UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

HABILITATION THESIS

Physiopathology – a bridge, not a wall from molecular mechanisms to clinical signs

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ABBREVIATION LIST

•NO – nitric oxide
•NO2- nitrogen dioxide
•O2- superoxide anion
•OH – hydroxyl radical
•RO2 – peroxyl radical
55. LDL – low density lipoprotein
7NOS – nitric oxide synthase
AA – arachidonic acid
ABBREVIATION LIST
ADP – adenosindiphosphate
AGE – advanced glycosylated end products
AI – atherogenic index
AL – alpha linoleic acid
ALA - alpha linoleic acid
Ang II - angiotensin II
BGM – basal glomerular membrane
b-NADPH2 - b- Nicotinamide adenine dinucleotide phosphate
BSA – bovin serum albumin
Chol.T – total cholesterol
CKF – chronic kidney failure
CoQ10 –Q10 coenzime
COX – cyclooxygenase
CRP – C reactive protein
CsA - Cyclosporine A
DHA – docosahexaenoic acid
DMFS - distant metastasis-free survival
DPD - dihydropyrimidine dehydrogenase
DTNB - 5,5¢ - ditiobis
DZ – diabetes mellitus
ECAI – angiotensin converting enzyme inhibitors
ECs – endothelial cell
EC-SOD – extracellular superoxide dismutase
ED – endothelial dysfunction
EDHF – endothelial derived hyperpolarizing factor
EDTA – Ethylenediaminetetraacetic Acid Tetrasodium Salt
EGFR – epidermal growth factor
ELAM – endothelium-leukocyte adhesion molecule
EPA –eicosapentaenoic acid
EPI - epinephrine
FA – fatty acids
FFA – free fatty acids
GSH-Px - glutathione peroxidase
H_2O_2 – hydrogen peroxide
Hb - hemoglobin
HbA1c – glycosylated hemoglobin
HCl – chlorhidric acid
HDL – high density lipoprotein
HE - hematoxylin and eosin
HMGA - 3-hydroxy-3metilglutaric acid
HMG-CoA - 3-hydroxi-3-methilglutaril-coenzime A reductase
HNSCC - head and neck squamous cell carcinoma
HPLC – high performance liquid chromatography
HPV - Human papillomaviruses
HPV-OPC - oropharyngeal cancers associated with human papilloma virus
Ht - hematocrit
HT – hypertension
ICAM-1 – intercellular adhesion molecule
ICI - immune checkpoint inhibitors
IDL – intermediate density lipoprotein
Ig – immunoglobulin
IGF-I – insulin-like growth factor
IL – interleukin
IP3R - inositol phosphate 3 receptor
LA – linoleic acid
LC PUFA – long chain polyunsaturated fatty acids
LCAT - lecithin-cholesterol acyl transferase
LDH - lactic dehydrogenase
LFFS - local failure-free survival
LP – lipoproteins
LPC - lysophosphatidylcholines
LT – leukotriene
LysoPC - lysophosphatide choline
MAPK - mitogen-activated protein kinase
MCP-1 – monocyte chemotactic protein
MCSF – macrophage colony-stimulating factor
MDA – malondialdehyde
MPA - metaphosphoric acid
mPTP - mitochondria permeability transition pore
MUFA – monounsaturated fatty acids
n3 FA – omega-3 fatty acids
n6 FA – omega-6 fatty acids
NBT - nitro blue tetrazolium
NFD – diabetic nephropathy
NF-kB – nuclear transcription factor
NGS - next generation sequencing technique
O_3- ozone

GAG - glycosaminoglycan
GAP - gliceraldehid-3-phosphate
GP – glycoproteins
GSH – reduced glutathione
OCSCC - oral cavity squamous cellular cancer
OD – optical density
ONOO-- peroxinitrite
OPMDs - oral potentially malignant disorders
Orc – orceine
O-Rd - oil red
OS - median overall survival
OS - overall survival
OSCCs - oral squamous cell carcinomas
oxLDL –oxidized LDL
PA – tisular plasminogen activator
PAF – platelet activator factor
PAI-1 – plasminogen activator inhibitor
PAS - periodic acid Schiff
PBS – phosphate buffer saline
PD - disease progression
PD-1 - programmed death ligand
PDGF – platelet derived growth factor
PEPCK – phosphoenolpyruvate carboxykinase
PG – prostaglandins
PGI_2 – prostacyclin
PKC - protein kinase C
PKC – protein kinase C
PLA_2 – phospholipase A2
PMN – polymorphonuclear neutrophils
PMP - platelet derivate microparticles
POD - peroxidase
PP2A - protein phosphatase
PPAR - peroxisome proliferator activated receptor
PR - partial response
PRP –platelet rich plasma
PS - Phosphatidylserine
PUFA – polyunsaturated fatty acids
PUFA n3 - polyunsaturated omega-3 fatty acids
PUFA n6 - polyunsaturated omega-6 fatty acids
RAGE – AGE receptor
RFFS - regional failurefree survival
RL – free radical
ROS – reactive oxygen species
SFA – saturated fatty acids
SIII - Sudan III
SMC – smooth muscular cells
SOD – superoxide dismutase
SREBP - sterol regulatory element binding protein
SRN – nitrogen reactive species
SRO – oxygen reactive species
STZ – streptozotocine
sVCAM-1 – vascular cell adhesion mollecule-1
SDG - secoisolariciresinol diglucoside
SDH - sorbitol dehydrogenase
sdLDL – small dense LDL
SECO - secoisolariciresinol diglucoside TAS – total antioxidant status
TBA –thiobarbituric acid
TBARS – thiobarbituric acid reactive substances
TCA –tricloracetic acid
TEA - trietanolamin-hydrocloride
TG - triglycerides
TGF – tumor growth factor
THP - thapsigargin
TM - thrombomodulin
TNF – tumor necrosis factor
TP - Thymidine phosphorylase
TPM - mitochondrial permeability transition
TRAP – total peroxyl radical trapping antioxidant parameter
TS - Thymidylate synthase
Tx – tromboxan
UPR - unfolded protein response
VCAM-1 – vascular cell adhesion molecule
VG - Van Gieson
VLDL – very low density lipoprotein
VSMC - vascular smooth muscle cells
vWF – von Willebrand factor

ABSTRACT

The habilitation thesis briefly presents the most important professional, academic and especially scientific achievements obtained after the completion of the doctoral studies (2006) but also some of the future projects for the years to come. The structuring of this thesis is carried out in accordance with the recommendations of the National Council for Attestation of University Titles, Diplomas and Certificates (CNATDCU) and the methodology of the Doctoral School of the "Grigore T. Popa" University in Iasi. The skill thesis consists of three sections as follows.

Section I is presenting postdoctoral professional, academic and scientific achievements. After this, Section I concentrates the reference results of own research activity completed by publishing the data in specialized journals. Chapter I represents the summary of some studies related to the pathogenesis of the atherosclerotic process. Since the first years of my activity, my scientific curiosity has been related to the interaction of some basic physiopathological processes: inflammation, coagulation and atherosclerosis. Atherosclerosis, an extremely widespread condition, occurs as a consequence of proliferative inflammation of the endothelium, through the gradual accumulation of cholesterol in macrophages and foam cells. As it progresses, these deposits narrow the arterial lumen, the rupture of the plaque complicated by thrombosis completes the obstruction. Platelets participate not only in the thrombotic complication of the atheromatous lesion but also in the initiation and progression of atherosclerotic plaques. Basically, platelets represent "a bridge" between two pathological processes inflammation and thrombosis and are strongly involved in the pathogenesis of atherosclerosis. Inflammation is characterized by interactions between platelets, leukocytes and endothelial cells, processes that lead to the recruitment of leukocytes into the vascular wall. Chronic platelet-induced inflammatory processes in the vascular wall result in the development of atherosclerotic lesions and atherothrombosis. The interaction between platelets and endothelial cells occurs in two ways: activated platelets can adhere to the unactivated endothelial cell or resting platelets can adhere to the activated endothelial cell. At the same time, the interaction between the activated platelet and the activated endothelial cell may occur. This involves complex interactions between membrane glycoproteins (adhesion molecules) on both endothelial cells P-selectin (CD62P), E-selectin, ICAM-1 and VCAM β3 integrins, ICAM-1 and the platelet surface receptors GPIbα and PSGL-1, GPIb/IX/V, GP IIbIIIa, platelet factor 4 (PF-4), a member of the C-X-C subfamily of chemokines that induces monocyte chemotaxis by promoting low-density lipoprotein (LDL) retention. Also, inflammation induces an oxidative stress responsible for the production of LDLox. At the same time, PDGF released by platelet granules stimulates the proliferation of muscle cells and produces hyperplasia (another cause that reduces the diameter of the vascular wall).

The experiments performed addressed the study of the endothelial cell in the inflammation-atherosclerosis interaction. The scientific and practical deepening of the experimental methods for the investigation of platelets (within the doctoral studies) continued with an experimental activity related to the functional involvement of platelets in the atherosclerosis process. Experimental studies on the interaction between platelet and endothelium in the atherosclerosis process are described in subchapters I.1, I.4, I.6. Chapter I.2 refers to the interaction between different platelet morphological and functional parameters and subchapters I.3 and I.5 respectively represent extensive experimental studies related to the influence of factors such as diet (enriched with lipids, with polyunsaturated fatty acids -PUFA, vitamin E and whole grains) and hormonal status on aspects of the development of atherosclerosis (lipid status, platelet function, and anatomical-pathological aspects).

Chapter II focuses on oromaxillofacial cancer as the main axis of research, starting from molecular and subcellular studies of tumor progression mechanisms and continuing with clinical aspects in patients with cancer of the oromaxillofacial sphere. The interest in this subject began with my inclusion as a researcher in the study carried out by Contract grant CNCSIS 61GR/16.05.2006, Title - "ASSESSMENT OF THE ANTI-TUMOR EFFECT OF VITAMIN D IN PATIENTS WITH HEAD AND NECK CARCINOMA" Director - Prof. Dr. Veronica Mocanu. Being a teaching staff of the Faculty of Dental Medicine, the interest in this subject continued afterwards.

The "biological era" of cancer treatment began in the 1950s after the discovery of the structure of DNA by Watson and Crick. Subsequent studies have led to a deep understanding of the molecular mechanisms that drive neoplastic transformation, progression and response to therapy. Cancer treatments focused on specific molecular targets and pathways are becoming more common.

Chapter II begins with an overview of some aspects related to the only known pathogen involved in the pathogenesis of oral cancer, *Human Papillomavirus* (HPV) and the ways through which infection with it mediates oral carcinogenesis (II.1). The following subchapters refer to other aspects related to the involvement of subcellular pathways and structures in the mediation of oral carcinogenesis, but also their use as targets in the modern, multimodal treatment of oral cancer.

Subchapter II.2 deals with the EGFR pathway, a recognized oncogene whose overexpression or mutation in oral cancer is much debated. EGFR immunotherapy was the first "biological target" approved for oral cancer. Cetuximab is a monoclonal antibody that targets the extracellular ligand-binding domain of EGFR. One of the proposed mechanisms of Cetuximab resistance is the expression of the tumor-specific EGFR deletion mutant EGFRvIII. Next-generation sequencing (NGS) assays are used to detect EGFR exon 20 insertions, mutations that may confer distinct therapeutic features compared to exon 19 deletions. Amivantamab, a dual-acting antibody on both EGFR mutation and receptor activity mesenchymal transition factor (MET) and on immune cells as well as Pozitinib, a next-generation tyrosine kinase inhibitor for targeting exon 20 aberration in EGFR appear to be new therapeutic promises in oral cancer.

Micro-RNAs represent evolutionarily conserved non-coding RNA fragments evaluated as potential biomarkers in numerous diseases, including cancer, but also with the potential of intrinsic predictors in concurrent or sequential systemic treatment. Chapter II.3 represents a meta-analysis related to the "signature" of Micro-RNAs in understanding the different response to treatment, with a special focus on the differentiation of HPV+ and HPV- subtypes of oral cancers. Anticipating the risk of toxicity associated with radiochemotherapy, the possibility of obtaining loco-regional control after treatment and assessing the risk of recurrence and distant metastasis represent the advantages of knowing the epigenetic mechanisms of cancerogenesis.

p53, initially considered a tumor suppressor was the subject of research in chapter II.4. The scientific knowledge of the current molecular biology has made a decisive contribution to the understanding of the mechanisms involved in the p53 pathway by identifying an increasing number of post-transcriptional targets but also to the understanding of the apoptotic mechanisms mediated by p53. The possibility of using HPV status, p53, and miRNA as biomarkers for therapy selection, as well as the updated interest in tumor metabolism as a possible target involving the restoration of p53 function may benefit therapeutic response.

