 2019], we did not find any significant correlation between BMI or AC and weekly PA in the analysed domains.

Study of Lee et al. indicated that while males perform a higher amount of moderate-vigorous daily effort (mostly occupational and leisure related), females spend less time performing PA, generally in the household related area [Lee et al., 2019]. Even though it was reported that gender substantially influences the amount and type of PA, both males and females from our study group performed similar physical effort, as assessed via the IPAQ questionnaire. In our study, moderate PA is the most common activity form for both genders, and although males reported a higher number of weekly METS than females, the difference did not achieve statistical significance. Although females per-formed more moderate activities inside their home, the total number of METS reported in the domestic and garden domains were not statistically significant. The difference between the amount of vigorous PA performed by males versus females in the domes-tic and garden domain reached borderline correlation.

In comparison to the other domains, the IPAQ considers time spent on leisure activities to be the most reliable [Tali et al., 2016]. Leisure time physical activity exhibits a north to south decline in the European Union, ranging from 24 MET – hours / week in Sweden to less than 10 MET – hours / week in southern countries such as Portugal, Spain, Italy and Greece. The average leisure time physical activity in our study group was 512.14 (± 1049.75) MET – minutes / week, similar to other southern European countries [Tali et al., 2016; Martínez-González et al., 2001].

Nolan et al. examined the effectiveness of IPAQ - L in individuals with type 2 diabetes, both with and without peripheral neuropathy [Nolan et al., 2016]. The authors demonstrated that patients with type 2 diabetes and peripheral neuropathy were significantly less active than people with type 2 diabetes alone ($p= 0.04$). A recent study comparing direct assessment of PA with IPAQ and indirect assessment of PA with the accelerometer in patients with type 2 diabetes showed that there was no significant difference between the 2 methods ($p< 0.05$). Therefore, their findings indicate that the IPAQ may serve as a potential tool for PA assessment in patients with type 2 diabetes [Mynarski et al., 2012].

In patients with hypertension, Riegel et al. showed a low agreement between self-report of adherence to PA in clinical routine and IPAQ interview [Riegel et al., 2019]. Additionally, the authors noted that the PA recommendation has a low association with BP control in clinical setting, suggesting that medical advice alone is not able to translate the effectiveness of supervised PA demonstrated in clinical trials to clinical practice. In sedentary hypertensive female, Bravo et. al [Bravo et al., 2012] proved that self-reported PA using IPAQ is predominantly related to domestic ($p= 0.018$) and work activities ($p= 0.001$) at moderate intensity. Therefore, IPAQ appears as an adequate instrument to assess the energy expenditure of hypertensive patients and its impact on their aerobic capacity.

Despite the fact that previous studies have found an age-related decrease in PA [Caban-Martinez et al., 2007; Pratt et al., 1999], the correlation only reached borderline value in our analysis. While several authors previously reported a natural inverse relation between physical activity and excess weight [Martínez-González et al., 2001; Tucker et al., 2011], other studies

found an uncommon positive association between total PA and BMI (especially among females), which could be explained by health consciousness or apparent motivation [von Lengerke et al., 2012; Lemon et al., 2009; Liang et al., 1999; Fan et al., 2015]. Fan et al. [Fan et al., 2015] reported a strong negative association between middle-age (40-49 years) and total PA in their study group, but also failed to show a substantial association between BMI and total PA. Although the authors showed that male sex as well as the 30-39 years old age group are associated with a higher total sitting time, we found no significant differences regarding sitting time between males and females or within the 3 analysed age-groups.

CPET represents a clinical method that allows for a global assessment of cardiorespiratory function to determine exercise capacity. It allows objective measurement of both sub-maximal and peak exercise responses using measures of respiratory oxygen uptake, carbon dioxide output, and ventilatory measures. Previous reports highlight that prior to major surgery, the CPET is often used to assess functional potential in patients to aid clinical decision-making and risk assessment [Jones et al., 2021]. Moreover, recent data assesses the utility of CPET in patients recovering from Covid-19, suggesting it as a potentially useful method for detecting ventilatory and cardiovascular changes in COVID-19 [Dorelli et al., 2021]. Thus, in subjects admitted to a cardiovascular and pulmonary rehabilitation program, CPET can be useful as a screening device for exercise capacity and cardio-ventilatory limitations. According to our results, the association of IPAQ - L to CPET could offer valuable information in specific populational groups.

A study performed in 2018 reported a moderate correlation between IPAQ short form results and treadmill stress test performance [Loprinzi et al., 2018]. Our analysis showed that MTCF as a measure of fitness level exhibited a significant positive correlation with total MET – minutes / week at work and a borderline correlation with total physical activity MET- minutes / week. Yet, a study of a larger population is required to confirm the latter correlation.

In a recent study, IPAQ was not appropriate for assessing PA in cardiovascular disease patients, because the subjects often recorded severe PA values, resulting in non-homogeneous outcomes [Fournier et al., 2018]. Though the IPAQ questionnaire can provide a comprehensive image of a patient's PA pattern on an individual basis, we cannot suggest using IPAQ - L in the study of a wider community of subjects with various cardiovascular disease due to the broad variability of responses reported by our patients (similar to those of Fournier et al. [Fournier et al., 2018]). Although other authors have used the IPAQ questionnaire in the analysis of PA among Romanian students [Leuciuc, 2018; Fagaras et al., 2015; Badicu et al., 2018] and in the geriatric population [Herghelegiu et al., 2017], to our knowledge, this is the first analysis of PA levels using the IPAQ - L and CPET in Romanian patients with cardiovascular comorbidities.

Limitations of the study

Our study had some limitations. First of all, we consider the small number of patients that underwent the CPET (18 participants), but despite this limited subgroup, we found some statistically significant correlations between the parameters that evaluate the PA and the ones that evaluate the functional capacity through the CPET. Another drawback is the lack of published studies involving patients who attended CPET, evaluated also using IPAQ - L; we were unable to find any data focused on patients who were admitted to a CR program. In addition, another limitation is considered to be the fact that the patients were evaluated given

that we do not have an evaluation of the effect of PA over time. Moreover, this is a cross-sectional study that does not allow us to explain causal relationships, our results being descriptive ones and cannot explain biological links.

I.3.4.6. Conclusions

IPAQ - L is useful for the evaluation of individual PA levels within a CR program. IPAQ-L is a suitable tool for measuring PA in order to develop public health policy recommendations or to optimize public health interventions at a very low cost. Currently, in this pandemic situation, the public health services are delayed. Thus, it would be auspicious to monitor the PA of the patient that needs CR in the outpatient department through the IPAQ-L questionnaire. With reference to the secondary outcomes, the IPAQ - L results in the patients with HFrEF are characterized by opposite values and high variability. Obesity, hypertension and type 2 diabetes were highly prevalent in our study group, but did not influence IPAQ - L results. The data from this study regarding the relationship between IPAQ - L questionnaire and CPET parameters were encouraging. Thus, while vigorous physical activity was correlated with VO2max%, moderate physical activity and walking correlated with HRR. However, the questionnaire cannot substitute the importance of the CPET in the assessment of effort performance.

The paper opens up new horizons for further research that requires larger groups of patients to certify the applicability of this tool in assessing patients with HFrEF. Additionally, IPAQ – L questionnaire is a possible instrument that should be used in patients with HFrEF that cannot perform cycle ergometer stress test, but are candidates for CR.

Chapter II. New approaches for refining pain management

Pain affects over 1.5 billion people globally and is recognized as a major public health issue [Goldberg, McGee, 2011; Dueas et al., 2016]. Moreover, chronic pain interferes with various components of a patient's life, negatively influencing their daily activities, physical and mental health, family and social relationships, and workplace interactions. In addition, it is linked to a considerable economic and social cost [Breivik et al., 2006; Dueas et al., 2016; Kela et al., 2021].

Pain has a significant economic burden on patients, which is either indirect (inability to work) or direct (treatment-related costs) [Ababei et al., 2014; Domenichiello AF, Ramsden, 2019]. The annual government investment for pain-related problems in the United States range between $560 and $635 billion, or nearly $2,000 per person. In addition, overall annual costs for pain care are higher than those for heart disease ($309 billion), cancer ($243 billion), and diabetes ($188 billion) [Gaskin, Richard, 2012]. For a better outcome and compliance in pain medication, health practitioners should be trained to properly administer pharmaceuticals through harmonization of therapeutics and pharmacology education [Brinkman et al., 2017; Bild et al., 2017].

Similar to other diseases, chronic pain is the consequence of a sequence of events. Usually a single triggering event such as an accident occurs in the development of chronic pain, but a number of factors influence the duration, intensity, and impact (psychological, physical, social, and emotional) of the pain [Diatchenko et al., 2013]. In the development, duration, and effect of chronic pain, health-related behaviours and their results are the most important modifiable risk factors [Mills, Nicolson, and Smith, 2019].

There are various types of medications available at the time, ranging from mild to severe analgesics. In some circumstances, analgesics association can improve difficult pain or lower the opioids need. Furthermore, multiple pharmaceuticals that target different pain-related pathways might be combined for a synergistic effect [Salvemini, 2010; Yam et al., 2018]. Finally, non-pharmacologic pain management options have been increasingly studied in recent years, with some strategies proving to be beneficial in certain situations [Lewis et al., 2018].

The physician faces frequently with a difficult selection between several treatment options. Despite the availability of numerous efficient medications, some types of pain remain difficult to treat. Furthermore, new treatments and drug combinations can be expensive, and in a society where medication coverage is not always available, this should be considered when choosing a long-term treatment. Moreover, chronic pain in people suffering from addiction and dependency typically develops into a complex disabling illness involving pain in various locations, psychosocial dysfunctions, medical and mental diseases, polypharmacy, and polysubstance use, all of which interact in complicated ways [Manhapra, Becker, 2018].

Consequently, basic strategies should be prioritized, with innovative combinations (such as those involving trace elements or heavy metals and analgesics) or plant extracts (such as those from the *Laminaceae* family) being tested in preclinical models with the ultimate goal of translating the results to the bedside. The utility of current analgesics could be significantly aided utilizing these methods, resulting in a reduction in analgesic use and, as a result, the negative side effects associated with opioid treatment [Peptu et al., 2015].

II.1. Therapeutic developments in pain management

II.1.1. Scientific context

Finding innovative techniques for administering analgesics, a topic where nanotechnology and nanoparticles have been increasingly investigated in recent years, is another potential strategy for better pain control. However, because of the pro-inflammatory effect that silver nanoparticles can have on normal and inflamed tissue, nanotechnology-based techniques should be utilized with prudence.

Empirical formulations of therapeutic mixtures, such as creams, ointments, pastes, powders, and liquid extracts of animal or vegetal origins, have been developed and perfected over centuries [Pastore et al., 2015]. The method of transdermal drug delivery systems has piqued the interest of numerous academics since transdermal medication administration offers many advantages over oral therapies or injections. Transdermal medication delivery aims to achieve local and surface effects, as well as target deeper tissues or even systemic circulation [Al Hanbali et al., 2019]. The study of such drug carriers should take into account skin features, drug type and biological activity, and drug-skin interactions due to the complexity of such systems, all of them being related to the drug's biological availability in a given time frame, with the therapeutic impact following as a result [Peptu et al., 2015]. In addition, knowing the general mechanism of action of these substances is essential in clinical practice when selecting the appropriate therapy.

Nowadays, nanotechnology has the potential to improve transdermal drug delivery. The fact that smaller drug carriers can improve the transport of therapeutic drugs over the skin is one of the advantages. Controlling the release of active chemicals and prolonging the length of retention on the skin can be used to modulate drug permeation/penetration. It also ensures that the stratum corneum and skin appendages are in direct and tight touch. Finally, the medication can be isolated against chemical or physical interactions with the skin [Benson et al., 2019].

The skin has simply evolved as the primary barrier against the external environment. As a result, finding appropriate techniques to overcome this barrier is the fundamental challenge in topical drug administration.

Topical anesthetics have significant benefits, such as a high safety profile and ease of administration, but they also have limitations, such as reduced absorption, local operations, delayed onset of anesthesia, local side effects, and expensive medication costs [Choi et al., 2020].