Fundamental studies were related to aspects of apoptosis in neoplastic cells and to the relationships between cytosolic and mitochondrial calcium concentration in the apoptotic process as well as the response of the endoplasmic reticulum to stress as an adaptive pathway

in carcinomatous progression. These studies were the subject of chapters II.5 and II.6. We also studied the influence of the calcium ionophore on the mitochondrial permeability pore (PTM) in normal, treated cells as well as in a cancer cell line. The following study is related to the genetic polymorphism of the proinflammatory cytokines TNFα (tumor necrosis factor α) and interleukin 1 (IL1) in potentially malignant oral inflammatory lesions. The action of cytosporin B, a NUR77 agonist, on the apoptosis of pro-B lymphocytes (a murine pro-B lymphocyte cell line dependent on interleukin-3) in the presence of gingival fibroblasts in culture was the subject of a subsequent experiment.

Subchapter II.7 presents new theoretical and experimental aspects of capecitabine and its much better known metabolite, 5FU (5 Fluorouracil) in tumor progression. An experimental model on the *in situ* release of 5FU from adapted formulas, through the theoretical multifractal model revealed a different release trend, depending on the crosslink density of the support reaching 96% for a high crosslink density.

In section II, future projects are presented in professional, academic and research terms

The thesis is completed by a selection of bibliographic references cited in this habilitation thesis.

REZUMAT

Teza de abilitare prezintă pe scurt cele mai importante realizări profesionale, academice și în special științifice obținute după finalizarea studiilor doctorale (1998-2006) dar și o parte din proiectele de viitor pentru următori. Structurarea acestei teze este realizată în conformitate cu recomandările Consiliului Naţional de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare (CNATDCU) și a metodologiei Școlii Doctorale a Universității "Grigore T. Popa" din Iași. Teza de abilitare este alcătuită din trei secţiuni după cum urmează.

Secțiunea I prezintă realizările profesionale, academice și științifice obținute postdoctoral și concentrează rezultatele de referință ale activității proprii de cercetare finallizate prin publicarea datelor în reviste de specialitate. Capitolul I reprezintă sumarul unor studii legate de patogenia procesului aterosclerotic. Încă din primii ani de activitate curiozitatea mea științifică a fost legată de interacțiunea unor procese fiziopatologice de bază: inflamația, coagularea și ateroscleroza. Ateroscleroza, afecțiune extrem de răspândită apare ca o consecință a inflamației proliferative a endoteliului, prin acumularea treptată a colesterolului în macrofage și celulele spumoase. Pe măsura evoluției, aceste depuneri îngustează lumenul arterial, ruptura plăcii complicate de tromboză completează obstrucția. Trombocitele nu participă doar la complicarea trombotică a leziunii ateromatoase ci și la inițierea și progresia plăcilor aterosclerotice. Practic, trombocitele reprezintă „o punte de legătură" între două procese patologice inflamație și tromboză și sunt puternic implicate în patogenia aterosclerozei. Inflamația se caracterizează prin interacțiuni între trombocite, leucocite și celule endoteliale, procese care duc la recrutarea leucocitelor în peretele vascular. Procesele inflamatorii cronice induse de trombocite la nivelul peretelui vascular au ca rezultat dezvoltarea leziunilor aterosclerotice și aterotrombozei. Interacțiunea dintre trombocite și celulele endoteliale are loc în două moduri: trombocitele activate pot adera la celula endotelială neactivată sau trombocitele în repaus pot adera la celula endotelială activată. În același timp poate apărea interacțiunea dintre trombocitele activate și celula endotelială activată. Aceasta implică interacțiuni complexe între glicoproteine membranare (molecule de adeziune) de pe ambele celule endotelială P-selectina (CD62P), E-selectină, ICAM-1 și VCAM integrinele β3, ICAM-1 și receptorii de suprafață plachetar GPIbα și PSGL-1, GPIb/IX/V, GP IIbIIIa, factorul trombocitar 4 (PF-4), un membru al subfamiliei C-X-C a chemokinelor care induce chemotaxia monocitelor promovând retenția de lipoproteine cu densitate joasă (LDL). De asemenea, inflamația induce un stres oxidativ responsabil și de producerea de LDLox. În același timp, PDGF eliberat de granulele trombocite stimulează proliferarea celulelor musculare și produce hiperplazie (o altă cauză care reduce diametrul peretelui vascular).

Cercetările s-au adresat studiului rolului celulelei endoteliale în interacțiunea inflamație-ateroscleoză. Aprofundarea din punct de vedere științific dar și practic a metodelor experimentale de investigarea a trombocitelor (in cadrul studiilor doctorale) a continuat cu o activitate experimentală legată de implicarea plachetelor din punct de vedere funcțional în procesul aterosclerozei. Studii experimentale privind interacțiunea dintre trombocit și endoteliu în procesul aterosclerozei sunt descrise în subcapitolele I.1, I.4, I.6. Capitolul I.2 se referă interacțiunea dintre diferiți parametri morfologici și funcționali plachetari iar subcapitolele I.3 respectiv I.5 reprezintă studii experimentale extinse legate de influența unor factori cum ar fi dieta (îmbogățită cu lipide, cu acizi grași polinesaturați-PUFA, vitamina E și cereale integrale) și statusul hormonal asupra aspectelor dezvoltării aterosclerozei (status lipidic, funcție plachetară, și aspecte anatomo-patologice).

Capitolul II vizează cancerul oromaxilofacial ca ax principal al cercetării plecând de la studii moleculare și subcelulare ale mecanismelor de progresie tumorală. Interesul pentru acest subiect a debutat cu includerea mea ca cercetător în studiul desfășurat prin Contract grant CNCSIS 61GR/16.05.2006, Title - "ASSESSMENT OF THE ANTI-TUMOR EFFECT OF VITAMIN D IN PATIENTS WITH HEAD AND NECK CARCINOMA" Director - Prof. Dr. Veronica Mocanu. Fiind cadru didactic al facultății de Medicină Dentară interesul pentru acest subiect a continuat și ulterior.

„Era biologică" a tratamentului cancerului a debutat în anii 1950 după descoperirea structurii ADN-ului de către Watson și Crick. Studiile ulterioare au condus la o înțelegere profundă a mecanismelor moleculare care conduc la transformarea, progresia și răspunsul la terapie neoplazice. Tratamentele pentru cancer axate pe ținte și căi moleculare specifice devin din ce în ce mai frecvente.

Capitolul II debutează cu o punere la punct a unor aspecte legate de singurul patogen cunoscut implicat în patogeneza cancerului oral, *Human Papillomavirus* (HPV) și căile prin care infecția cu acesta mediază carcinogeneza orală (II.1). Subcapitolele următoare se referă la alte aspecte de legate de implicarea unor căi și structuri subcelulare în medierea a carginogenezei orale dar și folosirea acestora ca ținte în tratamentul modern, multimodal al cancerului oral.

Subcapitolul II.2 se referă la calea EGFR, o oncogenă recunoscută a cărei supraexpresie sau mutație în cancerul oral este mult discutată. Imunoterapia EGFR a fost prima "țintă biologică" aprobată pentru cancerul oral. Cetuximab este un anticorp monoclonal care vizează domeniul extracelular de legare a ligandului EGFR. Unul dintre mecanismele propuse de rezistență la Cetuximab este expresia mutantului de deleție EGFR specific tumorii EGFRvIII. Testele de secvențiere de ultimă generație (NGS) sunt folosite pentru a detecta inserțiile exonului 20 EGFR, mutații care pot conferi caracteristici terapeutice deosebite în comparație cu delețiile exonului 19. Amivantamab, un anticorp cu dublă acțiune atât asupra mutației EGFR, cât și asupra activității receptorului factorului de tranziție epitelial mezenchimal (MET) și asupra celulelor imune precum și Pozitinib, un inhibitor de tirozinkinază de ultimă generație pentru țintirea aberației exonului 20 în EGFR par a a fi noi promisiuni terapeutice în cancerul oral.

Micro-RNAs reprezintă fragmente de ARN necodant conservate evolutiv evaluate ca potențiali biomarkeri în numeroase boli, inclusiv cancer dar și cu potențialul de predictor intrinsec în tratamentului sistemic concomitent sau secvenţial. Capitolul II.3 reprezintă o metaanaliză legată de "semnătură" *Micro-RNAs* în înțelegerea răspunsului diferit la tratament, cu un accent special pe diferențierea subtipurilor HPV+ și HPV- de cancerele orale. Anticiparea riscul de toxicitate asociat radiochimioterapiei, posibilitatea de a obține un control loco-regional după tratament și aprecierea riscul de recidivă și metastază la distanță reprezintă avantajele cunoașterii mecanismelor epigenetice ale cancerogenezei.

p53, considerat inițial un supresor tumoral a făcut obiectul cercetărilor în capitolul II.4. Cunoștințele științifice ale biologiei moleculare actuale au adus o contribuție decisivă la înțelegerea mecanismelor implicate în calea p53 prin identificarea unui număr tot mai mare de ținte post-transcripționale dar și pentru înțelegerea mecanismelor apoptotice mediate de p53. Posibilitatea utilizării statutusului HPV, p53 și miARN ca biomarkeri pentru selecția terapiei, precum și actualizarea interesului pentru metabolismul tumoral ca o posibilă țintă care implică restabilirea funcției p53 pot aduce beneficii în răspunsul terapeutic.

Studiile fundamentale au fost legate de aspecte ale apoptozei în celulele neoplazice și de relațiile dintre concentrația calciului citosolic și mitocondrial în procesul apoptotic precum și răspunsul reticolului endoplasmic la stres ca și cale adaptativă în progresia carcinomatoasă. Aceste studii au făcut obiectul capitolelor II.5 și II.6. Am studiat de asemenea influența ionoforului de calciu asupra porului de permeabilitate mitocondrială (PTM) în celule

normale, tratate precum și pe o linie de celule canceroase. Studiul următor este legat de polimorfismul genetic al citokinelor proinflamatorii TNFα (factorul de necroză tumorală α) respectiv interleukina1 (IL1) în leziunile inflamtorii orale potențial maligne. Acțiunea cytosporinei B, un agonist NUR77, asupra apoptozei limfocitelor pro B (o linie celulară murină de limfocite pro-B dependentă de interleukina-3) în prezența fibroblastelor gingivale în cultură a facut obiectul unui experiment ulterior.

Subcapitolul II.7 prezintă noi aspecte teoretice dar și experimentale ale capecitabinei dar și a mult mai cunoscutului său metabolit, 5FU (5 Fluorouracilul) în progresia tumorală. Un model experimental asupra eliberării *in situ* a 5FU din formule adaptate, prin modelul teoretic multifractal a relevat o tendință diferită de eliberare, în funcție de densitatea rețelei reticulare a suportului ajungând la 96 % pentru o densitate mare de reticulare.

În secțiunea a II-a sunt prezentate proiectele de viitor în plan profesional, academic și de cercetare.

Teza este completată de referințele bibliografice citate în această teză de abilitare.

Section I

SUMMARY OF PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACCOMPLISHMENTS

In essence, career development is regarded as one of the most important aspects of human development. This is seen as a dynamic, interactive process. A multitude of factors of an educational, physical, economic, psychological nature and even those related to chance act on the individual in the context in which he shapes his career. Career development does not start from a fixed moment in life, but, having a procedural nature, covers the past, present and future of the person. What characterizes any career today is the dynamism, the approach to several related fields of activity. Currently, there is an increased emphasis on career development, but especially on career education, precisely so that people become capable of managing their own careers.

In our profession, the career represents a combination of the three types of activity, deeply interrelated teaching activity, research activity and medical activity.

Teaching activity

I started my academic career in March 1998 when I was admitted by contest as a junior assistant at Pathophysiology Discipline, faculty of Dentistry at the University of Medicine and Pharmacy "Grigore T. Popa" of Iași. My academic career continued so that in 2002 I was promoted by contest as Assistant Professor. This initial period meant an intense stage of my professional and human development for acquiring new knowledge in the field of physiopathology, continuous improvement of the methodology of teaching students and starting the research activity within the doctorate. I continued the theoretical and practical training and this work was made concrete through the publication of support materials for practical works (in 2008 of a book chapter of practical works in the Romanian language and in 2014 of a compendium of physiopathology in French "Physiopathologie - points a comprendre", Ed. "Gr. T. Popa" of UMF Iasi). In 2014, I have been appointed as Senior lecturer, by contest. I continued to teach Pathophysiology to the second-year dentistry student's Romanian and French section, but also seminars and courses on scientific research methodology, which constituted an opportunity for my research activity. Since 2018 I introduced and held an optional course "Coagulopathy in dentistry" dedicated to fourth-year dental medicine students. In 2020, I was promoted by contest from the position of Senior Lecturer to that of Associate Professor and during the "pandemic era" I continued the didactic activity in a manner that represented both a challenge and a life lesson, especially for the medical field.

Medical activity

I graduated from the "Grigore T. Popa" University of Medicine and Pharmacy of Iasi in 1995 with the highest grade, and in the same year I was admitted as a resident in the family medicine specialty following the national residency competition. Then I was trained for 3 years and I completed 6 internships (Internal medicine - "St. Spiridon" Hospital, Surgery – Emergency Hospital, Iasi; Obstetrics-gynecology – "Cuza Voda" Clinical Hospital, Iasi; Pediatrics – "Sf. Maria" Clinical Hospital, Iasi, Infectious disease and Dermatology). It was a very good medical experience as I worked in 6 different specialties, which prepared me for my next step, becoming a specialist in the same specialty in 1998. In 2000, being already a university teacher, I chose to continue my professional training by becoming a resident doctor in laboratory medicine, a specialty that I graduated in 2005. This specialization was extremely useful for me in acquiring technical knowledge and helpful manual skills for my

research activity and of course it was the support of my medical activity. I am currently a primary physician in laboratory medicine in the laboratory of "St. Spiridon Hospital" in Iași.

Research activity

The research activity started once I became member of the teaching staff of the Physiopathology Department, in 1998. Right from the beginning, I was included in the research projects run by my colleagues and fellows. I started to communicate the results at the 3rd International Congress of Pathophysiology (Lahti, Finland, 1998).

In the same year (1998), I was admitted as PhD fellow, under the guidance of Prof. dr. Mihai Nechifor. The research activity carried out during the doctorate was focused on the morphological and functional characteristics of the platelet, drugs that influence platelet function and their mechanisms of action. I investigated platelet functions: adhesiveness, aggregability, malondialdehyde platelet level (MDA), adapting a method for determining platelet adhesiveness (according to Bellavite et al.) and a method for determining platelet aggregability (according to Bednar et al) in the laboratory with a microplate reader. Finally I demonstrated its suitability for purpose through an index significant Pearson correlation (r = 0.87) between the experimental method and the one accredited in the laboratory (classic aggregometry with the ChronoLog aggregometer). I started the study of platelet aggregability on the ChronoLog aggregometer during an internship in the Hemostasis Laboratory of the Fundeni Hospital and the subsequent I applied the knowledge gained by performing aggregometric determinations for diagnostic purposes in the Medical Analysis Laboratory of the "Sf. Spiridon" Iasi. The study continuated by the investigation of the morphological and functional platelet parameters in patients with acute and chronic liver disease, but also in laboratory animals in which we induced an acute/chronic toxic hepatopathy with CCl4. These researches led to the completion of a study on platelet functionality embodied in a doctoral thesis with the title "Research on platelet parameters in some acute and chronic liver disease", the first doctoral thesis in the field of platelet morphology and function completed in Iasi, under the guidance of Prof. Dr. Mihai Nechifor**.** The research I accomplished contributed in finalizing the PhD thesis (OMER no. 4871 / 07.08.2006.) and, also, publishing articles in important scientific journals.

I also continued the research activity within the pathophysiology discipline in the following fields: the use of liposomes as intracellular targets, the study of the mechanisms of smooth muscle contraction (tracheal, bronchial and vascular), polyamines and their functional effects, experimentally induced apoptosis, the role of mitochondria and the endoplasmic reticulum, the role of the diet enriched with flax seeds and respectively vitamin E in changing the cell membrane composition and its effects on the parameters of oxidative stress in different pathologies and respectively on the platelet function, the antitumor role of vitamin D. Starting with 2001, I became member of many multidisciplinary research teams running national grant projects. Most of these studies were carried out as a team member in the following research projects:

Projects, research grants

- "The effect of supplementation with flax seeds in experimental atherosclerosis", funded by the Nutrigrup Association, held during the period 2002-2003, Director - Prof. Dr. Veronica Mocanu
- "The role of linseed in the pathogenesis of cardiovascular complications from diabetes", financed by the Nutrigrup Association, carried out during 2003-2004, Director - Prof. Dr. Veronica Mocanu

National research grants

- Contract grant CNCSIS 61GR/16.05.2006, Title - "Assessment of the anti-tumor effect of vitamin d in patients with head and neck carcinoma" Director - Prof. Dr. Veronica Mocanu,
- Grant contract CNCSIS GR215/15.09.2006-8, project type A, code 1478, Title - "Integration of the molecular mechanisms of b prolymphocyte apoptosis", Director - Prof. Dr. COSTULEANU MARCEL
- Grant contract, A Type code 1128, 2003-2005, Title - "Relations of cytosolic and mitochondrial calculation with apoptosis", CNCSIS financier, Director, Prof. Dr. Marcel Costuleanu
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- Contract grant AT type, cod 206/2001,Titlu - „Development studies of applicability in respiratory therapy", CNCSIS financier, Director, Prof. Dr. Marcel Costuleanu

I am also the author of ten books and book chapters, two of which were awarded by the Romanian Academy of Scientists, respectively the "Carol Davila" University of Medicine and Pharmacy Bucharest.

I. **ELEMENTE DE FIZIOPATOLOGIE PRACTICĂ**, coordonator Veronica Colev Luca Editura "Gr. T. Popa", U.M.F. Iaşi, **2008**, ISBN: 978-973-7682-42-0, co-autor - **Roxana Iancu**

II. **PHYSIOPATHOLOGIE, Points à comprendre**, **Roxana Irina Iancu**, Editura "Gr. T. Popa", U.M.F. Iaşi, **2014**, ISBN: 978-9606-544-281-8

III. **CLASIC SI MODERN IN FIZIOPATOLOGIE – O ABORDARE INTEGRATIVA IN EDUCATIE SI CERCETARE,** sub redactia Magda Badescu – Ed. „Gr. T. Popa" UMF Iasi, **2015,** ISBN 978-606-544-310-5

IV. **CEREALELE INTEGRALE SI MICROBIOTA IN PREVENTIA OBEZITATII LA VARSTA COPILARIEI – RECOMANDARI SI BUNE PRECTICI** – coordonator Veronica Mocanu, Ed. „Gr. T. Popa" UMF Iasi, **2018**, ISBN 978-606-544-521-5

V. **TRANSLATIONAL RESEARCH IN CANCER**, Edited by Sivapatham Sundaresan and Yeun-Hwa Gu, Camil Ciprian Mirestean, Călin Gheorghe Buzea, Roxana Irina Iancu, Dragoș Petru Teodor Iancu Implications of Radiosensitizer and Radioprotector Factors in Refining the Dose-Volume Constraints and Radiobiological Models in IntechOpen, **2019** ISBN 978-1-83880-535-7

VI. **RADIOMICA, FUNDAMENTE ȘI APLICAȚII**, Călin Buzea, Viorel-Puiu Păun, Lucian Eva, Maricel Agop, Ionel Daniel Cojocaru, **Roxana Irina Iancu**, Dragoș Teodor Iancu –Editura Academiei Române, București, **2020**, ISBN:978-973-27-3206-9

VII. **PROBLEME ACTUALE ALE MEDICINEI MODERNE DIN PERSPECTIVA INTELIGENȚEI ARTIFICIALE**, Călin Buzea, Lucian Eva, Bogdan Doroftei, Corina Lupașcu, Dragoș T. P. Iancu, **Roxana Irina Iancu**, Ionuț Cojocaru, Maricel Agop, Ed. Ars Longa, **2020**, ISBN978-973-148-336-8

VIII. **BIOMEDICAL ENGINEERING TOOLS FOR MANAGEMENT FOR PATIENTS WITH COVID-19** Edited by: Valentina E. Balas, Oana Geman, Guojun Wang, Muhammad Arif, Octavian Postolache, Chapter 3 - Radiotherapy challenges in COVID era, Ciprian Mireștean, Maricel Agop, Călin Buzea, Marius Mihai Cazacu,

Marius Prelipceanu, **Roxana Irina Iancu**, Dragos Teodor Iancu, ISBN 978-0-12-824473-9, Academic Press, **2021** Elsevier Inc.

IX. **BIOMEDICAL ENGINEERING APPLICATIONS FOR PEOPLE WITH DISABILITIES AND THE ELDERLY IN THE COVID-19 PANDEMIC AND BEYOND** Edited by: Valentina Emilia Balas and Oana Geman, Chapter 14 - Radiotherapy for pelvic malignancies in a COVID-19 pandemic scenario: Focus on rectal and cervical cancers Ciprian Mireștean, Maricel Agop, Calin Gheorghe Buzea, Marius Mihai Cazacu, Marius Prelipceanu, **Roxana Irina Iancu**, Dragos Teodor Iancu, ISBN 978-0-323-85174-9. Academic Press, Copyright © **2022** Elsevier Inc.

X. **CARDIOTOXICITY INDUCED BY RADIOTHERAPY AND/OR CHEMOTHERAPY AFTER CANCER TREATMENT, Edited** by Virginie Monceau, Omid Azimzadeh, Marjan Boerma and Nadia Pasinetti**,** Chapter - Hypofractionated Whole-Breast Irradiation Focus on Coronary Arteries and Cardiac Toxicity—A Narrative Review, ISBN 978-2-83250-953-1, 2022, Published in Frontiers in Oncology, Avenue du Tribunal-Fédéral 34 1005 Lausanne, Switzerland, frontiersin.org

As a consequence, my international visibility is reflected in the following indexes: Web of Science Clarivate Analytics H-index: 6, *in extenso* ISI papers: 27, *in extenso* IDB papers: 51, FCIAP=58,241.

Chapter I

IS THE PLATELET THE WEAK LINK OF ATHEROSCLEROSIS?

I.1. The "*Canonical*" Role Of Platelets In Atherosclerosis.

Scientific context

Platelets were discovered by Giulio Bizzozero in 1882 and for many years knowledge related to the dynamic and multifunctional nature of platelets rested they did not represent a lively field of interest. Platelets are the smallest blood particles, non-nucleate, discoid there are fragments of megakaryocytes. Primarily they are known only for their hemostatic role but hemostasis or blood coagulation is not the sole function of platelets; rather it is employed in several multifunctional attributes monitoring the homeostasis of the body. While keeping interactions with leukocytes and endothelial cells, it restores its behavior as an important inflammatory marker (figure I.1.1). Platelet reactivity for different pathogenesis is widely dependent upon some biologically active markers like CD36, CD41, CD42a, CD42b, and CD61. These include some active surface receptors and platelet secretory products. Platelet tends to alter the expression and signaling of these markers in different disease diagnosis and prognosis, providing a huge field to explore disease progression (Ghoshal, 2014).