Topical anesthetics are already available and are applied to the skin or mucosal sites such as the mouth, conjunctiva, or genitalia [Peptu et al., 2015; Choi et al., 2020]. The penetration of topical anesthetics applied to the skin is a difficult issue due to the complex structure of the skin. Although solid drugs are only superficially absorbed when applied to intact skin, eutectic mixtures assure larger concentrations of the active principle because they melt at lower temperatures than their individual components, resulting in improved local anesthesia. Several liposomal preparations, nanovehicles, iontophoresis, epidermal needling, or occlusion methods have been used to try to overcome this [Benson et al., 2019].

The FDA has approved a huge number of topical anesthetics. New agents are continually being created as these drugs aim to prove their usefulness. The use of nanoformulation to enhance topical anaesthetics' skin penetration is a promising method. The use of a lidocaine

nanoformulation has yielded positive results recently [Peptu et al., 2015; Jiang et al., 2021].

Topical pain therapy that integrates nanotechnologies as penetration enhancers, particularly nanocarriers, macromolecular, and cyclodextrin-based enhancers, has proven to be a viable alternative to active penetration methods that rely on physical methods to create a transdermal passage for drug diffusion [Jiang et al., 2021]. Hydrogels and cyclodextrins appear to have complementarities with other enhancers or drug molecules and provide enough advantages in drug delivery design among the universally integrated components. Polymer architectures based on cyclodextrin cores are likely to be used in the development of transdermal drug delivery systems for pain management, keeping their complexation properties [Peptu et al., 2015; Yang et al., 2019].

Part of the preoccupations related to pain management were synthesized in the following articles:

ISI ARTICLES

1. Peptu C, Rotaru R, Ignat L, Humelnicu AC, Harabagiu V, Peptu CA, Leon MM, Mitu F, **Cojocaru E**, Boca A, Tamba BI. Nanotechnology approaches for pain therapy through transdermal drug delivery. Curr Pharm Des. 2015;21(42):6125-39. **IF: 3.052** http://www.ncbi.nlm.nih.gov/pubmed/26503147
2. Luca A, Mihai CT, Stanciu GD, Bild V, **Cojocaru E**, Ancuceanu R, Harabagiu V, Peptu C, Peptu CA, Leon Constantin MM, Alexa-Stratulat T. In-vivo safety and efficacy evaluation of a novel polymeric based lidocaine formulation for topical analgesia. Farmacia 2019; 67 (1):117-125. **IF: 1.607** http://www.revistafarmacia.ro/201901/issue12019art16.html
3. Leon-Constantin MM, Alexa-Stratulat T, Luca A, Tamba BI, Trandafir LM, Harabagiu V, **Cojocaru E**. The morphofunctional impact of topical Lidocaine formulation in inflammatory pain - experimental study. Rom J Morphol Embryol 2019; 60 (3):869–874. **IF: 1.411** http://www.rjme.ro/RJME/resources/files/600319869874.pdf
4. Tamba BI, Stanciu GD, Urîtu CM, Rezus E, Stefanescu R, Mihai CT, Luca A, Rusu-Zota G, Leon-Constantin MM, **Cojocaru E**, Gafton B, Alexa-Stratulat T. Challenges and Opportunities in Preclinical Research of Synthetic Cannabinoids for Pain Therapy. Medicina (Kaunas). 2020; 56(1):24. **IF: 2.430** https://www.mdpi.com/1010-660X/56/1/24

BOOK CHAPTERS

1. **Cojocaru E**. Neurobiologia durerii. În: Leon MM, Mungiu O (eds). Terapia durerii - aspecte actuale. Iași: Editura “Grigore T. Popa”, 2014; 31-37. ISBN: 978-606-544-264-1.

PROJECTS

1. *Innovative education project for cancer pain management in the second largest oncology hospital in Romania (INECAPOR)*, an educational project funded by the International Association for the Study of Pain. Contractor: UMF „Grigore T. Popa“, Iași (2013-2014);
2. *Complex formulations based on liposomes and cyclodextrin for transdermal pain therapy (NANODERMA)* (2014-2017), funded by UEFISCDI. Project coordinator: "Petru Poni Institute of Macromolecular Chemistry", Iași. UMF partner "Grigore T. Popa", Iasi (2014-2017).

II.1.2. A new polymeric-based lidocaine formulation for topical analgesia in laboratory animals

II.1.2.1. Introduction

Although some countries of the world deal with the ever-increasing use of opioids, new approaches to acute and chronic pain management are needed. Polypragmasia and polypharmacy are becoming more common as the number of elderly people with several comorbidities increases, necessitating multiple types of medicines and hence complex management [Giummarra et al., 2015; Uritu et al., 2018; Nwadiugwu, 2021]. As a result, topical analgesics have become a common choice for treating a variety of painful diseases. Local skin delivery allows topical formulations to exert their effects close to the application site, with only limited systemic uptake and diffusion. The pharmacokinetics of degradable drugs are enhanced and the frequency of side effects is reduced when utilizing the topical route [Sawynok, 2014]. Furthermore, these medications are simple to use and monitor, making this drug delivery method appropriate for vulnerable groups such as the elderly and children [Alexa et al., 2013; Choi et al., 2020].

Non-steroidal anti-inflammatory medications, lidocaine, capsaicin, amitriptyline, glyceryl trinitrate, opioids, menthol, pimecrolimus, or phenytoin are currently accessible topical analgesics with indications in different types of acute or chronic pain [Argoff, 2013; Derry et al., 2015]. Commercially accessible topical lidocaine-based analgesics are available as a cream [Wigerblad et al., 2017] or a patch [Mick et al., 2012].

Even tough the literature evidence suggests that they relieve a variety of acute and chronic pain, still not all clinical research support the efficacy of contemporary lidocaine formulations. Furthermore, the response rate for topical lidocaine varies significantly between studies, pain kinds, and anatomical region of application, implying that, while lidocaine is an efficient topical analgesic, its composition still needs to be improved.

Because of the challenges associated in adjusting the dose to skin penetration by evaluating and reproducing the precise concentration of medication required for reaching the skin layers at the appropriate depth [Draganescu et al., 2015; Santini et al., 2015], topical administration of a drug continues to be a challenge in pharmaceutics [Peptu et al., 2015]. Furthermore, because different medications penetrate at different rates, a proper formulation is critical for effective skin penetration [Nagai et al., 2017].

The cost and availability of topical analgesics [De Lima et al., 2014] are another concern in low- and middle-income nations. The majority of present pharmaceuticals are very expensive, which is a critical challenge when considering that the people who require these drugs (the elderly and/or those with many comorbidities) are the ones who cannot afford them [Alexa et al., 2012]. As a result, lower-cost, locally produced products are desirable.

II.1.2.2. Aim

The purpose of this study was to assess the efficacy of an innovative lidocaine-based spray on nociceptive and inflammatory experimental pain. The analgesic effects obtained in experimental animals by delivering lidocaine in various forms may be comparable to a certain level to the therapeutic effects in patients, which could be a starting point for clinical

investigation of new anti-nociceptive drugs discovered in laboratory experiments.

II.1.2.3. Material and methods

Adult Balb/c mice (25 ± 2g) were used for toxicity assessment and adult male Wistar rats (180- 200 g) were used for efficacy assessment. All animals were purchased were purchased from the Animal Source Unit, Bucharest and housed at 21 ± 2°C under a 12-h light/dark cycle with access to food and water *ad libitum*.

Animals were acclimatized to the testing room and equipment for five days prior to each experiment. The number of animals and the intensity of the painful stimuli used were the minimum needed to show that the pharmacological treatments had consistent effects. The study was conducted in accordance with the recommendations of the NIH Guide for the Care and the Use of Laboratory Animals. The protocol received the ethical approval from the ethics committee of the University of Medicine and Pharmacy "Gr. T. Popa", Iaşi.

Drugs

CX001 was synthesized by "Petru Poni" Institute and AB Pharm Romania. The formula was delivered as a powder and diluted in saline solution in order to create a spray that was applied by means of a commercially-available disperser. CX001 is made up of a mixture of innovative polymers and lidocaine. The manner in which lidocaine is included in this compound significantly influences the skin-compound interaction and enhances topical drug diffusion. CX001 aims to surpass the existing topical lidocaine-based analgesics by increasing lidocaine solubility and skin permeability, improving target control and decreasing side-effects and toxicity.

EMLA© cream (lidocaine 2.5% and prilocaine 2.5%) (Astra Zeneca) was purchased and used as a reference drug for the efficacy of CX001.

λ-carrageenan (CG) diluted in fresh saline solution (Sigma-Adrich Germany) was administered subcutaneously in order to produce the model of inflammation.

Tests

The Hot Plate (HP) test was carried out in accordance with the procedure described by Woolfe and MacDonald with some minor adjustments [Tamba et al., 2013]. The rats were individually positioned on a hot plate maintained at 55°C (± 0.1°C) (Hot Plate Ugo Basile, DS 37, Italy) and the time elapsed before the first sign of discomfort (licking, shaking of hind paws or jumping off the surface) was measured - paw withdrawal latency (PWL). Cut-off time was set at 12s to prevent tissue injury.

The Cold Plate (CP) test was performed according to the method described by Wal *et al* with some minor modifications [van der Wal et al., 2015]. Briefly, the animals are placed on a 5°C thermostatically-maintained plate (Ugo Basile Cold Plate 35100) and the discomfort-related behaviour in five minutes is quantified. The results were expressed as the number of movements per 300 seconds.

The Randall-Selitto Method was used for evaluating the response to mechanical stimuli. The Analgesy-Meter (7200; Ugo Basile, Italy) progressively applies a force that increases by 16 grams/second; the animal's paw is placed on a small plinth under a cone-shaped pusher with a rounded tip. Paw withdrawal occurs when the pressure becomes

painful for the animal. The time elapsed until withdrawal was recorded [Jeong et al., 2012]. Cut-off time was set at 320 g (20 seconds).

*Carrageenan-induced inflammation:*100 μL of 1% λ- carrageenan were subcutaneously injected in the ventral portion of the right hind paw of rats to test the effectiveness of the new topical analgesic on inflammatory pain [Wigerblad et al., 2017]. After injection, the animals were immediatelyplaced in acrylic boxes for observation. The local inflammatory status was monitored by periodic comparison of the two paws (with and without λ- carrageenan). Inflammation was considered at its peak approximately two hours and forty minutes after injection.

Study design and Safety assessment

We divided adult male BALB/c mice (25 ± 2g) into six groups (n = 6/group). Three of the groups received topical administration of EMLA©, CX or saline solution on the right hind paw. The other three groups first received a subcutaneous injection of λ-carrageenan into the right hind paw and topical application of EMLA©, CX or saline 2 hours and forty minutes after. The mice were sacrificed 15 minutes after the topical applications and the liver and the skin of the hind paw were removed and fixed in a 10% formalin solution for additional tests.

Liver and skin samples were stained with both hematoxylin and eosin (HE) and Szekely tricromic stains (SZ) for the histological examination.

Nociceptive pain assessment

Rats were divided in three groups (n = 6/group) and each group received topical administration of a thin layer of EMLA© cream - group En, two puffs of saline solution - group Sn and two puffs of CX001 spray - group CXn). HP and Randall-Selitto assessments were performed at baseline and 5, 15, 30, 45, 60, 120, 180 and respectively 240 minutes after administration. The response to cold stimuli was assessed by means of the CP test that was performed at baseline, 30 minutes after topical administration and hourly after that over a four hours period of time.

Inflammatory pain assessment

At the beginning of the study, all rats were examined using the HP, CP, and Randall-Selitto methods. Following that, each animal received 10 μL 1% λ-carrageenan into the right hind paw. Two hours and forty minutes after intraplantar administration, rats were divided into three groups (n = 6/group) receiving either EMLA© (group Ei), saline solution (group Si) or CX001 (group CXi). HP, CP and the Randall-Selitto assessments were performed with the same frequency as for the nociceptive pain assessment.