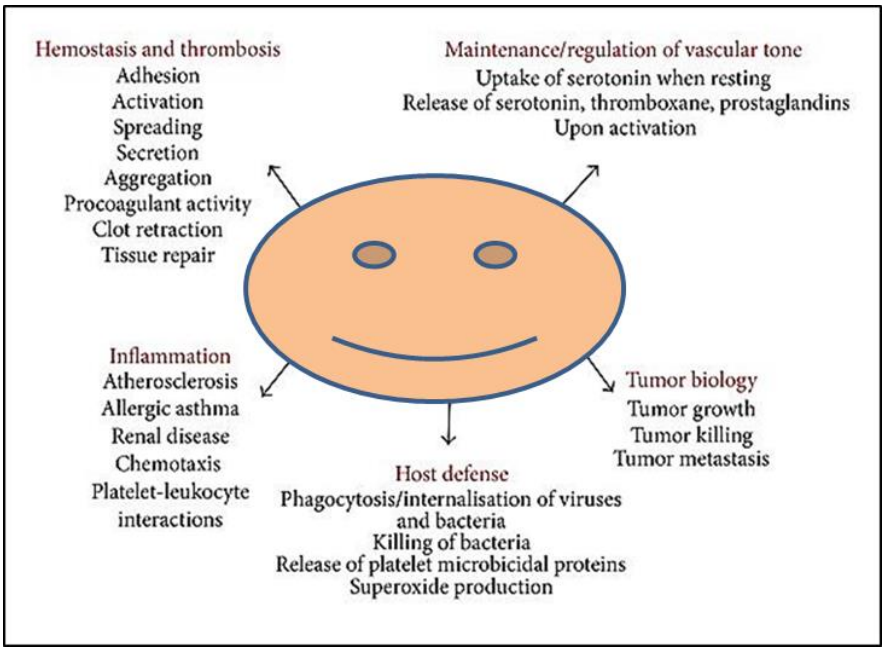

Figure I.1.1
The multifunctional role of platelet, adapted from Ghoshal, 2014

Atherosclerosis is a pathology which occurs due to established factors as a consequence of the proliferate inflammation of the endothelium, classically known as the enlargement of the arterial endothelium through the gradual accumulation of cholesterol in macrophages and foamy cell. As time passes, these deposits become tough and narrow the arterial lumen, which may progressive block blood flow or more frequently the blockage id the result of the plaque rupture complicated by thrombosis that complete the obstruction.

Platelets do not only participate in thrombus complications of atheromatosus injury, but also in the initiation and progression of atherosclerotic plaques (Willeit, 2000).

Practically, platelets represent connecting element, ”a connecting bridge” between two pathological processes inflammation and thrombosis, strongly involved in atherosclerosis pathogenesis (Figure I.1.2).

Inflammation is characterized by interactions among platelets, leukocytes, and endothelial cells, processes that lead to leukocyte recruitment into the vascular wall. Platelet-induced chronic inflammatory processes at the vascular wall result in development of atherosclerotic lesions and atherothrombosis (Gawaz, 2005).

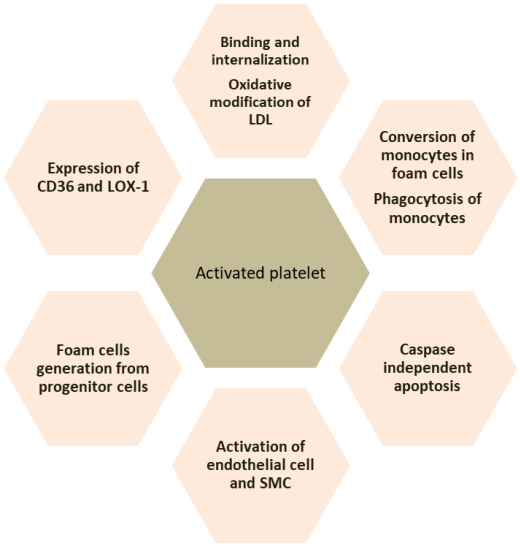

Figure I.1.2
Potential mechanisms from platelets contribution to atherosclerosis process
adapted from Siegel-Axel D. et al., 2008

I.1.1. Platelet and endothelial cell in atherosclerosis

The interaction between the platelets and the endothelial cells occurs in two ways: activated platelets can adhere to non-activated endothelial cell, or resting platelets can adhere to activated endothelial cell. In the same time interaction between activated platelets and activated endothelial cell can occur.

I.1.1.a. Platelet adhesion to non-activated endothelial cell

The circulation of activated platelets has been shown to be one of the initial causes of atherosclerosis development. Activated endothelium surface expresses P-selectin. Platelet surface receptors GPIbα and PSGL-1 interact with endothelial P-selectin (CD62P) and mediate platelet rolling. The interaction between P-selectin and PSGL-1 or GPIb/IX/V is rapidly reversible and insufficient for stable adhesion. Subsequent firm adhesion is mediated through β3 integrins, ICAM-1 in ECs which bind the platelets to the endothelium (Huo Y, 2003). Animal models have also demonstrated the important role of P-selectin in the development of atherogenesis. Animals which lose P-selectin expression show a decrease in atherosclerotic injury (Blann, 2003). In vivo, firm platelet adhesion to the endothelium can be inhibited by anti-αIIbβ3 mAb, and platelets defective in αIIbβ3 do not firmly adhere to activated ECs (Massberg, 1999).

After platelets activation, they release from α granules, pro-inflammatory molecules that alter the functions of the endothelium. The molecules express by activated platelet induce an increase of the expression of molecule adhesion cells (MACs), E-selectin, ICAM-1 and

VCAM and stimulated the release IL-8 and monocyte chemo attractant protein 1 (MCP-1). The endothelial cell release cytokines who favors the beginning of leukocyte recruitment. The protein expressed on platelet membrane after activation also induces the expression and liberation of metalloproteinases which disintegrate extracellular matrix proteins.

The activated platelets release also other molecules like IL-1ß (principal activator of the endothelial cell), IL-6 and IL-8 (involved in inflammatory response). They cause chemotaxis of monocytes and other leukocytes promoting low-density lipoprotein (LDL) retention favoring the formation of atheroma.

Platelet factor 4 (PF-4), a member of the C-X-C subfamily of chemokine, is derived by limited proteolysis from platelet basic protein. PF-4 causes chemotaxis of monocytes and other leukocytes. Nassar (2003 provide more evidence for the involvement of PF4 in the development of atherosclerosis. PF-4 enhances the binding of oxidized low-density lipoprotein (oxLDL) to vascular wall cells, including endothelial cells and smooth muscle cells. PF-4, localized with oxLDL in atherosclerotic lesions, especially in macrophage-derived foam cells, is able to dramatically increase oxLDL esterification by macrophages.

The principals membrane glycoprotein GPIb and GP IIbIIIa may be less relevant to the development of atherosclerosis because neither GPIb monoclonal antibody blockade nor GPIIbIIIa drug blockade are protective for atherosclerosis (Massberg, 1999, Shpilberg, 2002).

Likewise, inflammation induces an oxidative stress responsible for production of LDLox also. In the same time PDGF released by platelet granules stimulates the proliferation of muscular cells and produces hyperplasia, another cause who reduces the diameter of the vascular wall (Huo, 2004).

I.1.1.b. Platelet adhesion to activated endothelial cell

Under physiological conditions endothelial cell himself is capable to control vascular tone, leukocyte adhesion, smooth muscular cell growth and platelet aggregation by molecules including prostacyclin and nitric oxide. These alterations of the endothelium known like endothelial dysfunction may occur very early in a life time. The first evidence of this is the decrease in nitric oxide synthesis, causing an increase of the vascular tone and an increase in permeability and endothelial adhesion. Leukocyte adhesion at the site of the lesion represents the earliest histological evidence of plaque development. Normally, the endothelium is a non-adherent surface. However, during EC apoptosis, pro-adhesive processes are initiated that may allow for the joining of non-activated platelets (Frenette, 1995). Thus, activated or dysfunctional endothelium is a determining factor in platelet adhesion.

I.1.1.c Microparticles derivated from platelet and atherosclerosis

As a result of the platelet activation process the thrombocyte undergoes changes in shape (from a disk to a sphere with extensions) it releases the materials existing in the granules and, in addition, forms the so-called *microvesicles derived from platelets* which are nothing more than fragments of the platelet membrane that externalize phosphatidylserine residues. Being fragments of the platelet membrane, they express glycoproteins with a role in initiating adhesion to the endothelial cell, monocyte or even to other platelets, like an activated platelet. So, platelet microparticles, released from activated platelets, contain most of the platelet adhesive molecules and proinflammatory factors, and cause a variety of inflammatory reactions, as do activated platelets. The role of activated platelets in the development of atherosclerosis may be partially attributed to platelet microparticles.

I.1.2. Platelets and lipids metabolism in atherosclerosis

Congenital or acquired dyslipidemia represents a major risk factor in atherosclerosis. The more serious it is or with a longer evolution, the higher is the prognosis of death due to

cardiovascular or stroke. The effects of chronic hyperlipidemia are complex; it causes lipid deposition in atherosclerotic lesions, primary endothelial damage, and increased platelet reactivity. Thus, the mechanism leading to enhanced platelet reactivity is one of the key points in the treatment of vascular diseases. Evidence suggests that lipoprotein–platelet interaction plays an important role in atherogenesis.

It is well known that platelets, through specific binding receptors, affect low-density lipoprotein (LDL) per se triggers platelet activation, and enhances platelet aggregation and secretion, whereas HDL desensitizes platelets, underlining the anti-atherosclerotic properties of high-density lipoprotein (HDL) (Surya, 1993). Human scavenger receptor B1 (known to be a receptor for 'protective' HDL) was described on the surface and inside human platelets also (table I.1.1).

The lectin-like oxidized LDL receptor-1 (LOX-1) has also been identified on platelets. As a C-type lectin family member it belongs to the scavenger receptor class E and is a type-II membrane protein that is translocated to the membrane upon stimulation by fusion of alpha-granule membranes with the plasma membrane. Experiments with blocking antibodies against CD36 indicated that, beside LOX-1, CD36 is responsible for one part of the overall binding of OxLDL in human platelets (Relou, 2003).

Platelet factor 4, PF-4, released from granules enhances the binding of oxidized low-density lipoprotein (oxLDL) to vascular wall cells, including endothelial cells and smooth muscle cells. PF-4, colocalized with oxLDL in atherosclerotic lesions, especially in macrophage-derived foam cells, is able to dramatically increase oxLDL esterification by macrophages (Huo, 2004).

Human platelets express LDL receptor-related protein 8 (LRP8), (Riddell, 1999). LDL binding to LDL receptors on platelets promotes lipid exchange between LDL particles and platelet plasma membrane, initiates phosphorylation of focal adhesion kinase (FAK), enhances the binding of fibrinogen and integrin αIIbβ3, and induces an increase in intracellular Ca2+ triggering the release of materials from the granules and platelet aggregation with the formation of white thrombus.

Native HDL regulates platelet signaling pathways by binding to platelet HDL receptors (such as SR-BI and apoER20), as well as by balancing the cholesterol content in platelets to prevent platelet hyper-responsiveness (van der Stoep, 2014).

OxLDL binding to CD36 (a class B scavenger receptor also called platelet glycoprotein IV), on actives platelet and increases exposure of platelet P-selectin and activates integrin αIIbβ3, which may involve the signal pathway including SRC family kinases, Syk, and phospholipase C-γ. Furthermore, activated platelets internalize oxLDL, while platelets loaded with oxLDL further activate the endothelium, release chemokine to recruit monocytes, and promote foam cell development (Wang, 2020). Native LDL particles affect platelet function, they can induce the synthesis or translocation of membrane phospholipids or favor the insertion of phospholipids altering the phospholipid composition of the platelet membrane. In contrast, binding between oxidized LDL particles and the platelet surface induces activation, morphological changes, and platelet aggregation, thereby contributing to the formation of thrombi, particularly after plaque rupture (Badimón, 2009).

Table I.1.1
Effects of cholesterol fractions on platelets, adaped from Siegel-Axel D. et al., 2008

Native LDL	− Binding and activation of platelets − Increase of platelet sensitivity to platelet-activating agents − Change of the composition of platelet membrane phospholipids − Transfer of lipids to other cells

	– lipid peroxidation (transformation into OxLDL by platelet ROS)
Oxidized LDL	– binding to platelets via SR-B, CD36, LOX-1 – platelet hyperreactivation – induction of platelet shape change inducing aggregation – cholesterol release by activated platelets to macrophages – foam cell formation after platelet phagocytosis by macrophages induction of foam cell formation in platelet/progenitor cell co-culture
HDL	– anti-atherosclerotic effects by platelet desensitization

Lipoproteins and atherogenesis

The circulating cholesterol is carried in mostly in LDL which is the lipoprotein most closely associated with the development of atherosclerosis. Physiologically LDL may pass from the plasma into the subendothelial space and return to the liver to be removed from the circulation. In this way it has performed its transport functions without being included by macrophages and form foam. However, in case of endothelial dysfunction or hypercholesterolemia the retention of the LDL in the endothelial space is increased, due to endothelial injury or if removal of LDL from the circulation is delayed, it can become damaged by oxidation or modified in other ways.