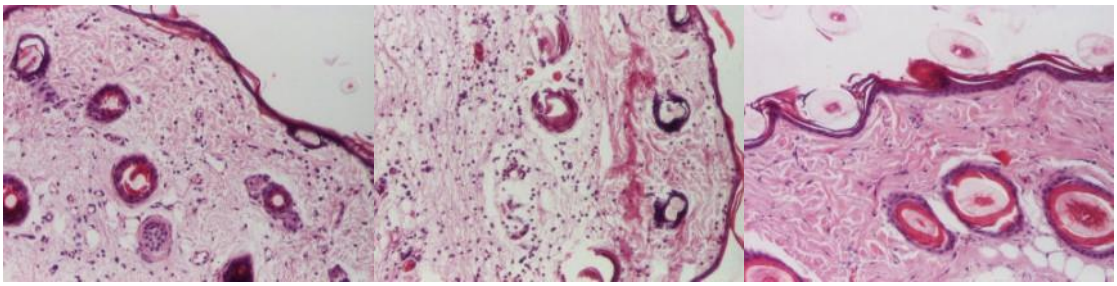
Data analysis

The statistical assessment was performed by means of SPSS v.20 and GraphPad Prism 6.0 software. ANOVA was used to assess time and substance effect. Post-hoc comparisons were performed by using Tukey`s test. The significance level was set a priori at $p < 0.05$.

II.1.2.4. Results and discussion

Safety of the CX001 topical analgesic

The histological profile of skin samples from mice who had only received EMLA©/CX001/saline topical application was normal. No other differences were noted between groups. Histological evidence of inflammation, including neutrophil infiltration, vascular congestion, and oedema, were found in the skin tissue of mice that received a subcutaneous carrageenan injection prior to topical medication delivery (Figure 38). No differences were noted between EMLA© and CX001-treated groups, although lidocaine in topical administration (in either EMLA© or CX001 formula) was associated with less inflammation.



A B C

Figure 38. *HE coloration - microscopy (x 100) assessment of skin tissue in the inflammation groups. A - EMLA©, B - CX001, C - saline solution*

It is known that lidocaine acts as a nonselective blocker of Na+ channels. Furthermore, some investigations have found that lidocaine's analgesic impact is related to its interaction with resident cells (keratinocytes and immune cells), which results in an anti-inflammatory effect [Cassuto et al., 2006; Tikhonov et al., 2017]. This effect could account for the results we have obtained in rats with induced inflammation - i.e. less inflammatory cells in EMLA© and CX001-treated groups. There were no changes in the hepatic architecture, according to histological study of liver tissue. Our findings are in line with previous evidence from other lidocaine formulations [Ji et al., 2015; Negi et al., 2015], as well as other experimental data obtained in our lab by incorporating analgesics in similar ways [David et al., 2010; Iurea et al., 2013]. The novel CX001 chemical is a novel drug with good pre-clinical safety and effectiveness properties.

In a clinical trial that included both clinical and imaging assessments of pain, Hashmi et al reported no difference between the lidocaine patch and placebo in terms of pain reduction [Hashmi et al., 2012]. Other research [Grosse-Steffen et al., 2017] indicated that topical lidocaine/prilocaine cream has no effect on post-operative discomfort in women following caesarean section and no significant difference in pain intensity following dental injection between a lidocaine/prilocaine mixture and applying local pressure [Milani et al., 2016].

Hot Plate: There were no significant differences at baseline in the nociceptive pain group, with an average PWL of 3.35 ± 0.28 s in the Sn group, 3.48 ± 0.37 s in the En group and 3.48 ± 0.27 s in the CXn group. Five minutes after topical analgesic/saline administration, EMLA© treated animals had an increase in PWL when compared with saline solution (5.01 ± 0.47 s *vs*. 3.43 ± 0.22 s) or CX001 treated animals (5.01 ± 0.47 s

vs. 3.16 ± 0.29 s). PWLs started to increase in the CX001 treated group after this time point and both drugs weresuperior to saline solution, 15 minutes after administration. The effect remained consistent at 30, 45 and 60 minutes after administration ($p < 0.005$) (Figure 39). PWLs in all groups were similar at the end of the experiment (after four hours). ANOVA repeated measures identified that there isa significant effect throughout the experiment, with $p = 0.0058$ ($F(2, 10) = 9.022$) and significance in time ($p = 0.0095$).

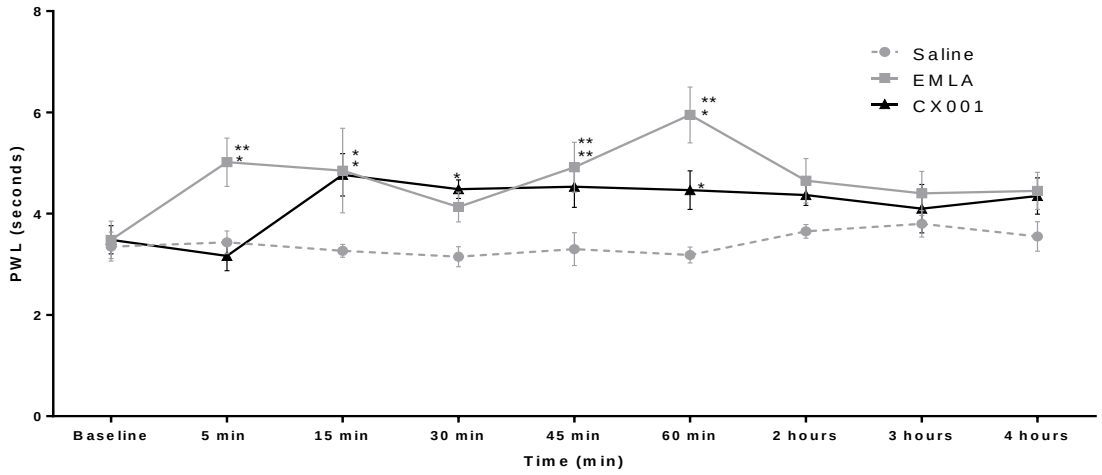


Figure 39. *Hot Plate PWLs in the nociceptive pain group. * = < 0.005 versus control group; ** = < 0.005 versusEMLA©/CX001 group*

There were no significant differences between groups at baseline in the inflammatory pain group. All animals showed evidence of discomfort after receiving CG injections, avoiding placing the injected hind paw on the ground and licking/scratching the damaged area. When compared to baseline, saline-treated animals had a lower PWL five minutes following topical analgesic/saline application (2.66 ± 0.40 s), whereasCXi and Ei groups showed no such modifications. Furthermore, there was a statistically significant difference between the Si and CXi groups ($p=0.05$), but no significant difference between the Si and Ei groups (Figure 40).

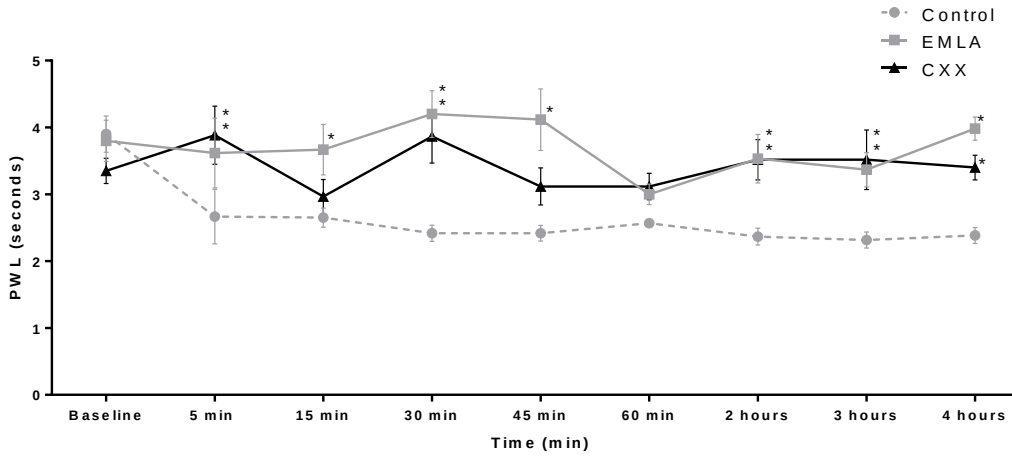


Figure 40. *Hot Plate PWLs in the inflammatory pain group. * = < 0.005 versus control group; ** = < 0.005 versusEMLA©/CX001 group*

All the way through the experiment, both CX001 and EMLA© topical administration led to increased PWLs as compared to saline administration. ANOVA repeated measures identified that there isa significant effect throughout the experiment, with $p < 0.0001$ ($F(2, 10) = 26.86$) with significance in time ($p = 0.0097$). There were no significant differences between Ei and CXi groups at any time point.

The Hot Plate test evaluates both spinal and supra-spinal responses and is commonly used to evaluate both nociceptive and inflammatory pain [Gunn et al., 2011]. In numerous forms of delivery and formulation, lidocaine has been shown to increase Hot Plate latency [Er et al., 2017], so our results are in line with available data. CX001 and EMLA© have similar efficacy in terms of Hot Plate PWLs, only CX001 becomes effective approximately 15 minutes after EMLA©, most likely due to the fact that it is liquid and it only contains lidocaine, not a combination between lidocaine and prilocaine.

Cold Plate

In terms of nociceptive pain response, neither EMLA© nor CX001 produced any significant variation in the number of cold-evoked movements in 300 seconds over the course of the experiment. There was no significant substance or time effect, according to ANOVA repeated measurements. These findings are consistent with prior research that looked at the effect of a 5% lidocaine patch on healthy human volunteers and showed that lidocaine had no effect on the threshold for heat and cold-induced pain when applied to non-inflamed tissue [Wehrfritzl et al., 2011].

There were no significant differences between groups at baseline in the inflammatory pain group, with an average number of cold-evoked movements of 8.75 ± 0.47 s in the Si group, 7.75 ± 0.48 s in the Ei group and 7.75 ± 0.47 s in the CXi group. Thirty minutes after topical analgesic/saline solution administration, saline treated animals expressed more cold/pressure related discomfort, with an average of 36 ± 1.58 s movements in 300 seconds, whereas rats in the Ei and CXi groups had an average of 22 ± 2.27 s respectively 19.75 ± 2.05 s movements (Figure 41).

When compared to saline administration, both CX001 and EMLA© topical administration were linked with fewer discomfort-related movements throughout the experiment ($p < 0.005$ for CXi and Ei at 30, 60 and 120 minutes).

Throughout the experiment, ANOVA repeated measures revealed that there is a significant influence, with $p = 0.04$ ($F(2, 6) = 5.756$) and that the effect is significant in time ($p = 0.0076$). There were nostatistically significant differences between Ei and CXi groups at any time point.

EMLA© and CX001 were more effective in decreasing the number of cold induced discomfort movements in the inflammatory model than in the nociceptive model. In the saline group, there was also a difference in baseline and post-CG measurements (when compared to the differences noted in other tests). The fact that cold can function as a pain inhibitor by directly altering epidermal nervous terminations and lowering sensitivity in the inflamed area [Barkin, 2013] is one possible reason for this finding.

Additional explanation for the fact that bothEMLA© and CX001 are more effective in the inflammatory model is that lidocaine primarily acts on Aδ and C fibers that have

abnormal excitation, a situation that occurs in both inflammatory and neuropathic pain [Hashmi et al., 2012].

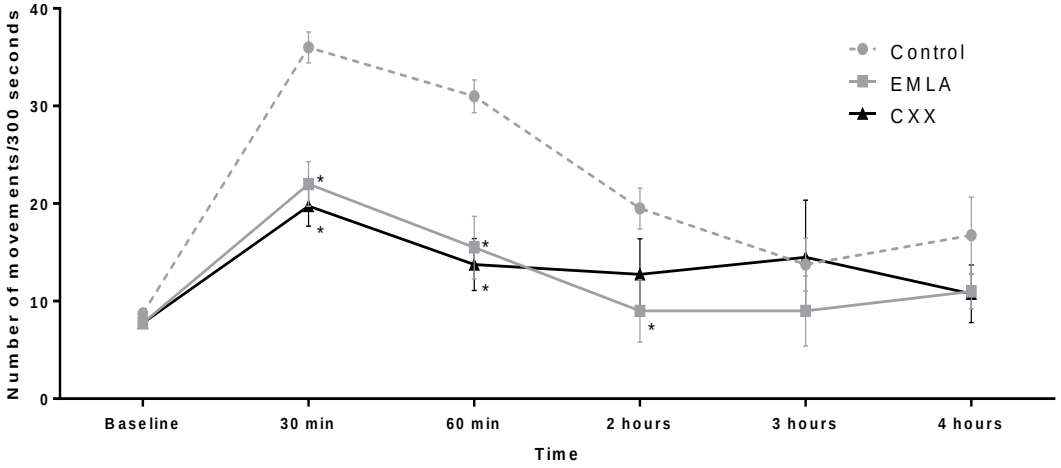


Figure 41. Hot Plate PWLs in the inflammatory pain group. * = < 0.005 *versus* control group; ** = < 0.005 *versus*EMLA©/CX001 group

The Randall-Selitto Method

Concerning *the nociceptive pain response*, there were no significant differences between groups at baseline, with an average PWL of 6.66 ± 0.49 s in the Sn group, 7.33 ± 0.66 s in the En group and 6.33 ± 0.62 s in the CXn group. Five minutes after topical application, the EMLA©-treated group had significantly increased PWLs, with an average of 13.58 ± 2.43 s when compared with CXn (8.00 ±1.01 s) and Sn (7.00 ± 0.85 s) groups.

CX001-treated animals, however, experienced a slow progressive increase in PWL and surpassed the PWLs of the EMLA© group at 30 and 45 minutes (Figure 42).

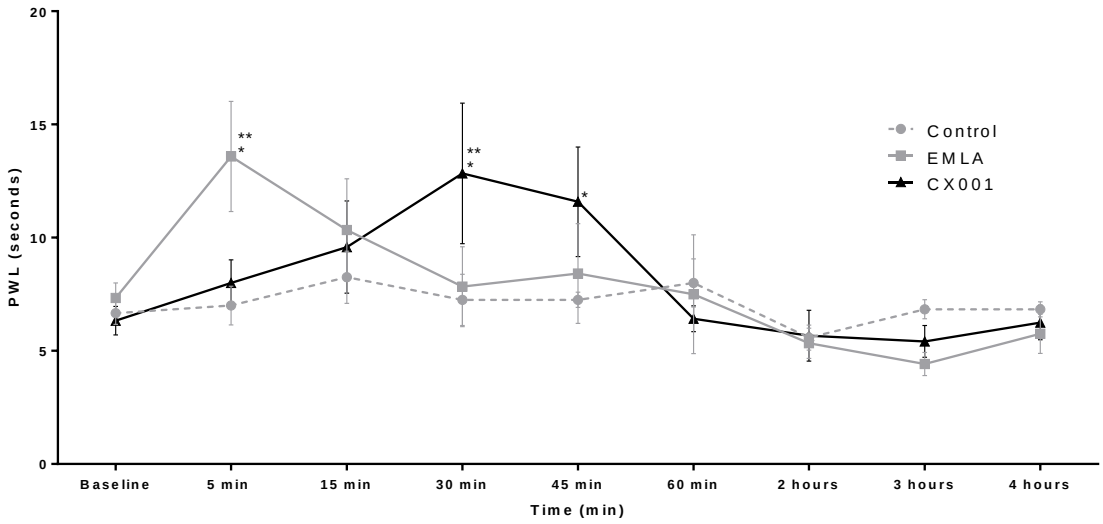


Figure 42. *Mechanical PWLs in the nociceptive pain group. * = < 0.005 versus control group; ** = < 0.005 versusEMLA©/CX001 group*

One hour after administration, all groups had PWLs similar to those at baseline, a result that was consistent over the two, three and four hour assessments. ANOVA repeated measures identified that there is a significant effect with p = 0.008 and F (16, 80) = 2.279.

In *the inflammatory pain group*, there were also no significant differences between groups at baseline, with an average PWL of 8.33 ± 1.08 s in the Si group, 7.08 ± 0.68 s in the Ei group and 8.33 ± 0.92 s in the CXi group. Five minutes after EMLA©/CX001 topical applications, both formulations led to a statistically significant increase in PWL when compared to saline. Fifteen minutes after topical application, the CXi group had longer PWLs when compared to the Ei group (12.41 ± 1.72 s vs. 5.16 ± 0.45 s), a significant difference that lasted up to two hours after topical drug administration (Figure 43).

Throughout the experiment, ANOVA repeated measures revealed that there is a significant influence, with p < 0.0001 (F (2, 10) = 93.16) and that it is significant in time (p < 0.0001). At the end of the experiment (4 hours after administration), all groups had similar PWLs (4.08± 0.20 s in the Si group, 4.75 ± 1.17 s in the Ei group and 6.08 ± 0.15 s in the CXi group).

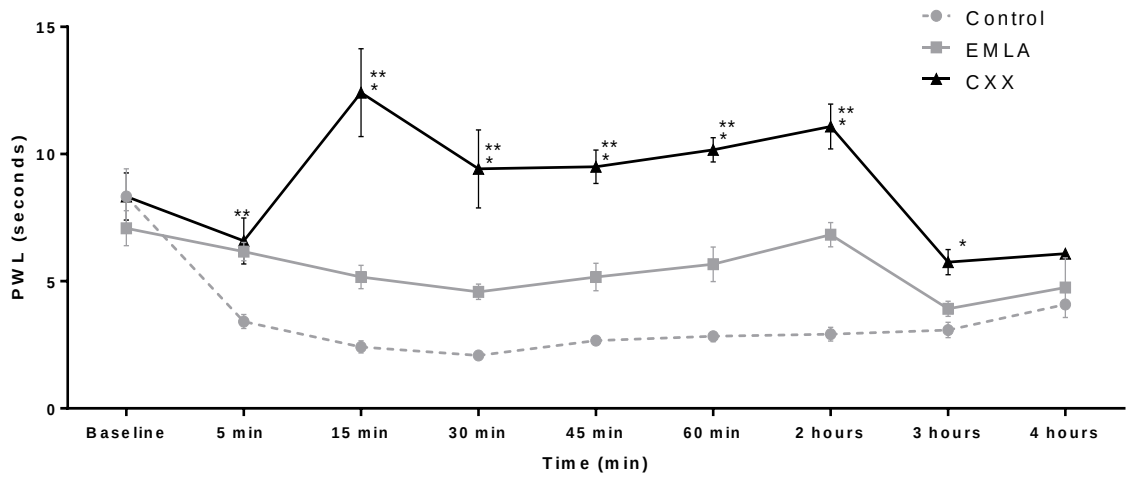


Figure 43. *Mechanical PWLs in the inflammatory pain group. * = < 0.005 versus control group; ** = < 0.005 versus EMLA©/CX001 group*

Our findings are consistent with the literature, which indicates that topical EMLA© treatment is only marginally helpful in lowering tactile sensitivity in newborn rats [Strain et al., 2014]. According to a previously published clinical trial, EMLA© partially decreases pain during tympanocentesis in less than a third of the patients [Jyväkorpi, 1996]. The findings of this study are especially important because the perinatal period is thought to be the most effective time for topical analgesics [Hardman et al., 1998]. Due to its improved formula, CX001 is significantly superior to EMLA© as assessed by the Randall-Selitto test, a method that primarily evaluates the response to tactical stimuli.

II.1.2.5. Conclusions

The CX001 lidocaine-based topical formulation is a revolutionary system for topical drugs delivery. CX001 has a very low toxicity, with no serious side effects and no liver toxicity. CX001 dissolves easily in saline to produce a spray that can be applied

topically with simplicity. The new formula is as effective as the lidocaine 2.5%-prilocaine 2.5% reference cream in improving nociceptive pain and is more effective in pressure-induced inflammatory pain. The findings suggest that CX001 has the potential to reduce pain and could be used topically to treat pressure-related discomfort.

II.1.3. Experimental study on the morphofunctional impact of topical Lidocaine formulation in inflammatory pain

II.1.3.1. Introduction

Pain management in pediatric and adult patients continues to be a poorly elucidated problem worldwide [Anitescu et al., 2013; Argoff, 2013], considering the three features involved in its pathogenesis: nociception [Barkin, 2013; Breivik et al., 2013], emotional factors [Cassuto et al., 2006; Draganescu et al., 2015], and behavioral factors [Birol Muhammet et al., 2016; Grosse-Steffen et al., 2017]. Physicians are nowadays confronted with challenges in pain management, such as medication cognitive impairment due to central effects, analgesic partial efficacy, and systemic effects, all of which limit treatment effect [Hardman et al., 1998; Gunn et al., 2011; Hashmi et al., 2012].

Many researchers have become interested in transdermal drug delivery methods because they provide many advantages over oral therapies or injections. The purpose of transdermal drug delivery is to achieve local and surface effects, as well as to target deeper tissues or even systemic circulation.

Recent findings on topical anesthetic drugs, as well as ways for overcoming the skin barrier and delivering the medicine efficiently, have been reported [Peptu et al., 2015]. Local anesthetics applied to the skin are known to enter through the intercellular network, specifically the lipid domains of the epidermis (the stratum corneum) However, the stratum corneum reduces local anesthetic penetration and is the primary barrier to their practical use [Zhao et al., 2018]. The essential target of topical preparations development is the improvement of patient compliance to medical treatment, by providing efficient pain relief with less central nervous system effects and minimal drug regimen burden [Jyväkorpi, 1996; Ji et al., 2012; Mick, Correa-Illanes, 2012].

II.1.3.2. Aim

Skin permeation enhancers improve skin permeability by improving the permeability of the stratum corneum. The purpose of the study was to identify new transdermal Lidocaine delivery systems that will ensure a controlled release, and an improved availability and will finally enhance local pain control.

II.1.3.3. Material and methods

Animals: Our experimental study involved male adult Wistar rats, weighing between 180– 200 g, purchased from "Victor Babeş" National Institute for Research and Development in Pathology and Biomedical Sciences, Bucharest, Romania. The animals were kept in a room with controlled temperature (21±2°C), with circadian rhythm 12 hours light/12 hours dark, and

were allowed to accommodate in this climate for at least 24 hours before the experiment. There was one rat per cage, with free access to food and water. The study was conducted after receiving the approval from the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania, and the testing chemical compound from the "Petru Poni" Institute of Macromolecular Chemistry, Iaşi. We respected all recommendations of the European Community (EC) regarding the use of medicinal products in preclinical studies, in conformity with the conventions of good laboratory practice, as well as in accordance with clinical norms and protocols regarding the testing of drugs.

The nociceptive tests were performed, meeting the standards recommended by the Ethics Committee of the International Association for the Study of Pain (IASP): the recommendations of the "Declaration of Helsinki"; "Guiding Principles in the Care and Use of Animals" approved by the American Physiological Society (published in Journal of Neuro-physiology); and "Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals" [Sawynok, 2014; Strain et al., 2014; Peptu et al., 2015].

Methods

We divided the rats into three groups10 animals each (two controls and eight experimental animals): group C (λ-carrageenan), group E+C [eutectic mixture of local anesthetics (EMLA) and λ-carrageenan] and group T+C (tested substance and λ-carrageenan). A λ-carrageenan injection was used to induce inflammatory hyperalgesia at the start of the study. The tested substance containing Lidocaine or EMLA was applied to the hind paw once the paw edema was developed. Immediately after, the rats were subjected to a battery of nociception tests: algesimeter, cold plate and hot plate. All the assessments were performed at specified intervals (5, 15, 30 minutes, one hour, two hours). The paw withdrawal thresholds (PWTs) for mechanical (algesimeter) and thermal (cold plate and hot plate) stimuli were analysed to measure the antinociceptive effect of these two compounds. The tests were carried out after the animal's hair removal.

Euthanasia of laboratory animals

The animals were euthanized at the end of experiment without physical pain. The use of a volatile anesthetic (Isoflurane) resulted in unconsciousness in a matter of seconds. Tissue samples were taken after certifying the death. The following drugs were used in the experiment: λ-carrageenan, which is a high molecular weight sulphated polysaccharide obtained from red algae with inflammatory properties; the tested substance, which was synthesized by "Petru Poni" Institute and delivered as a powder, which is soluble in saline solution; it is formed by a mixture of polymers and Lidocaine, which facilitate penetration of drug in the skin; EMLA – a eutectic mixture of 2.5% Lidocaine and 2.5% Prilocaine formulated as an oil in water emulsion, known for its analgesic properties [Milani et al., 2016].

Histopathological assessment

The collection of samples was performed after euthanasia of animals and from the area

where the substance was administered. They were specifically processed, by paraffin inclusion, microtome sectioning (3–5 μm), and usual staining – Hematoxylin–Eosin (HE) and Goldner–Szekely (GS) trichrome [Mondal, 2017; Suvarna et al., 2019].