Hypercholesterolemia promotes endothelial dysfunction by reducing the production of NO, thus favoring leukocytes adhesion. When NO decreases, it creates an oxidative environment that oxidizes LDL in transit from the plasma to the site of the endothelial lesion. There, the presence of lisophosphatidylcholin induces the expression of VCAM-1 in the endothelial cell (Palomo, 2008).

Oxidized or otherwise modified LDL are retained in the subendothelial space and included by monocyte-derived macrophages *via the scavenger receptor* leading to the formation of *foam cells*. The accumulation of LDL causes arterial sub-endothelial fatty streaks who are the precursor of atheroma. Small dense LDL particles (associated with postprandial hypertriglyceridemia and VLDL increase) appear to be more susceptible to oxidation which may make them more atherogenic (figure I.1.3).

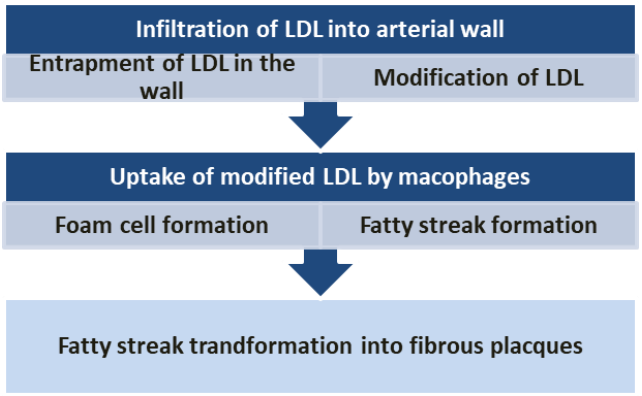

Figure I.1.3
Steps in atherogenesis, adaped from BJC, 2021

In addition, there is an interaction between dyslipidemia and inflammation, the pro-inflammatory pathway can directly affect lipid metabolism, including elevated level of triglyceride-rich very low-density lipoproteins (VLDL), triglyceride (TG) and free fatty acids (FFA). The oxidized lipoproteins deposit in the sub endothelial space generates an inflammatory reaction – the 'response to retention' hypothesis of atherosclerosis. At high TG levels status, VLDL particles are initially converted to large LDL particles and subsequently

converted to small dense LDL, resulting in an increase of small dense LDL particles level in circulation. Inversely, lipids can also directly induce an inflammatory reaction, and cholesterol feeding can promote the inflammatory reaction, which in turn may contribute to the development of metabolic syndrome. Partially metabolised remnants of triglyceride-rich lipoproteins (remnant lipoproteins) that appear post-prandially are able to induce foam cell formation without modification. These are considered the most highly atherogenic of all.

Other atherogenic lipoproteins readily retained in the subendothelial space include glycated LDL and lipoprotein(a). Apolipoproteins are proteins that bind lipids to form lipoproteins. They transport lipids through the lymphatic and circulatory systems. They also serve as enzyme cofactors, receptor ligands, and lipid transfer carriers that regulate the metabolism of lipoproteins and their uptake in tissues. HDL are, however, able to penetrate deep into the subendothelial space and are able to remove oxidised lipid from macrophages and prevent foam cell formation, in addition to having a protective effect on the endothelium. Reduction of HDL particle numbers or functional activity is therefore pro-atherogenic.

PERSONAL CONTRIBUTION RELATED TO THE ROLES OF PLATELETS IN ATHEROSCLEROSIS WAS SYNTHESIZED IN THE FOLLOWING PAPERS:

	Articles
1.	**Haliga RE, Iancu RI, Butcovan D., Mocanu V, Flaxseed prevents leukocyte and platelet adhesion to endothelial cells in experimental atherosclerosis by reducing sVCAM-1 and vWF, The Scientific World Journal, 2013, Vol 2013, 303950**
2.	**Mocanu V, Haliga R, Paduraru O, Baran D, Badoi D, Iancu R, Oboroceanu T, Balanica A, Badescu M A diet rich in whole grain flaxseeds has antithrombotic effects without increasing oxidative stress in experimental atherosclerosis Journal of Biologically Active Products From Nature , 2011, 1 (3), 144-159**
3.	**Iancu RI, Iancu D, Nechifor M, Costuleanu M, Research on interrelationships between morphological and functional parameters of platelet. Romanian Journal of Functional & Clinical, Macro- & Microscopical Anatomy, 2010, Vol. 9 Issue 4, p442-446.**
4.	**Chelariu R, Dumitriu IL, Iancu RI, Chelaru L, Slătineanu SM, Petrescu G, Costuleanu M Interactions between angiotensin ii and polyamines incorporated in liposomes in experimental inflammation. Annals of the Romanian Society for Cell Biology 2011, 16 (1)**
5.	**Chelaru L, Iancu RI, Slătineanu SM, Petrescu G, Costuleanu M Targeting of subendothelial space using liposomes. Annals of the Romanian Society for Cell Biology 2011, 16 (1)**
6.	**Iancu RI, Toader PM, Mocanu M, Mirestean CC, Iancu DT Malondialdehyde (MDA) as a Marker of Platelet Function in Rats with CCl4-Induced Toxic Hepatopathy , Revista de Chimie (Rev. Chim.), 2020, 71 (5), 315-320**
7.	**Iancu RI, Haliga R, Luca V, Stitt P.A., Mocanu V, Modularea funcţiei plachetare prin suplimentare alimentară cu seminţe de in şi vitamină E la hamsterii diabetici Revista Medico-Chirurgicală, 110 (4)2006, 962-968,**

8.	**Ciurea E, Mocanu V, Iancu R, Haliga R, Pricop F Luca V, https://sogr.ro/revista-sogr/platelets-activity-changes-in-estrogen-deficiency-and-the-effects-of-dietary-flaxseed-supplementation/ Rev Soc de Obst si Ginecol, 2010 (1)**
9.	**Balanica A, Luca V, Haliga R, Iancu R, Oboroceanu T, Mocanu V, Efectul vitaminei E asupra plachetelor la hamsterii femele ovariectomizate, Rev Med Chir Soc Med Nat Iasi, 2007; supl, I**

I.2. Research on interrelationships between morphological and functional parameters of platelet

I.2.1. Introduction

Platelets are the smallest blood cells with role in thrombus formation. Initially, their study consisted only in counting and observing them under a microscope (layout size, "platelet aggregates") in the evaluation process of hemostasis. Later, investigation of morphology and platelet function was improved without abandoning the traditional methods in it's entirely. The hematology analyzer can establish a number of parameters that characterize the overall and specific platelet morphology. Platelet function tests have been performed since the early twentieth century aimed mainly adhesion, platelet aggregability and certain enzymes. The structure and function is a whole in achieving the role of platelet coagulation, affecting one or another of this complex process induced disturbances.

Typical platelet function tests range from assessment of their primary hemostatic function including measurement of granule secretion using lumi-aggregometry to whole blood shear based assays, which measure platelet function under flow conditions. Platelet function can be tested in washed platelets, whole blood or platelet rich plasma.

Platelets pathology often refers to reduce the number or impaired platelet function as two separate things. The aim of our study was to investigate if, into a lot of healthy people, there is any correlation between morphology and platelet function. No correlations were found between morphological and functional platelet parameters; some strong correlation have been emphasized separately from some morphological parameters and separate from all functional parameters too.

I.2.2. Platelet indices

Platelet indices are useful as inexpensive and non-invasive biomarkers for assessing platelet activation (Budak, 2016) Platelet indices are straightforwardly measured by semi-automated counters in complete blood counts (CBC) and usually include four factors; platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), and depending on the analyser, platelet large cell ratio (P-LCR). PLT is a universal indicator of haemostasis in a clinical setting and is utilised as a sensitive biomarker for a range of diseases. High PLT, even within the physiological range of 150–450 μl is associated with a greater risk of thrombosis and CVD suggesting that enhanced PLT encourage platelet hyperactivity and a pro-inflammatory state. However, the consequence of high platelet numbers that are still within physiologic ranges remains unclear (Twomey, 2018)

MPV reflects the average platelet size while PDW reflects the volume variability in platelet size the volume of circulating platelets is heterogeneous with subsequent functional differences. Some authors suggest that larger platelets are metabolically more active than smaller platelets, that they have faster rates of aggregation and release higher quantities of pro-thrombotic elements such as TxA_2 and ADP (Mangalpally, 2010) and PDW levels can be altered in several diseases including (Berger, 2010), and in this regard they have been suggested as markers of subclinical platelet activation.

P-LCR and PCT may serve as sensitive biomarkers of platelet health PLCR indicates the percentage of large platelets present in blood. PLCR is significantly higher in subjects with dyslipidaemia compared to healthy subjects. Moreover, Rechcinski (2013) have hypothesized that PLCR has the potential to be a prognostic biomarker importantly; thrombogenicity of large platelets may put individuals at higher risk of acute cardiovascular events.

PCT is the volume of blood occupied by platelets as a percentage, similar to the erythrocyte measurement of hematocrit (HCT). PCT reflects total platelet mass and is calculated as PLT × MPV/10^7, providing comprehensive information about platelet activity. PCT has been proposed as a novel predictor of cardiovascular risk and higher PCT is associated with the risk of re-infarction and long-term mortality in CVD patients. However, the clinical significance, reference values and efficacy of some of these parameters are still under exploration.

In peripheral blood, there is ample interplay between RBCs, WBCs and platelets and altered levels of blood cells and their morphology have been associated with CVD. Platelet adhesion and aggregate size, is influenced by platelet indices, RBC and WBC. RBCs encourage platelets towards the vessel wall, which can affect platelet adhesion and aggregation. In this context, it is important to investigate the associations between the various indices of each blood cell to interpret the multicellular contribution to both thrombogenesis and CVD risk (Twomey, 2018).

The aim of this study was to determine the morphological and functional parameters of platelet and establish possible correlations between them.

I.2.3. Material and methods

The control group consisted of 23 patients both sexes, aged between 28 and 68 years old, 11 males and 12 females. They were hospitalized in the "St. Parascheva" Infectious Disease Hospital Iași for other diseases not involving impaired platelet function. Each patient underwent a complete clinical examination consisting of clinical examination and laboratory tests that included screening tests: determination of amino-transferase, bilirubin, protein determination, ESR, complete blood count and liver ultrasound. Patients receiving medications or supplements that affect platelet function or with a history of some disease (hepatitis, thromboembolic disease, diabetes, pregnant women or those who use oral contraceptives, patients with major hyperlipidemia – which is opalescent sera, patients which inflammatory disease – fibrinogen > 400 mg/dL) were excluded from this study.

We determined a series of 11 parameters (Table. II), which we intuitively divided into "morphological" and functional parameters. "Morphological parameters", related to the number and size of platelet production, characterized indirectly bone marrow production of platelets as well as platelet function. Functional parameters (platelet adhesively and aggregation) and the ability of eicosanoids synthesis characterize directly platelet function.

Morphological parameters determined by MICROS 45 OT analyzer, were:

1. PLT, platelet count per µl in circulation. The principle involves measuring the impedance variation. Normal values range is between 150000-4000000/µl.
2. MPV, the average platelet volume is indicated by numerous studies as having a clinical relevance. Normal values range, related to the technique used in this study are from (7.23 – 9.03) in males and (8.18 – 9.6) fL in women. However, a diagram is needed to determine whether MPV is normal or not concerning the number of platelets.
3. PCT, percentage platelet volume, with normal values in the technique that we use is between 0.100 to 0.500%. Interpretation is extremely difficult because limits are wide.
4. PDW, "platelet distribution width", is a marker of the degree of platelet anizocytozis.