Data analysis

Statistical assessment was performed by means of Statistical Package for the Social Sciences (SPSS)v.20 and GraphPad Prism 6.0 software. Information were expressed as mean ± standard error of the mean (SEM). Repeated measures analysis of variance (ANOVA) was used to assess time and substance effect. The significance level was set a priori at $p<0.05$.

II.1.3.4. Results

Pharmacological testing of the researched substance

The algesimetry results showed that the effect was present 15 minutes after injection (at 5 minutes none of the substances had effects). In comparison to EMLA cream, the tested drug had a stronger effect 15 minutes after administration. (2.5% Lidocaine and 2.5% Prilocaine). After 30 minutes, the effect was still present (statistically significant), but it diminished before the experiment ended (Figure 44).

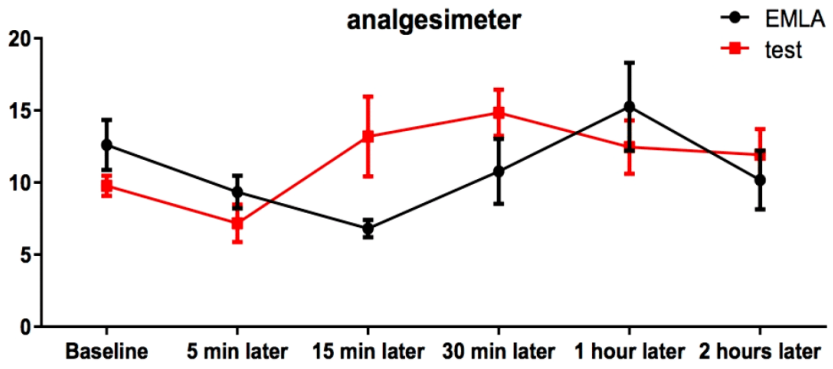


Figure 44. *Algesimeter testing of the researched substance vs. EMLA. EMLA: Eutectic mixture of local anesthetics.*

The effect of the tested drug was less effective than EMLA's after one hour, but it became stronger after two hours, and was nearly identical to the effect achieved 15 minutes after administration. As a result, the start of action was within 15 minutes after administration, the peak effect was within 15–30 minutes, and the effect gradually faded but did not disappear after two hours.

The cold plate test confirmed the results obtained with the algesimeter, showing that the therapeutic action of the tested drug was much higher than that of EMLA after 15 minutes, then began to decline, becoming similar to EMLA's after one hour. When using the cold plate, the peak effect was 30 minutes after delivery (Figure 45).

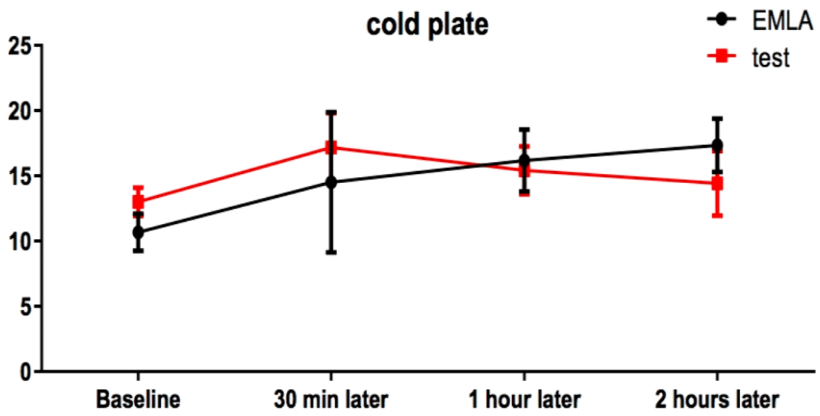


Figure 45. *Cold plate testing of the researched substance vs. EMLA. EMLA: Eutectic mixture of local anesthetics.*

The results revealed that the tested chemical had a more powerful effect at the 5, 15, and 30 minute evaluations when we performed the hot plate test. The effects of the two drugs were similar in the one-hour test, as they had been in the earlier tests. After two hours, the effect of the substance we examined started to diminish (Figure 46).

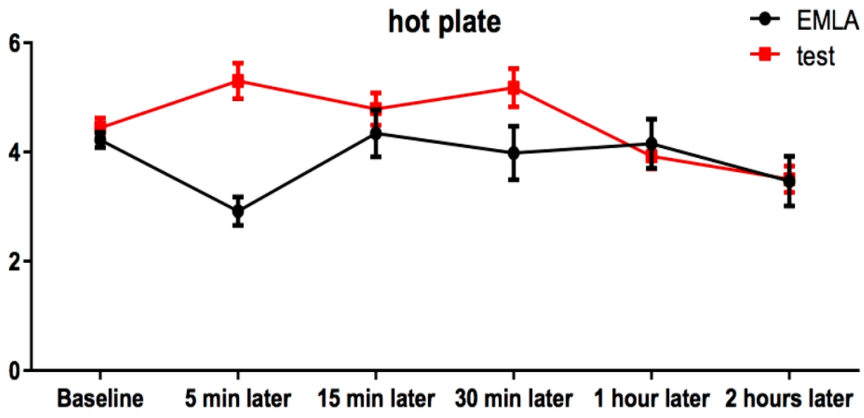


Figure 46. *Hot plate testing of the researched substance vs. EMLA.*

EMLA: Eutectic mixture of local anesthetics.

According to the results of the performed tests, the action of the tested substance settles within 15 minutes, reaches the peak effect at 30 minutes and then falls of, persisting at a low intensity at the last testing, at two hours after administration.

Results of the histopathological assessment

Skin samples collected from control animals demonstrated a normal architecture in both HE and GS trichrome staining: epidermis (thin keratinized stratified squamocellular epithelium), superficial dermis (loose connective tissue), and deep dermis (dense connective tissue). There were no pathological alterations in the pilosebaceous, apocrine, or eccrine glands (Figure 47, a–c). Rare inflammatory elements represented by polymorphonuclear neutrophils (PMN), discrete edema in the superficial and deep dermis, and minor vascular congestion were seen in the skin samples collected from group C, indicating a minimal acute inflammatory reaction (Figure 48 a and b).

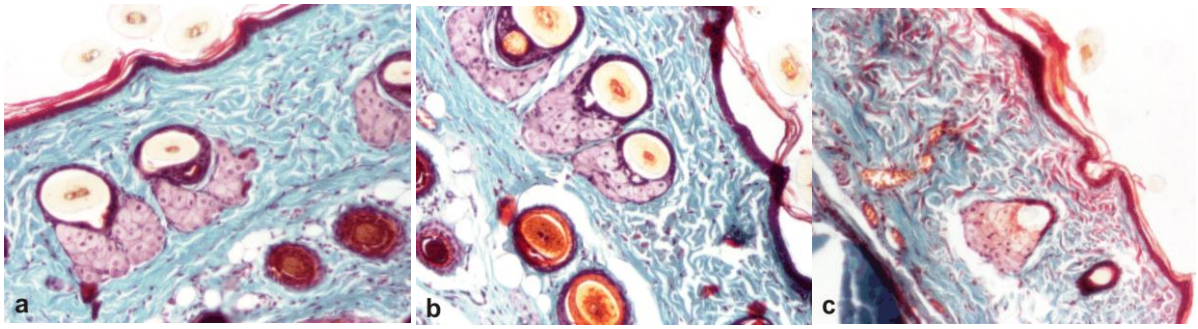


Figure 47. *Control. Normal skin, general view: (a) Group C; (b) Group E+C; (c) Group T+C. GS trichrome staining: (a–c) ×100. C: λ-Carrageenan; E: Eutectic mixture of local anesthetics (EMLA); T: Tested substance; GS: Goldner– Szekely.*

The skin samples from the E+C group revealed a more important acute inflammatory response. PMN represented most of the inflammatory elements, with diffuse distribution in the superficial and deep dermis, abundant in the deep dermis (the injection site), with perivascular disposition. There was also edema as well as vascular congestion, with leukocytosis (Figure 49, a–c).

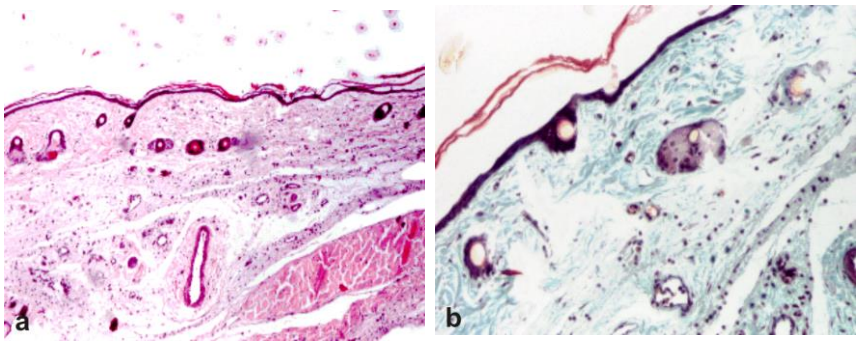


Figure 48. *Experimental Group C. Skin with mild acute inflammatory reaction, discrete edema in the superficial and deep dermis and mild vascular congestion: (a) Mild inflammation in the dermis; (b) Mild inflammation, edema and vascular congestion in the dermis. HE staining: (a) ×40. GS trichrome staining: (b) ×100. C: λ-Carrageenan; HE: Hematoxylin– Eosin; GS: Goldner–Szekely.*

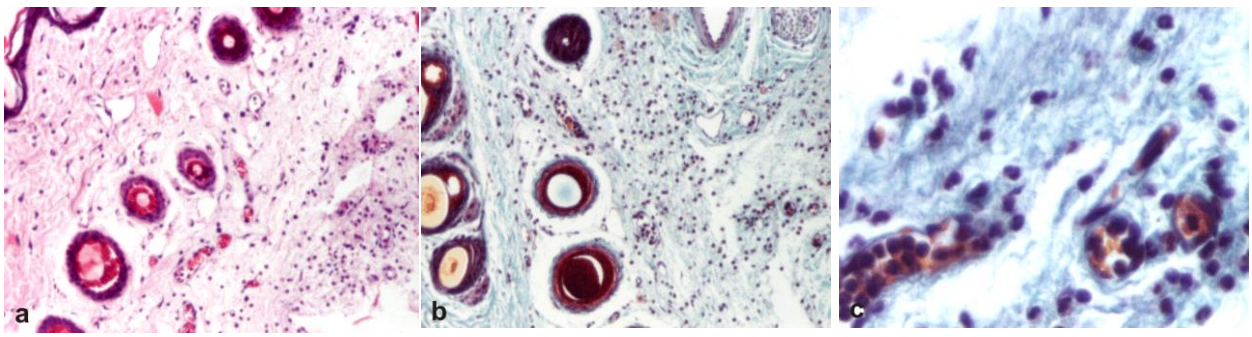


Figure 49. *Experimental group E+C. Skin with inflammatory reaction, with diffuse disposition in the superficial anddeep dermis (the place of injection), with perivascular disposition, edema and vascular congestion with leukocytosis: (a)Diffuse inflammation in superficial and deep dermis; (b) Diffuse and perivascular inflammation in deep dermis; (c) Vascular congestion with leukocytosis. HE staining: (a) ×100. GS trichrome staining: (b) ×100; (c) ×400. E: Eutectic mixture of local anesthetics (EMLA); C: λ-Carrageenan; HE: Hematoxylin–Eosin; GS: Goldner–Szekely.*

Frequent PMN, especially in the deep dermis, with perivascular and perineural distribution, vascular congestion with leukocytosis, interstitial edema, and focal sites of microhemorrhages were found in the T+C group (Figure 50, a–d).