Platelet functional parameters investigated in this study were:

1. ***Platelet aggregation*** by two methods:

- using a Chronolog Aggro/Link® witch expresses the aggregation index - IA% (aggregation amplitude) as a percentage of platelet aggregates after adding reagent (ADP and epinephrine in 10μM final concentration cell), expressed in percentages as final optical shaft transmission, considering 0% transmission for platelet-rich plasma (RPR) and 100% transmission in platelet poor plasma (PPP). The slope (Slope) is determined by drawing a tangent to the apex aggregation curve steeper;
- ADP aggregation uses a microplate reader, (Beddnar method (1995), modified by Robert C. Chadderon and Michael Cappello (1999) determining the tangent to the curve of aggregation expressed as mOD/minute. This method establishes the decrease of optical density/minute in the process of aggregation to ADP 10μM, thanks to the formation of the large platelet aggregates.

2. ***Platelet adhesion*** to extracellular matrix components is a component of platelet function. The method used in our study was described by P. Bellavitte (1994).
3. ***Determination of MDA*** in platelet-rich plasma PRP) after stimulation with ADP. We used TBARS reagent method Buege, (1978). Levels of MDA (malondialdehyde A) are a way to measure the level of platelet prostaglandin synthesis.

The data were loaded and processed using statistical functions in Excel and EPIINFO using Student t-test significance threshold of 95% is generally accepted that p = 0.05) and $\chi 2$ test and Pearson correlation coefficient. The intensity of the relationship between variables is measured by the correlation coefficient, which must take values between $\mathbf{-1 \leq r \leq 1}$, as is closer to 1 is even stronger correlation, either direct or reverse if r = -1.

Table I.2.1
Platelets parameters

PLT	Platelet count per μl in circulation
PCT%	Percentage platelet volume
MPV	Platelet medium volum
PDW %	Platelet distribution width
ADP IA%	Aggregation amplitude to ADP (%)
ADP SLP	The slope of aggregation to ADP
Epi IA %	Aggregation amplitude to epi (%)
Epi SLP	The slope of aggregation to epi
mOD/min	The slope of aggregation to ADP on microplate reader
MDA	Malondialdehyde A (nmoli/mL)
adez%	Platelet adhesion (%)

I.2.4. Results and discussion

Mean age, expressed as mean ± standard deviation in the control group was 41.35 years ± 12.73 years of age and gender distribution of the female average vs. male average was 36.08 ± 7.63 *vs.* 47.09 ± 14.94.

Distribution of patients by gender reveals approximately equal frequency between the two sexes in the control group (female 52.2% *vs.* male 47.8%). The parameters values were approximately equal between sexes, which may not induce statistical significance between sexes (we didn't find statistically significant differences in our group). We considered r > ±0.5 correlated, and r > ± 0.6 significantly correlated.

To summarize, Table III highlights the strong links between the parameters investigated for the study group.

Table I.2.2
Interdependence between investigated parameters Pearson coefficient correlation r

	r		**r**
PLT - PCT%	0,94	ADP-IA% - ADP-SLP	0,53
PLT - MPV	-0,52	ADP-IA% - EPI-IA%	0,47
PLT - PDW%	0,51	ADP-IA% - mOD/min	0,87
PCT% - MPV	-0,42	ADP-IA% - MDA	0,92
PCT% - PDW%	0,54	ADP-IA% - ADEZ%	0,81
EPI-IA% - EPI-SLP	0,90	ADP-SLP - MDA	0,45
EPI-IA% - MDA	0,42	ADP-SLP - ADEZ%	0,56
mOD/min - MDA	0,88	MDA - ADEZ%	0,78
mOD/min - ADEZ%	0,83		

PLT (platelet count per µl in blood circulation) is worthy of interest in our study because represents a balance between production and use versus loss and destruction on the other side.

MPV (the medium platelet volume), indicated by numerous studies to have clinical relevance (Bessman JD 1985, Baynes 1988, Jackson, 1993) improve diagnostic accuracy of platelet disorders. Normally there is a nonlinear inverse relationship between MPV and PLT, relationship that persists in the case of thrombocytosis or thrombocytopenia provided that bone marrow function is normal. Park's study (2002) suggests that MPV may be considered as an indicator of platelet activation in vivo. Older studies (Jorgensen, 1984) argue that in hepatic diseases both MPV and PLT decreased, so, in this context, the inverse correlation between MPV and PLT pass away. Other studies (Fusegawa, 2002) show that platelets are activated in vivo in chronic hepatitis C virus which could be expressed through increased MPV. However, a possible explanation given by Jorgensen (1984) shows that the platelet activation in vivo would complete the loss of platelet granules (which explains the scarcity of aggregation) and increased platelet consumption. Data from this study is consistent with other studies (Jorgensen, 1984, Jackson, 1993, dos Santos 2004, Baynes, 1988). All this studies show a nonlinear inverse correlation between PLT and MPV, with values close to those obtained by us (0.52 in our study vs. 0.49) in Baynes (1988) study.

PCT (percentage platelet volume) has normal values range from 0.100 to 0.500%. The limits are broad, the interpretation extremely difficult and the literature does not comment it, unlike other platelet parameters.

PDW (platelet distribution width) is a marker of the degree of platelet anizocytosis. Some studies (Ogura, 1995) claim a correlation between PDW, MPV and P-LCR (percentage of reticulated platelets, undetermined parameter in this study).The same study claims that there is a significant correlation between PDW and platelet survival time and between PDW and platelet's turnover rate. Luzatto's study (1988) concludes that the PDW (with MTI - megathrombocytic index, percentage of large platelets) are faithful markers of thrombopoietic function and better indicators of altered platelet homeostasis than the MPV. PDW increase is explained mostly by increasing the percentage of large platelets (Panasiuk, 2001), reticulated platelets and platelet-derived microparticles. Microparticles are a marker of platelet activation which can be detected by the technique of monoclonal antibodies (Fusegawa, 2002).

Data obtained by us in terms of correlations between MPV and PLT are consistent with those published by Jorgensen (2004). He argues that there is a nonlinear inverse correlation between MPV and PLT (we also obtained a correlation coefficient 0,52 into the control group subjects). Jorgensen (2004) argues that this correlation no longer held in conditions of liver dysfunction and we will pursue this in our study. Another study (Baynes 1988) found a

correlation coefficient of - 0.49 PLT- MPV (value close to that found by us) in patients with rheumatoid arthritis and concluded that the disproportion between MPV and PLT can be the basis for diagnosis of rare congenital syndromes (such as "giant platelets syndrome").

Another study concerning platelets, dos Santos (2004) show correlations to levels approaching those found here, with a correlation coefficient r = - 0.35 PLT - MPV in healthy pregnant women. Jackson (1993) discusses the importance of MPV in diagnosis, because this parameter depends on the time after harvest, storage or method anticoagulant laboratory standardization is extremely important. Our data were obtained in a single laboratory, the Central Laboratory of Hematology, "St. Spiridon "University Hospital Iasi. When working conditions are standard, MPV changes in correlation with PLT can be extremely useful in monitoring and diagnosis of disease (Jackson, 1993).

In terms of the relationship between morphological and functional platelet parameters our study did not find any correlation, fact suggested also by other studies (Budak. 2016). They conclude there is no correlation between the platelets level (PLT rates) and aggregation (IA). In our study too, no correlation was found between the level of aggregation to ADP (ADP-IA expressed that mOD/min) and epinephrine (EPI-IA) and platelet count (PLT), r index values are PLT-mOD/min r =- 0.17, PLT-ADP -IA% =- 0.002 and =- 0.29% PLT. Data from this study is consistent with other studies Jorgensen (1984) (Jackcson, 1993, dos Santos, 2004, Baynes, 1988) studies show a nonlinear inverse correlation PLT- MPV, values close to those obtained by us 0.49 (Baynes, 1988), *vs.* 0.52 in our study.

I.2.5. Conclusions

Concerning platelets parameters there are no statistically significant differences between sexes. In terms of interdependence between some parameters:

1. We found a direct correlation, significant highly statistically, between platelet aggregation (both ADP and epinephrine), platelet adhesion and MDA.
2. There is a highly significant correlation (r = 0.87) between the two distinct methods - turbidimetric aggregation (Chronolog Aggro) and aggregation through microplate reader (TECAN microplate reader) - to determine platelet aggregability.
3. We found also a highly significant nonlinear correlation (r = 0.94) between the total value of platelets (PLT) and percentage platelet volume (PCT).
4. Lower intensity correlation existed between other parameters (see Table nr. III) of which mention the correlation between platelet morphological parameters, inverse correlation between the average platelet volume (MPV) and PLT, and inverse correlation between the degree of platelet anizocytosis (PDW%) and plachetocrit (PCT).
5. Any correlation was found between morphological parameters (PLT, MPV, PCT% PDW%) and platelet functional parameters (ADP-IA%, SL-ADP, EPI-IA%, EPI-SLP, mOD/min, MDA, ADHES%).

I.3. Effects of dietary supplements on endothelial dysfunction

I.3.1. Introduction

Atherosclerosis is a systemic disease. The formation of fibro-fatty lesions in the arterial wall causes much morbidity and mortality worldwide, including most myocardial infarctions and many strokes, as well as disabling peripheral artery disease. Development of atherosclerotic lesions requires low-density lipoprotein, a particle that carries cholesterol through the blood. Other risk factors for atherosclerosis and its thrombotic complications include hypertension, cigarette smoking and diabetes mellitus. Increasing evidence also points to a role of the immune system, as emerging risk factors include inflammation and clonal hematopoiesis. Studies of the cell and molecular biology of atherogenesis have

provided considerable insight into the mechanisms that link all these risk factors to atheroma development and the clinical manifestations of this disease.

Endothelial dysfunction plays a key role in the pathogenesis of cardiovascular diseases. Therefore the identification and investigation of certain biomarkers of early endothelial dysfunction, in order to address prevention and therapy strategies, and thus the susceptibility of developing atherosclerosis, is one of the priority research areas nowadays. In this context we considered important to be familiar with less costly, and therefore easier to implement, methods of prevention and therapy of endothelial dysfunction. Currently there are no studies regarding dietary flax seeds supplements effects on platelets parameters. The literature's data supports the participation of the vascular wall in the atherogenesis process involving an inflammatory process, endothelial dysfunction and platelets hyper reactivity. Platelets receptors activation, platelet membrane fluidity alteration, and the membrane lipids composition change, contribute to platelets activation.

It has been shown that platelet activation is characterized by increased platelet adhesivity, increased platelet aggregation, particularly to ADP, but also to thrombin or collagen. If these changes are complemented by a decreased release of endothelial prostacyclin in the context of endothelial dysfunction, as noted in our ovariectomy group, an imbalance in TxA2-PGI2 in the platelet membrane occurs. The mechanisms of these changes are partially known

- Endothelial dysfunction is responsible for the decrease of the strongest vasodilator (PG I_2)
- Changes in platelet membrane composition, as a result of serum lipid profile changes, leads to the release of increased amounts of Tx A2,
- Oxidative stress with platelet activation by oxidized LDL.

By plateles activation ("platelet hyperfunction"), they become more adherents to endothelium, more sensitive to pro-aggregant agents, and also affect the interaction with other circulating proteins.

I.3.2. Scientific context

Currently there are no studies in the literature regarding integral flax seeds dietary supplements on platelet functional parameters in ovariectomy associated with hyperlipidic diet. However, studies have been conducted to compare the effects of supplementing the diet with ALA rich oils (C18:3, n-3) with LA rich oils (C18:2 n-6), on membrane composition and platelets function.

The results of this study showed that consumption of ALA rich oils may have important protective effects against cardiovascular disease, compared with LA rich oils, through the ability to reduce platelet aggregation (Allman, 1995). There is controversy regarding the effects of PUFA n3 (55-58% in flax seeds), on oxidative status in animals with n3 FA rich diet. Thus, Frenoux et al. (2001), found an increased antioxidant status in EPA (6 g/kg) and DHA (5 g/kg) rich diet. The same experiment observed that diets supplemented with PUFA n3 increased VLDL and LDL lipoprotein resistance to peroxidation and reduced platelet aggregation and plasma lipid concentrations.

The interest in flax seeds consumption results from its rich content in omega-3 polyunsaturated fatty acids, and also in fibers, proteins, and mucilage and phenol compounds. These components, by their pharmacological properties, can reduce the impact of some known cardiovascular risk factors including oxidative stress, dyslipidemia, hormonal deficiency in menopause and platelet activation and aggregation.

Estrogen atheroprotection is linked to the status of arterial endothelium and the production of nitric oxide (NO) by endothelial cells, stimulated by estrogen hormone type

estradiol (Strehlow, 2003), so it is considered that estrogen delays the development of endothelial dysfunction independently from plasmatic lipid modification (Lemay, 2002).

Due to beneficial effects on health, flax seeds are becoming more commonly incorporated into human diets (Parikh, 2019) and could give them the quality of nutritional supplement. Some of the components of flax seeds (n6 FA, lignans, and fibers) have pharmacological actions that may have influence on some risk factors for endothelial dysfunction, including dyslipidaemia, hyperglycaemia, oxidative stress, platelets hyperagregability. Flax seeds are known to contain 35- 40% fat, of which 55%, is represented by α-linolenic acid (omega-3 FA or n3 FA) and 15 - 18% linoleic acid (n6 FA) and their metabolites.

Another important component of flax seed are the lignans, one of the major groups of phytoestrogens; flax seed are the most important dietary source of secoisolariciresinol diglucoside (SDG) (Mandasescu, 2005), lignans and fibers. Integral flax seeds, through the lignans content represent an important source of phytoestrogens. Some studies have reported that phytoestrogens have protective effects on vascular endothelium, partially by improving lipid profile. Thus, dietary supplementation with 40 g of flax seeds in postmenopausal women was followed by reduced serum levels of LDL and serum TG, and also HDL-C, although without statistical significance (Libby, 2000).

Another motivation is that decreased alimentary intake of n3 FA from reduced fish consumption and industrial production of animal food rich in n6 FA; that explains the increased contents in n6 FA and poor in n3 FA. Grown vegetables also contain, less n3 FA than plants grown in the wild (Simopoulous, 2003). In the current diet, the ratio PUFA n-6/PUFA n-3 is 20-30/1, compared with 1-4/1 in the diet during the period in which human genetic code was established in relation with the diet. Flaxseed oil contains twice as many n3 FA, but contains no lignans or other fiber types found in integral flax seeds. Fish oils contains mostly arachidonic acid (AA), precursor of hormone-like substances (prostaglandins), known to negatively influence the symptoms of diseases associated with pain, inflammation and swelling. Other studies in rats have investigated the effects of flaxseed oil on serum lipids and oxidative stress showing that flaxseed oil does not influence the serum lipids or hypercholesterolemia atherosclerosis extension being also ineffective in influencing the level of oxidative stress (Keaney, 2002). Several studies have shown the effects of modulation of oxidative stress produced by n3 FA. They suppress the production of SRO and of IL-1, TNF and leukotrienes B4 (LT B4) by neutrophils and monocytes (AlRamadneh, 2022). Some studies have shown that SDG, predominant lignan from flax seeds and its metabolites, seicosolariciresinol, enterodiol, and enterolactone have antioxidant activity as well (Peter, 1997, Prasad, 1998). Flax seeds content in n3 FA and lignans explains their benefic effects and the possibility of being used for preventive and/or therapeutic purpose. Advantages of using flax seed are due to effects on multiple systems:

- Cardiovascular: reduced endothelial dysfunction by lipid-lowering effects (reduction of LDL-cholesterol) and antioxidant, lowering the blood pressure and the risk of stroke (Libby, 2000);
- Anti-inflammatory: n3 FA are metabolized to substances that can exert anti-inflammatory effects (Zhanga, 2019)
- Menopause: lignans from flax seeds are phytoestrogens, structurally similar to estrogens, moderately reduce the menopausal symptoms, but also have protective effects on endothelium reducing the increased cardiovascular risk in postmenopausal women (Yang Hu, 2021)
- Glycemic metabolism: fibers from the flax seeds increase the tolerance to carbohydrate intake, thereby helping to regulate glycaemia having a lipid-lowering effect as well (Villarreal Renteria, 2022).

The main objective of this study was to identify non-invasive diagnostic methods of endothelial dysfunction and evaluation factors, considered markers of endothelial dysfunction, together with endothelial activation, elements confirming the pre-lesional stage of atherogenesis process.

We studied as well the effects of ovarian hormones deficiency in surgical menopause (experimental ovariectomy), combined with a hyperlipidic diet (polyunsaturated fatty acids) as risk factors in the development and progression of endothelial dysfunction. The lack of endogenous estrogen distorts the lipid metabolism and reduces the antioxidant capacity and excess omega-6 polyunsaturated fatty acids causing hyperlipidemia and hypercholesterolemia with high pro-atherogenic potential.

Another objective of this study was to optimize strategies for prevention and improvement of endothelial dysfunction by supplementing the diet with a source rich in omega-3 fatty acids and lignans, contained in the flax seeds, known for their antioxidant effects. The experiment was conducted on Wistar female white rats. Flax seeds supplemented diet was given to both ovariectomy and control lots.

The ideal of this study was to try the transfer of knowledge and results of basic research to applied medicine, given the increasing interest in the ways to reduce morbidity and mortality from cardiovascular disease by dietary supplements. The motivation of the present study is to investigate less known aspects, but responding to national and international priorities in health and basic sciences, to identify circulating soluble markers of endothelial dysfunction, to develop minimally invasive methods of investigation, and to study the preventive use of vegetal products, rich in omega-3 polyunsaturated fatty acids, such as flax seeds (*Linum usitatissimum*), vitamin E (alpha-tocopherol) in prevention of atheroma plaque development.

The motivation of the experimental model choice to produce endothelial dysfunction - ovariectomy and/or hyperlipidic diet is based on the tradition and experience of Pathophysiology Department from UMF "Gr T. Popa" Iasi in basic research regarding the pathogenesis of angiopathy and the role of exogenous atherogenic risk factors (Ciobanu-Jurcuț, 2004). Moreover, diabetes and diabetic angiopathy has been the subject of many studies carried out in our discipline over the years.

In this sense, multiple studies were carried out in the pathophysiology department, under the coordination of Prof. Dr. Veronica Mocanu, studies that focused on the effects of various actions on the development of the athermanous lesion as well as on the parameters closely connected with it. I am referring here to lipid metabolism parameters (serum studies but also from tissue homogenate) as well as microscopic examination of large vessels and the determination of lipid content in vessels (per mg of tissue), oxidative stress parameters (serum and tissue) as well as studies on function platelet aggregation (platelet aggregation and adhesiveness) the last ones I performed directly. The levels of soluble serum molecules involved in platelet activation and aggregation (sVCAM-1 and vWF) were also studied.

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I.3.3. Material and methods

We used 42 female Wistar rats, weighing 180 ± 20 g, purchased from the animal farm of the University of Medicine and Pharmacy "Gr T. Popa" Iasi. There were kept in a room with controlled temperature (21 ± 2 ºC) in standard laboratory conditions with a normal light cycle of 12 hours. Rats were divided into cages with stainless steel mesh, provided with continuous manure cleaning with water; cages had an appropriate size, allowing keeping one animal in a cage, according to the guidelines. They were also provided with appropriate

ventilation and water *ad libitum*. All experimental procedures were in strict accordance with international ethical regulations and were approved by the Medical Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iasi, Romania. The experiment respected as well the instructions of the Guidelines on the Care and Use of Animals for Scientific Purposes, National Advisory committal for Laboratory Animal Research, 2004.

Diets

The animals were divided into two study groups: normal control animals (M) and ovariectomised animals (Ø) – to induce experimental menopause. Each group was further divided into three lots – each one consisting in 7 animals according to the three types of diet administered as follows:

1. Normal control animals (M):
    a. M - control group, consisting of 7 female Wistar rats, with standard diet;
    b. M + n6 - control group, consisting of 7 female Wistar rats, with standard diet + hyperlipidic diet 40 g / 100 g food) (PUFA n6);
    c. M + n6 + n3 - control group, consisting of 7 female Wistar rats with standard diet + hyperlipidic diet supplemented with flax seeds (flax seeds 15 g / 100 g food) (PUFA n3 and lignans);
2. Ovariectomised animals (Ø):
    a. Ø - ovariectomised group, consisting of 7 female Wistar rats, with standard diet;
    b. Ø + n6 - ovariectomizat group consisting of 7 female rats, Wistar race, which received standard diet supplemented with hyperlipidic diet (40 g/kg diet) (PUFA n6);
    c. Ø + n6 + n3 – ovariectomised group, consisting of 7 female Wistar rats with standard diet + hyperlipidic diet supplemented with integral flax seeds (flax seeds 15 g/100 g food) (PUFA n3 and lignans);

Tehnics.

The three types of diet were administered for 36 weeks (9 months) and then the animals were sacrificed after anesthesia with at a dose of 1 ml/100g, 0.01% thiopental solution. After the vital signs disappearance (breathing, heart contractions), the collection of organs samples for biochemical determinations and pathological examination was done:

- Liver samples for liver homogenate from which we determined:
    - Lipid metabolism evaluation parameters: cholesterol, triglycerides
    - Oxidative stress evaluation parameters: malondilaldehide (MDA), adapted from (Dobrian, 2001) and reduced gutathion (GSH) after Tietze (1969), compared with hepatic proteins levels (after Bradford, 1976).
- Heart and aorta samples for pathological exam:
    - Myocardium samples were fixed in formalin 10%, included in paraffin, cut to 4.5 microns with the microtome, and eventually colored standard, with hematoxylin and eosin (HE), periodic acid Schiff (PAS) and Van Gieson (VG),
    - Aorta was sampled as well and paraffin sections were performed; for intimal atherosclerotic changes assessments we used standard coloration HE, oil-red and Sudan III.

The following biochemical parameters were also determinate in serum (and tissues) for evaluate and compare the levels of oxidative stress and the lipid profile (malondialdehyde, MDA), reduced glutathione (GSH), total cholesterol, HDL cholesterol, non HDL cholesterol, triglycerides atherogenic index (AI).

Platelet activation and pro-thrombotic status were investigated by determination of seric levels of circulating soluble plasmatic molecules - vWF - VCAM-1 (strongly correlated with pro-thrombotic state and endothelial dysfunction also). Platelet function alone were

determinate by evaluation platelet aggregation and platelet adhesion by laboratory methods adapted after Bednar's method (1995), changed by Chadderdon and Cappello (1999), and was expressed in absolute value (mOD/min), which represent the mean decreasing of the optical density as a consequence of platelet aggregation induced by ADP and platelet adhesion to fibrinogen was performed also with the micro plate reader TECAN, according to Bellavite's method (1994).

Statistical methods. Data were loaded and processed using statistical functions of EPIINFO 6.0, SPSS 13.0 and Excel. t-Student test (significance threshold of 95% is generally accepted that p = 0.05) and Pearsons correlation were performed.

I.3.4. Results and discussions

I.3.4. a. Hyperlipidic PUFA n6 diet in estrogenic deficiency (ovariectomy)

Literature data on antioxidant defense systems are often contradictory; Kamalakkannan, (2006) reported significantly reduced levels of non-enzymatic antioxidants GSH, vitamins C and E in liver and kidney in diabetes while Kim HK (2006) have reported increased enzymatic antioxidant activity, hepatic GSH-reductase and SOD suggesting a possible compensatory reaction, in response to increased oxidative stress. The results of this study are consistent with the literature's data on oxidative stress markers in endothelial dysfunction. In our study, oxidative stress is due, on one side, to excess polyunsaturated fatty acids (PUFA), especially PUFA n6, which enhances the appearance of reactive oxygen species (ROS) and on the other side to reduced antioxidant defense in the absence of endogenous estrogen, induced by experimental menopause. After administration of 40% PUFA n6 diet, mean serum MDA values are higher both in control (M+n6) (4,94 *vs* 4,34 nmol/ml) and in ovariectomy group (Ø+n6) (5,48 *vs.* 5,08 nmol/ml) compared with the control group with standard diet, without statistical significance ($p > 0,05$);

In both the ovariectomy - hyperlipidic diet (Ø+n6) (5,48 *vs.* 4,94 nmol/ml serum), and the ovariectomy group with standard diet (Ø) (5,08 vs. 4,34 nmol/ml serum) have higher serum MDA values than controls, without statistical significance ($p > 0,05$).