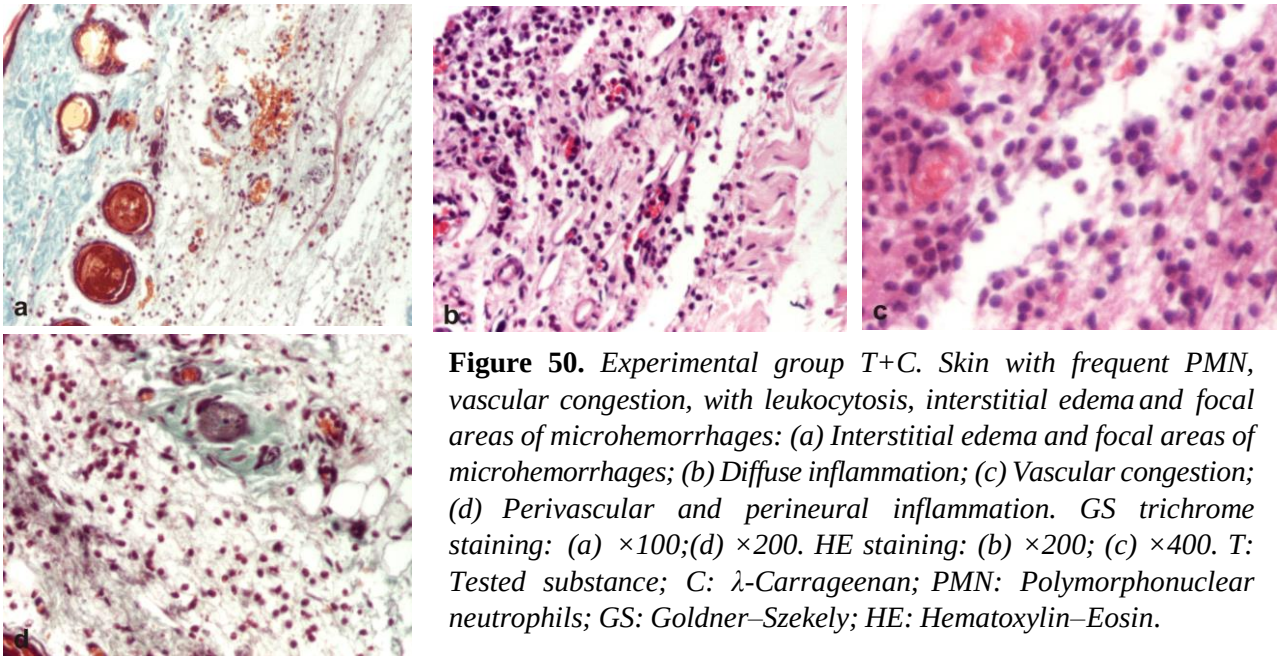


Figure 50. *Experimental group T+C. Skin with frequent PMN, vascular congestion, with leukocytosis, interstitial edema and focal areas of microhemorrhages: (a) Interstitial edema and focal areas of microhemorrhages; (b) Diffuse inflammation; (c) Vascular congestion; (d) Perivascular and perineural inflammation. GS trichrome staining: (a) ×100;(d) ×200. HE staining: (b) ×200; (c) ×400. T: Tested substance; C: λ-Carrageenan; PMN: Polymorphonuclear neutrophils; GS: Goldner–Szekely; HE: Hematoxylin–Eosin.*

II.1.3. 5. Discussions

Animal models are currently being studied in order to investigate the analgesic potential of various drugs as well as the inflammatory changes they induce. The most commonly used compound that causes inflammation is λ-carrageenan [Fehrenbacher et al., 2012]. We used this model in our research by injecting a saline solution of λ-carrageenan into the skin aiming to generate inflammatory pain.

To improve the absorption of topically applied drugs at the skin level, two methods have been investigated up to the date: the first uses diffusion enhancers, and the second uses a device or vehicle that takes advantage of drug delivery into the skin without unsettling the physical and chemical features of the superficial layer [Tadicherla, Berman, 2006]. A good enhancer should be non-allergenic or non-irritating, non-toxic, a good solvent for the drug, and pharmacologically inactive. Consequently, our research aims to present a molecule having the aforementioned qualities, as well as efficacy in pediatric and adult clinical practice.

EMLA is an oil-in-water emulsion that was created to anesthetize intact skin using two well-known local anesthetics, Lidocaine and Prilocaine. EMLA must be applied under occlusive bandages, which aids penetration into the skin's layers. The time of analgesia which is at least one hour, is controlled by the density of blood vessels in the limited area [Tadicherla, Berman, 2006; Biran et al., 2011].Our findings showed that the effect of the tested drug, which does not require occlusive bandages, was higher than that of EML after two hours of testing. The peak effect occurs between 15 and 30 minutes, after which it fades but does not disappear after two hours.

Local effects of EMLA include transitory blanching followed by erythema, as well as

edema and irritation. Many applications cause more intense local reactions, but they fade away once the treatment is stopped [Tadicherla, Berman, 2006]. Our study's histological examination demonstrated a more intense inflammatory response in the group of animals that got both λ-carrageenan and EMLA than in the group that only received λ-carrageenan, implying that EMLA has a pro-inflammatory local effect. In the group of animals when the tested drug was associated with λ-carrageenan, the results were comparable.

Our findings regarding the efficacy of topical EMLA administration vs other Lidocaine-containing compounds are in line with a lot of the literature data [Kargar et al., 2016; Ata et al., 2017; Latsios et al., 2017; Sun et al., 2019; Abbas et al., 2019], independent of the administration mode. Lidocaine is easier to administer via skin contact than via injection, and it has the same effect. In contrast to our findings, there is one study in the literature that indicates that Lidocaine alone has a smaller effect than EMLA [Horikiri et al., 2018]. On the other hand, Cozzi et al., in a study published in 2017, showed that warm Lidocaine administration has a superior effect than EMLA [Cozzi et al., 2017]. However, We believe that more research is needed in this area to fully understand the morpho-physiological characteristics of topical Lidocaine compounds delivery.

II.1.3.5. Conclusions

Over the last few years, significant research related to skin permeation mechanisms have been performed. Their findings are reflected in our days by the developments of new formulations and procedures that enhance drug delivery, essential for local and or systemic effects. Within our study, the results of the algesimeter, when compared to the results of the cold plate test and the hot plate test, revealed that the tested drug has superior analgesic effects than EMLA. The histopathological examination showed no significant differences regarding the morphological features associated to inflammatory reaction between the groups to which EMLA and the tested substance were associated, but further studies are needed in the future.

Section II. Future directions in academic, medical and scientific activities

This section briefly summarizes the professional competencies, that I hope to build as a result of the directions defined in the first part of the thesis. The information presented below lays out my professional field in terms of teaching, scientific research, administration, etc. and includes a thorough examination of personal goals, principles, priorities, and future professional responsibilities.

II.1. Developments in academic activity

The teaching profession, by definition, necessitates ongoing training and the learning of new knowledge. Furthermore, till the end of their careers, medical practitioners must continue to progress in their fields. Professional growth is important for teachers in order to discover efficient methods of communicating with the students. Thus, in order to provide the best possible teaching experience for students, residents, and PhD students, I strive to set an example for all of them by staying current on medical information and news while also making time to answer their questions.

In this regard, the goal is to maintain a professional development and improve the teaching process' quality. Continuous professional growth is usually intrinsically motivated, but it must be augmented by a moral obligation that instructors have in terms of training and progress. It demonstrates their appreciation for their vocation and for the students.

Specifically, in my future activity I want to contribute to the enhancement of instructional activities in the Morphopathology Discipline by utilizing cutting-edge technology tools. I will continue to develop the Morphopathology Discipline's research and teaching directions, as well as of the "Grigore T. Popa" University of Medicine and Pharmacy, Iasi. I consider that all educators should collaborate to design a strategy for scheduling educational activities and topics over the course of the year in order to satisfy the needs of students, residents, and PhD students while remaining compliant with current European criteria.

Implementing new and more interesting instructional methods can arouse students' enthusiasm for learning while also ensuring a high level of education. I intend to promote a dynamic classroom environment providing various teaching materials mainly by the e-learning platform, which allows students to access lectures, books, and various case reports prior to class, transforming our meetings to a lively dialogue. In this perspective, I endeavour to keep the courses for students up to date and to periodically check the acquisition of notions by different testing methods.

I will promote the study of various strategies for identifying the significant criteria for histopathological diagnosis. Furthermore, I aim to reach a definite independence of the student and I will try to introduce them into the critical thinking, with the enrolment of those with potential in research field since the early years of faculty. I will engage them in discussions, I will encourage them to address issues from different perspectives and to apply knowledge and skills in solving various problems. Another goal is to get students used to critical analysis of scientific papers, statistical data interpretation and even writing scientific materials in order to attend to national and international congresses.

My future concerns include the coordination of postgraduate courses in different areas of Pathology. I also plan to attend national, European, and international conferences, symposiums, and congresses. Developing and fostering relationships with thought professionals in the field provides the ideal environment for continual learning and knowledge renewal, which results into outstanding standards and quality in pedagogic and research activities, and also the foundation for ongoing personal development. In addition, all these will provide me with immediate access to knowledge that has a high likelihood of subsequently fulfilling in new projects and scientific subjects. Moreover, I will promote and stimulate the professional development of the younger members of the teaching team from the department I belong to, and I will continue to develop alongside them.

II.2. Developments in medical activity

Teaching and healthcare are inextricably linked, which is why it is critical for me to maintain and improve my medical abilities. Being a pathologist is a career that lasts a lifetime, in which you must constantly evolve due to the rapid expansion of technology and information.

Medical activity in a Pathology Laboratory requires a permanent need for training in order to be up to date with the latest developments in the field. There are several reasons why such ongoing improvement is needed. To begin with, you must be prepared to handle all cases as competently as possible, regardless of their difficulty. Second, by providing regular access to information and participation in training and specialty courses, the medical act will keep improving. The goals outlined in this plan are centred on a constant process of professional and personal growth, as well as the support given toward the training of future generations of high-quality human and professional specialists.

II.3. Developments in research activity

Scientific research is a critical component of any university's educational-formative development, and it is both a responsibility and a honour for any professor. Preceding scientific expertise, collaborative relationships acquired so far, and prior scientific achievements are all significant beginning points for my future scientific activity.

My research development strategy adheres to the notion of continuous improvement. My goals are to continue and develop research in the areas outlined in my thesis, as well as in other fields. The high level of academic collaboration that I have built in recent years will enable the research team that I am a part of to expand its interdisciplinarity, encouraging active participation of PhD students from different specialties. I will need to identify funding sources for these projects, such as internal grants from "Grigore T. Popa" University of Medicine and Pharmacy Iași, national grants from UEFISCDI, international grants from several international scientific societies, in order to carry out the research topics I propose.

- **Research studies in the field of atherosclerosis**

To begin with, I intend to continue my research activity in the field of atherosclerosis, a disease that I started to study more than 10 years ago in my doctoral thesis. With regard to this topic I will focus on the following directions:

1. The involvement of endothelial to mesenchymal transition in atherosclerosis development

According to the general hypothesis proposed in the last decade, atherosclerosis is thought to be caused by continue and repetitive local injury of the endothelium, a specialized subtype of epithelium, followed by excessive extracellular matrix production and collagen deposition, leading to aberrant epithelial and mesenchymal repair. Impaired fibroblast and/or myofibroblast activities, associated with smooth-muscle proliferation has been recognized as a key pathogenic event for vascular wall damage and unfavourable clinically events. Endothelial injury initiate intimal plaque formation, and stimulates matrix metalloproteinase (MMP), macrophages, or other inflammatory cells and fibrosis [Kovacic et al., 2019].

Endothelial cells may acquire a mesenchymal phenotype through EndMT (endothelial-to-mesenchymal transition) in the presence of oxidative hypoxia, Transforming Growth Factor-Beta (TGF-signalling), Bone Morphogenetic Proteins (BMPs - 2,4,6,9,10), epigenetic aberration, and inflammation [Levet et al., 2015]. EndMT is a type of EMT that involves endothelial cells and consists of a series of molecular pathways that have been linked to a variety of vascular diseases, promoting both early and severe ATS lesions [Evrard et al., 2016]. Moreover, through the process of EndMT, endothelial cells loss intercellular cellular adhesion with reduced expression of endothelial genes or proteins and change in phenotype toward a mesenchymal cell either myofibroblasts or smooth muscle cells differentiation, leading to valvular disease, pulmonary hypertension, fibroelastosis and other cardiovascular diseases (CVDs) [Kovacic et al., 2019].

It is known that EndMT has an indispensable role in vascular biology being involved in neointimal formation and vascular remodelling during embryonic development. This implies activation of the EndMT-associated transcription factors such as SNAI1, SNAI2, TWIST, LEF-1, ZEB1, ZEB2 and SMAD3, as well as additional EMT/MET-related autocrine or paracrine signalling molecules [Kovacic et al., 2019].

Challenging research proved that EndMT phenomenon can be detected via modulation of cellular phenotype by co-localization and/or co-positivity of individual cells or cell clusters, which can lead to EndMT gradations in: (i) complete EndMT; (ii) partial EndMT; (iii) reversible EndMT; (iv) transient EndMT [Manavski et al., 2018, Alvandi, Bischoff 2021]. Prior studies showed that endothelial cells located in the intima and neointimal tissues may be highlighted through endothelial biomarkers such as CD31, endothelial nitric oxide synthase (NOS3), VE-Cadherin, TIE1, and vWF, as well as through mesenchymal biomarkers such as α-smooth muscle actin (α-SMA), calponin, CD44, vimentin, FSP1, SM22α, versican) [Medici et al., 2012; Kovacic et al., 2019]. Nevertheless, it is very difficult to evaluate the interaction between these molecules; in addition, a clear cross-talk between them has not been fully demonstrated.

Interstitial vascular stromal cells, especially fibroblasts, play an important role in the fibrotic process of ATS via EndMT mechanism. In addition, TGF-β, Wnt/β-catenin, Notch signalling pathways, epigenetic changes, non-coding RNAs, including micro-, long and circular (miRNAs, lncRNAs, circRNAs) have also been demonstrated to activate and contribute to EndMT regulation. All of these together with other cytokines or additional receptors could stimulate EndMT and to mediate its biological functions. The expression of EndMT molecules as well as their biologic properties have been studied in ATS samples cultured *in vitro* as well as in ATS from mice and humans [Evrard et al., 2016, Xiong et al., 2018].

According to Edvard et al., 2016, accumulation of fibroblast clusters due to mesenchymal transition in advanced ATS is a hallmark of EndMT-fibroblast derived cells,

which has been linked to plaque instability and increased vulnerability [Evrard et al., 2016]. Recently, Dai et al., 2018 postulated that endothelial damage caused by the EndMT mechanism could be a key trigger for ER stress, plaque erosion, and subsequent acute coronary events determined by arterial thrombosis [Dai et al., 2018].

Based on recently proposed information, the directions for my future research projects regarding the **EndMT in ATS** aim the following aspects:

1. To demonstrate how endothelial cells undergo EndMT;
2. To demonstrate the role of EndMT in the development and progression of atherosclerotic disease.
3. To investigate by immunohistochemistry the importance of crosstalk between epithelial (CD31, NOS3, VE-Cadherin, vWF) *versus* mesenchymal markers (α-smooth muscle actin (α-SMA), calponin, CD44, vimentin, respectively).
4. To analyse the role of EndMT in plaque erosion.

2. The assessment of obesity complications and comorbidities in pediatric age

It is already known that obesity is has an important role in atherosclerosis and coronary artery disease. As stated by WHO, childhood obesity represents a global public health issues of our century, owing to two factors: first, the rapid rise in its prevalence over the world, and second, the serious repercussions it has on public health [WHO, 2018]. Since the 1970s, the number of obese children has risen dramatically, and it is likely to continue to increase if new global strategies for combating obesity are not created and implemented. According to epidemiological data, there are currently 381 million overweight or obese children in the world [McPhee, Singh, and Morrison, 2020].

Obesity appears to affect children under the age of five, with the World Health Organization (WHO) reporting that over 38 million children under the age of five were obese in 2017 [WHO, 2018]. Obesity incidences have recently increased in undeveloped countries, despite the fact that obesity was traditionally only seen in countries with a higher standard of life. As a result, governments have aimed to develop management strategies with a common objective: to prevent the rise in the number of overweight and obese children in all pediatric age groups by 2025 [WHO, 2018].

Obese children are likely to become obese as adults. Immediate complications include an increased risk of hypertension, CVD, IR, and T2DM non-alcoholic fatty liver disease, as well as psychological issues; additionally, novel risk factors linking obesity and increased CV risk from prenatal age to adulthood include the role of perinatal factors, diet, hyperuricemia, dyslipidemia, nutrigenomics, and nutri-epigenetics, and so on [Drozdz et al., 2021]. Accordingly, identifying hormonal and inflammatory markers as early as childhood is very important. A deep understanding of the inflammatory mechanisms that define obesity is essential in preventing the disease and its repercussions in obese pediatric patients.

Within this direction, I intend to continue the study of metabolic, cardiovascular and liver complications in obese children in order to contribute to the current understanding of the cardiovascular and metabolic risks in this category of patients. My interest in this subject has already been presented in the thesis content. Together with my colleagues from "Sfânta Maria" Emergency Clinical Hospital for Children, Iasi, where I carry out the integration activity, we

studied some predictive markers of early cardiovascular impairment and insulin resistance in obese pediatric patients. In concrete terms, we analysed inflammatory markers such as IL-6, Intercellular Adhesion Molecules 1(ICAM1), endotoxemia and their correlation with IR metabolic markers represented by insulinemia, HOMA index and plasma cortisol. The levels of IL-6, ICAM 1 and endotoxemia, were significantly higher in our patients, conducting to chronic and systemic inflammation. Accordingly, they could be considered as predictors of cardiometabolic diseases in this individuals. In addition, significant correlation between the HOMA index and the BMI percentile proved that obesity is a major risk factor for the development of IR. The BMI percentile definitely has significant predictive power for metabolic markers of insulin resistance. Nevertheless, our results emphasize the necessity for further research into the dynamics of obese pediatric patients by age group.

In my opinion, interdisciplinarity is essential for the research. Thus, I intend to develop various research projects regarding cardiometabolic complications of obesity within large research teams involving pathologists, pediatric specialists, residents, students and PhD students, as well as other Departments of "Grigore T. Popa" University of Medicine and Pharmacy, and other universities in the country and abroad.

3. Optimization of diagnosis and management in patients with hypercholesterolemia by assessing classic and additional cardiovascular risk factors

In this direction, together with colleagues from the 6th Medical Clinic of the Rehabilitation Hospital, Iasi, we plan to perform an ample investigation of cardiovascular risk factors in a group of patients with hypercholesterolemia. In addition, we will consider identifying cases with familial monogenic hypercholesterolemia so that we can conduct genetic testing and develop an effective preventive strategy.

Hypercholesterolemia can be monogenic or multifactorial, according to previous studies [Brautbar et al., 2015; Tada et al., 2019; Brandts et al., 2020]. Familial hypercholesterolemia is the most prevalent monogenic form (1/200 individuals) [Wiegman et al., 2015; Izar et al., 2021]. Mutations in the LDLR (60-80 %), APOB (1-5 %), and PCSK9 (0-3 %) genes cause the disorder, which is autosomal dominant, new mutations being extremely rare. In 20-40 % of cases, the defect is unknown. Differential diagnosis is made with hypercholesterolemia secondary to diabetes, obesity, drugs, hypothyroidism or kidney disease, situations in which the distribution of cases in the family is non-mendelian. A differential diagnosis is also made of autosomal recessive hypercholesterolemia (mutations in the LDLRAP1 gene), combined familial hyperlipidemia (associated with increased LDL-C and triglycerides, caused by mutations in the LPL gene), and polygenic hypercholesterolemia (caused by various genetic polymorphisms and adverse environmental action) [Youngblom et al., 2014; Tada et al., 2021]. Genetic testing is indicated in individuals with a family history of hypercholesterolemia and consists of complete sequencing (including 5 ', 3', and intron regions) of the LDLR, APOB, and PCSK9 genes (in that order). Modern testing (Next Generation Sequencing) can verify the sequence of the 3 genes simultaneously. In negative cases, the study of polymorphisms associated with hypercholesterolemia is recommended [Youngblom et al., 2014; Berberich et al., 2019].

The objectives of our research will consider, on the one hand, the evaluation of the cardiovascular genetic predisposition in patients with severe forms of the disease (associated pathology, severely affected young people, patients with a normal or supraponderal body mass index). Homocysteine will be tested in these categories and a CVD StripAssay will be performed. On the other hand, it is desired to evaluate specific polymorphisms related to the efficacy of statins in patients with their associated adverse effects. Specific polymorphisms in the CYP3A4, CYP7A1 and SLCO1B1 genes will be studied. In addition, a comparative study of statins will be performed. Last but not least, the study of families with familial hypercholesterolemia (high cholesterol values without other risk factors) will be carried out in order to achieve an optimal management plan for the patient and the family. The LDLR, APOB and PCSK9 genes will be tested in order.

Another purpose is to conduct a thorough investigation of specific cases of hypercholesterolemia in order to identify potential worsening factors. The following items will be assessed: thyroid hormones, intima-media thickness, arterial stiffness, DXA, and vitamin D. Patients who are young, have a normal or overweight BMI, or have statin side effects will be evaluated.

Epidemiological evidence has revealed that dyslipidemia, particularly hypercholesterolemia, plays a critical role in the development of cardiovascular disease. Because appropriate treatment is critical for these patients, we believe it is essential to monitor the side effects of cholesterol-lowering drugs and their links to specific deficiencies in drug metabolism genes.

Given the rise in the detection of genetic abnormalities in cardiovascular disease in Europe, we consider it would be useful to integrate simple genetic tests into Romanian medical practice in order to identify people with high cardiovascular risk who need specific management.

- **Research studies regarding chronic pain management**

I intend also to continue my research activity in the field of pain management initiated by collaboration with colleagues from other departments, from The Advanced Center for Research and Development in Medicine Experimental (CEMEX), and last but not least from the "Petru Poni" Institute of Macromolecular Chemistry, Iasi.

Chronic pain is a very important public health problem, with more and more individual suffering from different types of painful conditions [Nicholas et al., 2019]. In several European nations, pain medicine is now accepted as a specialty, sub-specialty, or competency-based training. Unfortunately, advanced pain management as a branch of modern medicine that necessitates specialized training and experience is still undervalued by health professionals and legislators, as well as within the medical community. This is because the full cost of pain is still unknown, as well as the fact that pain affects so many different areas of medicine [Breivik et al., 2013].

In Romania, pain assessment is one of the most challenging aspects of the therapeutic practice, and there are various reasons for this. To begin with, there is a paucity of pain-related medical training programs in medical schools and colleges, as well as a poor management of pain diagnosis and therapy in numerous clinical specialties. Moreover, few materials exist in

Romania that address the challenges of cancer pain management, methods, or specific treatment regimens. Both physicians and patients sometimes adopt an improper approach to the use of opioids or other powerful analgesics in the treatment of pain. Despite continuing efforts in recent years, Romania ranks at the top of the WHO world list for opioid consumption for medicinal purposes, owing to the limited availability of opioid medications on the Romanian market [Mosoiu, et al., 2007].

Another issue is the maintenance of treatment after discharge from the hospital. Patients and/or their families sometimes choose to adjust or interrupt their continuing pain therapy. All of these factors contribute to an ineffective pain management. Consequently, increasing medical professionals' broad understanding in the field of chronic pain is an essential component in the approach of this crucial health issue. With this regard, personal concerns have materialized in participating as a member in the team of an educational grant *Innovative education project for cancer pain management in the second largest oncology hospital in Romania* (INECAPOR), funded by the International Association for the Study of Pain, completed with the publication of a book - Leon MM, Mungiu O (eds). Pain therapy - current issues, "Grigore T. Popa" Iasi Publishing House in 2014.

In addition, my research interest focused on the experimental study of the effects of nanoparticles incorporating analgesic drugs on nociceptive sensitivity in laboratory animals. Personal concerns are reflected in my participation as a team member of a new grant *Complex liposome and cyclodextrin formulations for transdermal pain therapy (NANODERMA),* coordinated by "Petru Poni" Institute of Macromolecular Chemistry. The project objective was to improve the percutaneous pain therapy by developing new gel formulas with topical application for controlled transdermal drug delivery, with beneficial effect on pain control. We aimed to improve the functional model, the preparation methods and the efficiency of analgesic formulas as a gel form with topical application. Within the study, lidocaine was selected as the biologically active principle. Using a mixture of penetration enhancers, such as liposomes, the in vitro and in vivo release properties have been adjusted.

Several inactive compounds have also been included to ensure that the system's contents are stable and that the final product has the physical attributes required for application as a gel on the skin. The project is finalised, the substance has passed all the tests, and a patent for the new beta-ciclodextrine-based topical lidocaine formulation is on the way as the documentation has been submitted, and the response is expected. Currently, we are working on a research proposal to further refine our previous patent and translate our findings in clinical practice.

Together with my colleagues, our preoccupation is related to investigations regarding the design, characterization, acute toxicity, in vivo biocompatibility and pharmacodynamics effects of different nanoparticles / metals / compounds with analgesic effect. In addition, we will focus on experimental study of the effects of nanoparticles incorporating analgesic drugs on nociceptive sensitivity in laboratory animals.

The analgesic effects obtained in experimental animals by delivering lidocaine in various forms may be comparable to a certain level to the therapeutic effects in patients, which could be a starting point for clinical investigation of new anti-nociceptive drugs discovered in laboratory experiments.

- **Research studies regarding epithelial to mesenchymal transition in pediatric tumoral pathology**

The epithelial-to-mesenchymal transition (EMT) and its many roles in organ integrity are impacted by a variety of mechanisms that differs depending on the underlying physiological or pathological context [Lu, and Kang, 2019; Yu, Yustein, Xu, 2021]. EMT is thought to play a role in (i) organ development during embryo implantation (EMT type I); (ii) tissue injury/regeneration and fibrosis (EMT type II); and (iii) cancer progression (EMT type III). During EMT, epithelial cells undertake a mesenchymal morphological phenotype as well as distinct function with strong migratory potential [Dongre, Weinberg, 2019]. Furthermore, EMT leads to upregulation and expression of mesenchymal markers (N-cadherin, P-cadherin, matrix metalloproteinase (MMPs), fibroblast-specific protein 1, fibronectin, smooth muscle actin (SMA), or vimentin) by progressive suppression of epithelial markers (E-cadherin, zona occludens 1 (ZO-1), claudins, and cytokeratins). All of these events appear to be associated to extracellular matrix cell (EMC) secretion in addition to tissue environment rearrangement, transcription factor (TF) activation, or EMT-related gene suppression [Dongre, Weinberg, 2019].

The expression of neural cell adhesion molecule (NCAM), a key adhesion molecule, has been demonstrated to cause EMT by regulating tyrosine kinase receptor's activity. Active forms of NCAM have been found in direct association with a tyrosine kinase from the SRC family, and have been linked to EMT-mediated migration and invasiveness [Lamouille et al., 2014].

TGF-β, Notch, Wnt, Hedgehog, and tyrosine kinase receptors are some of the signalling pathways involved in EMT transition [Lu et al., 2019]. Throughout EMT, there is a general change in the expression of miRNAs, from miRNAs that inhibit EMT (miR-7, miR-30, miR-200, miR-205, etc.) to miRNAs that promote EMT (miR-7, miR-30, miR-200, miR-205, etc.) due to continuous signalling pathways regulated by many molecular players and increased metabolic requirements (miR-9, miR-27, miR-255, miR-222 etc) [Choi, Ng, 2017].

A reverse process, known as mesenchymal-epithelial transition (MET) involves dynamic molecular pathways mediated by transcription factors such as ZEB1, ZEB2, SNAI, SLUG, TWIST1, and TWIST2. Under the guidance of these EMT transcription factors, mesenchymal cells with a migratory, fibroblast-like phenotype can differentiate into other cellular lineages, such as epithelial cells with cell polarity and cell adhesion feature. Additionally, during the MET process, the cytoskeleton is reorganized in a complex manner by remodelling actin filaments. Sometimes, an 'intermediate state' or so-called "partial EMT" can develop between these two processes [Yu, Yustein, Xu, 2021].

Both EMT and TME express proteins with autocrine and paracrine function that are important regulators of cell plasticity in the carcinogenic process via epithelial cancer cells' invasiveness. EMT status has been extensively explored in carcinomas and epithelial tumour metastasis so far. The exact involvement of EMT-related processes in malignancies that arise from mesenchymal tissues, such as bone and soft-tissue sarcomas, is yet uncertain. According to growing evidence, many sarcomas can go through EMT-related processes, which may be linked to aggressive clinical behavior. Still, there are few studies in the literature regarding the

role of EMT-MET in tumoral pathology. In addition, MET mechanism in pediatric mesenchymal tumor it is even less studied.

Sarcomas represent approximately 21% of all pediatric solid tumors and fewer than 1% of all adult solid malignant cancers, according to the SEER program (Surveillance, Epidemiology, and End Results) [Burningham et al., 2012; Kahlert et al., 2017], being often divided into two categories: malignant bone tumors and soft tissue sarcomas.

Most cases of pediatric mesenchymal tumors are represented by osteosarcomas (OSs), Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET), and rabdomyosarcomas (RMSs) [Ehnman et al., 2019].

OSs account approximately 3.4 per million people and 27% deaths, with a survival of less than 5 years at patients with metastatic OS [Misaghi et al., 2018]. Every year, 800 to 900 new cases of OSs appear in the United States [Cokkinides et al., 2005]. Despite the fact that treatment (intensive chemotherapy) and surgery (often amputation) are helpful in the management of pediatric osteosarcoma, evolution and metastatic dissemination remain a challenge [Yu, Yustein, Xu, 2021].

The majority of osteosarcomas (OSs) arise from mesenchymal cells in the bone, and they are the most common sarcoma in young patients. There is a substantial link between microenvironment with the tumor risk and aggressive behavior of OSs [Wen et al., 2020]. OSs can be divided into several subtypes, but three major groups are most frequently: intramedullary, juxtacortical, and extraskeletal OSs [Kundu et al., 2014]. Intramedullary subtype accounts nearly 80% of all OSs. They develop from medullary cavity, and include several histopathological patterns as osteoblastic, chondroblastic, fibroblastic, small-cell, and epithelioid OSs [Yu, Yustein, Xu, 2021].

Recent studies postulate that links between mesenchymal-associated adhesion molecules and tumor microenvironment may provide insights into mechanisms of disease progression and metastasis in osteosarcoma. During MET tumor cells regain epithelial properties. In this context, markers of epithelial differentiation such as E-cadherin, α-catenin, catenins, and Claudin-1 were found to be expressed in various sarcoma. This phenomenon leads to acquisition of epithelial features in sarcoma cells which allow to increase cell migration and cellular protrusion by over-expression of E-cadherin. In addition, Notch signalling are involved in sarcoma progression. In OSs, the tumoral cells can reprogram their status and adopt various degrees of EMT or MET properties acquiring different histological subtypes (polygonal to spindle-shaped morphology) [Sannino et al., 2017].

It was demonstrated that the knockdown of EMT-promoting transcription factors (EMT-TFs), as SNAIL, SLUG, ZEB1, ZEB2 in MET-driven OSs significantly reduces cell invasiveness [Yu, Yustein, Xu, 2021]. TWIST1 expression is found in 32-56 % of OSs, which is associated with poor clinical outcomes and a high metastatic potential [Yu, Yustein, Xu, 2021]. In addition, ZEB2 activation causes bone development and subsequent poor outcome in fibrosarcoma and other mesenchymal tumors that produce bone tissue [Yu, Yustein, Xu, 2021].

Because E-cadherin upregulation occurs when EMT-TFs are knocked down, OSs cells lose their migratory and invasive capabilities. As a result, inhibiting EMT-TFs promotes the MET-like process, whereas activating EMT-TFs promotes the EMT-like process by maintaining the mesenchymal status of sarcomas [Yu, Yustein, Xu, 2021].

During sarcoma EMT/MET transition, there are different expression levels of epithelial and mesenchymal markers (either more epithelial or more mesenchymal), resulting in different plasticity states. It seems that patients with more epithelial-like carcinoma and high cytokeratins, claudin-1, and ZO-1 expression have a higher five-year overall survival rate compared to those with more mesenchymal-like carcinoma and low epithelial expression [Ehnman 2019; Yu, Yustein, Xu, 2021].

Following OSs, Ewing sarcoma/primitive neuroectodermal tumor (ES/PNET) is a heterogeneous malignancy that arises in either bone or soft tissue. It was demonstrated that in the presence of transcription factors, epithelial markers form an active heterocomplex that promotes transcription of MET-inducing genes and enhance expression of epithelial-like markers. The association of ZO-1 molecules (in 50% of ES/PNET) with cells transition mediators is known to be essential for partial epithelial differentiation, which is associated with better clinical outcomes [Yu, Yustein, Xu, 2021].

Rhabdomyosarcoma (RMS) develops from abnormally growing skeletal muscle cells in young patients. Induction of N-cadherin and alpha9-integrin by Notch signalling pathways have been shown to confer higher invasive phenotype on rhabdomyosarcoma cells, and thus involved in MET phenomenon [Ehnman et al., 2019]. Still, the molecular link between the Notch pathway and the process that leads to cell adhesion remains largely unclear.

In the context of MET-related mesenchymal tumor, leiomyosarcomas which develop in pediatric patients, are characterized by several molecules with high epithelial signature that trigger MET, by blocking EMT-TFs [Tian et al., 2013].

However, increased expression of epithelial-like markers such as E-cadherin defines the MET process in sarcoma, but typical mesenchymal markers such as vimentin remain prominently expressed in sarcoma cells.

It seems that the development of adherent junctions during MET in mesenchymal tumors results in higher stability of the cytoplasmic reserve of catenin, which is associated with a better prognosis. Moreover, the inhibition of Wnt/LDL receptor related protein 5 (LRP5) signalling, results in MET related osteosarcoma cells by upregulation of E-cadherin expression and downregulation of mesenchymal markers expression, such as N-cadherin. In addition to epithelial marker expression in sarcoma cells, inhibition of Wnt/ LRP5 signalling also regulates MET through downregulation of TWIST1 and SLUG that are known to be linked to mesenchymal status [Yu, Yustein, Xu, 2021].

A few recent reports have demonstrated that the decrease of transcriptional repression of EMT-TFs such as SNAIL and SLUG leads to increase in E-cadherin expression in translocated-synovial sarcoma X1 and 2 (SYT-SSX1/2) cells. This epithelial differentiation status of synovial sarcoma cells positively correlates with good prognosis [Yu, Yustein, Xu, 2021].

Furthermore, in osteosarcoma cells the expression of autocrine motility factor (AMF), known as phosphoglucose isomerase (PGI) and Ovo like zinc finger 2 (OVOL2), were associated with reduction of other EMT binding transcription factors such as SNAIL or ZEB1. The consequences were inhibition of cell migration and invasiveness, by promoting a MET process [Yu, Yustein, Xu, 2021].

However, the role of EMT signalling in conjunction with MET signalling pathways has not been explored during cellular transition of all sarcoma cell types. Additional research and

knowledge of the EMT/MET-regulatory mechanisms in OS cells could lead to the discovery of potent and selective molecular targets. Additionally, due to the complexity of EMT/MET-like regulatory networks and cancer cells' capabilities to respond to stress, focusing on a single protein or pathway may not be enough to completely stop EMT or activate MET.

In this general background, my future research developments will aim:

1. To investigate the importance of cross talking between EMT related transcription factors (EMT-TFs), namely SNAIL, SLUG, TWIST1 and ZEB1 during the molecular transition that induces MET.
2. Consequently, I am interested in examining the effects of EMT-TFs inhibition in sarcoma cells on MET-induced localization *versus* delocalization of E-cadherin and how this inhibition can generate epithelial cells.

To conclude, in terms of research, I intend to develop scientific activities line up with international standards, adhering to rigorous ethical guidelines.

Final remarks

After almost 20 years of activity I remain faithful to the idea that a career is built on teamwork abilities, in addition to a continuous learning, enthusiasm, proficiency, and personal ambition.

This habilitation thesis summarizes my accomplishments during the postdoctoral period (2011-2022) following the completion of my doctorate in medical science, as well as a partial view of the projects I have planned for future professional, academic/teaching, and scientific development. Attaining the habilitation certificate will give me the opportunity of mentoring PhD candidates as well as to continue my development as an academic professor and as a physician.

It is an honour to be part of the "Grigore T. Popa" University of Medicine and Pharmacy and I consider that all of my upcoming projects will not only provide my personal satisfaction, but also will contribute to the development of the discipline, department, and university to which I belong.

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