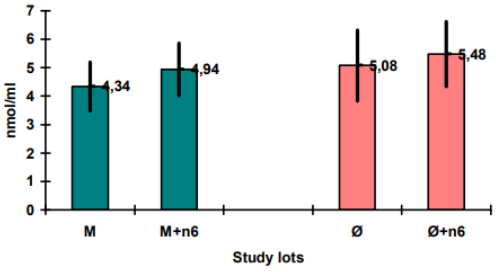


Figure I.3.1
MDA changes (serum) in hyperlipidic diet (n6 FA) ovariectomised female rats (Ø), mean values

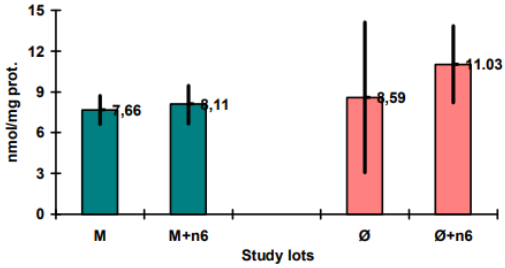


Figure I.3.2
MDA changes in hepatic homogenate in hyperlipidic diet (n6 FA) in ovariectomised female rats (Ø),mean values

Variations of MDA mean values in liver homogenate shows that in hyperlipidic diet (PUFA n6), mean values of hepatic MDA rise both in control (M + n6) (8.11 *vs.* 7.66 nmol/ml prot), and especially in ovariectomy group (Ø+n6) (11.03 *vs.* 8.59 nmol/ml prot), but the differences are not statistically significant ($p>0.05$).

The highest values of MDA in liver homogenate (11.03 nmol/mg prot) were observed when ovariectomy was associated with a hyperlipidic diet (Ø+n6) showing that oxidative stress is increased in case of cumulative risk factor. Increased hepatic and serum MDA in ovariectomised animals receiving PUFA n6-rich diet confirms that oxidative stress and lipid peroxidation was increased compared with standard diet animals.

These results support the hypothesis that lipid peroxidation, one of the most harmful effects of oxidative stress, increase substantially in the liver, being reflected in blood levels as well. In most reported studies, lipid peroxidation products in liver were increased, while in others were unchanged (Banerjee, 1993). We noticed a slight reduction of hepatic GSH levels in control group with hyperlipidic diet (M + n6) and especially in ovariectomy group (Ø). Our results confirmed the decrease in antioxidant defense by a reduced value of GSH, which contributes to increased oxidative stress, also confirmed by increased MDA levels (lipid peroxidation marker), in agreement with other studies findings.

The liver is the main organ involved in the synthesis of GSH who has a role in a variety of xenobiotic detoxification in the liver which unlike in erythrocytes have various metabolic functions; almost all major metabolic processes involve thiol redox system. GSH plays a key role in cellular resistance to oxidative damage, as a free radicals scavenger through ascorbate and tocopherol regeneration in the liver and erythrocytes. Seven, 2004 pointed out that antioxidant network representatives have to act synergistically to destroy activated oxygen species (ROS).

Endothelial dysfunction markers

Triglyceride-rich lipoproteins (VLDL) also increase oxidative stress leading to their oxidation and attracting macrophages. These two events are central to the accumulation of lipids in the prelesional (endothelial dysfunction) and in the lesion stage of atherogenesis (Figure I.3.3).

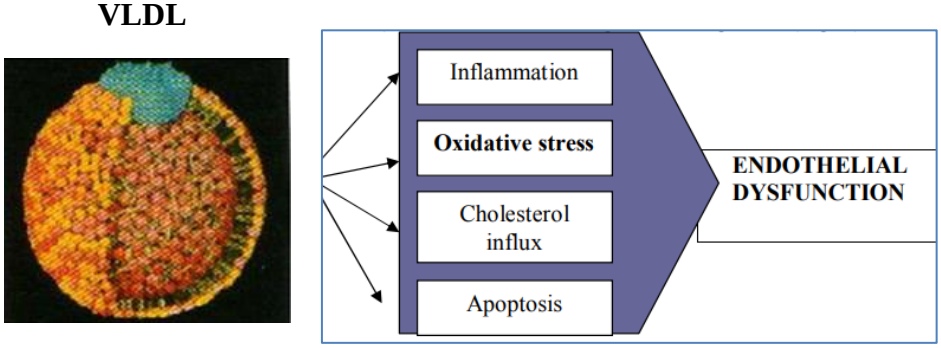


Figure I.3.3
Multiple effects mediated by VLDL, inducing and accelerating endothelial dysfunction (adapted from Zouzenkova, 2005)

It has been firmly established in the recent years that inflammation plays a key role not only in the initiation and progression of atherosclerosis pre-lesional changes but also in the lesion stage, of erosion, fissure and possibly in the atheroma plaque rupture (Renaud, 2001, Akiba, 2000). The observations that atherosclerosis has an important inflammatory component as evidenced by histological studies are derived primarily from experimental studies on animals. Thus, the animals receiving an atherogenic diet, showed an increased monocytes adhesion to endothelial cells, especially in vascular regions prone to atheroma development (Lee P, 2003).

Important data resulted from prospective clinical studies which identified some systemic markers of inflammation - predictors for future cardiovascular events, not only in healthy subjects but especially in patients with stable/unstable coronary heart disease. This represents indirect evidence regarding the level of the inflammatory process in the area affected by atherosclerosis. Thus, reduction of inflammation may have beneficial effects, delaying the development of endothelial dysfunction but also preventing accelerated atherogenesis under multiple risk factors. In the future, new investigations are needed to confirm this possibility.

The places where the endothelium produces adhesion molecules responsible for adhesion, migration and accumulation of monocytes and lymphocytes are specific arterial areas, branches, bifurcations and bends. Circulating leukocytes adhesion to endothelium occurs from the early stages of endothelial dysfunction. Under normal conditions, leukocytes do not adhere to the intact endothelium, but with the endothelial dysfunction their adhesivity increases. That is why adhesion molecules play a central role in this process.

Since the endothelial-origin adhesion molecules (sVCAM-1, ICAM-1, E-and P-selectines) are likely to be released into the blood, clinical studies have suggested that investigating circulating levels of these molecules can be used as a biomarker of cardiovascular disease and may have a predictive value (Mulvihil, 2002).

Antigens that promote the inflammatory response are incompletely known; out of these are discussed in the literature: oxidized LDL, infection with *Cytomegalovirus, C pneumoniae, H. pylori* (Ross, 1999). These data are only part of the histopathological and biochemical evidence that inflammation is the major pathogenic component of endothelial dysfunction and atherogenesis process.

Dyslipidemia, hyperlipidemia, estrogen deficiency, overweight, excess free fatty acids, and oxidized LDL increase, change the function of many cells, including endothelial cells, smooth muscle cells and platelets. Consequently, the endothelial synthesis of NO· is low, while increased superoxide anion level is acting directly, destroying NO·. In addition, it increases endothelial synthesis of endothelin-1 and angiotensin II (Jessup, 1997).

Increased endothelial production of vWF and of the strong procoagulant tissue factor, together with increased plasma levels of factor VII and declining endogenous anticoagulants, ant thrombin III and protein C, create a procoagulant status. Furthermor increased endothelial production of plasminogen activator inhibitor (PAI-1) decreases fibrinolysis.

sVCAM-1 and ICAM-1 are adhesion proteins produced by activated endothelial cells and are found on their surface, are needed for leukocytes adhesion to endothelium, by coupling with leukocyte-synthesized integrins. Soluble form of sVCAM-1 antigen was found increased in patients with atherogenic risk factors, even in the initial stages of endothelial dysfunction.

The results of our experimental study showed high levels of sVCAM-1 in hyperlipidic diet, which confirms the presence of a degree of endothelial dysfunction both in control animals and in those ovariectomised. With cumulative risk factors, in the ovariectomised group with decreased antioxidant defense and increased intake of PUFA n6 (Ø + n6), results in higher values of sVCAM1 compared with ovariectomised animals with standard diet (Ø) ($p < 0.05$). These data fully motivate the importance of inflammation study through specific biomarkers of endothelial origin (VCAM-1 and ICAM-1) when endothelial dysfunction is suspected.

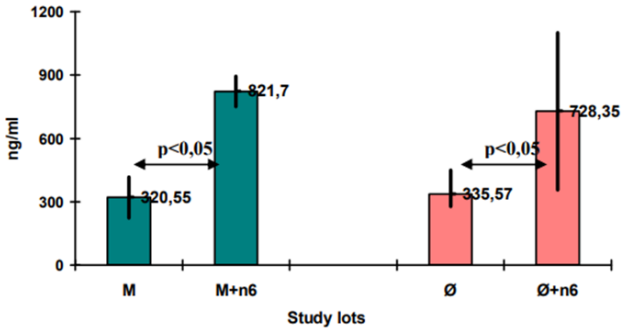


Figure I.3.4
sVCAM-1 changes in hyperlipidic diet (n6 FA) in ovariectomised female rats (Ø), mean values

vWF, a circulating glycoprotein (0,45-12 million daltons) is synthesized primarily by endothelial cells and released by exocytosis from the Weibel-Palade body; vWF is produced by megakaryocytes as well and by sub-endothelial connective tissue. The largest molecules are more thrombogenic due to increased platelet adhesivity and aggregation. The plasmatic circulating form derives mainly from the endothelial cells. This form has an important role in platelet adhesion to the sub-endothelial tissue in case of endothelial detachment due to the presence of glycoprotein at the platelet membrane level. Clinical studies have shown that the presence of major risk factors of endothelial dysfunction (hypercholesterolemia, diabetes mellitus, hypertension and smoking) is characterized by increased circulating vWF level. These data are suggesting the important role of vWF as endothelial dysfunction biomarker, but also the role played by vWF in mediating the thrombotic complications of atherosclerosis.

In our study, the largest increase, statistically significant, of vWF (160.29% vs. 101.59%) was observed when ovariectomy was associated with hyperlipidic diet ($p < 0,05$) – see Figure I.3.5. The results confirmed the presence of endothelial dysfunction in ovariectomised animals due to endogenous estrogen deficiency, combined with a second risk factor, hyperlipemia resulting from the diet.

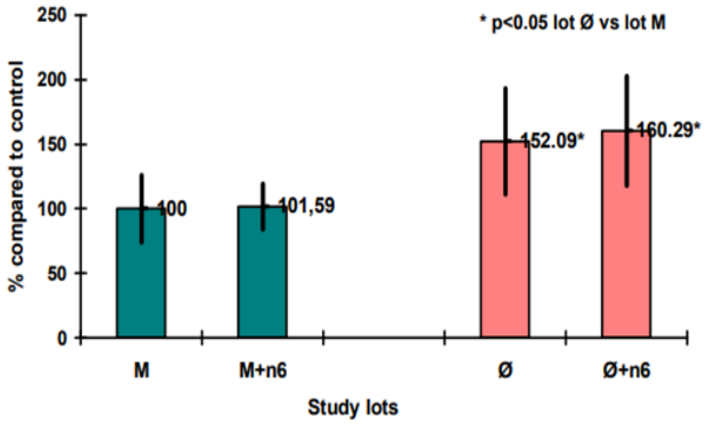


Figure I.3.5
vWF changes in hyperlipidic diet (n6 FA) in ovariectomised female rats (Ø) (mean values)

Effects on lipid metabolism

Diet is one of the most important regulatory factors of lipid metabolism. Pathological diet may be the result of excess calories or food quality imbalance (hyperlipidic, hyperglucidic) or both, in different ways. The effect depends on genetic predisposition and preexisting metabolic disorders.

In our study, by assessing the mean values of serum and liver lipids in ovariectomised rats with standard diet compared with healthy controls with the same diet, we found that ovariectomy produced a statistically significant increase in serum cholesterol ($p < 0,002$) and high liver triglycerides ($p < 0,05$), changes that have been exacerbated by hyperlipidic diet (PUFA n6) – see Figure I.3.6.

Our results confirmed impaired lipid metabolism, characterized by high triglycerides, consistent with the literature data, characteristic to physiological or surgical menopause. In our study we observed a decrease in serum HDL-C after administration of hyperlipidic diet (n6 FA) in healthy animals (49.96 ± 11.47 mg/dl) compared with those who received standard diet ($58, 23 \pm 11.47$ mg/dl), suggesting a decreased transport capacity of cholesterol from vascular tissue to the liver according to other observations in literature and also a

decreased inhibition capacity of LDL oxidation. It is known that most of serum triglycerides are transported especially by VLDL fractions, which explains non HDL-C changes.

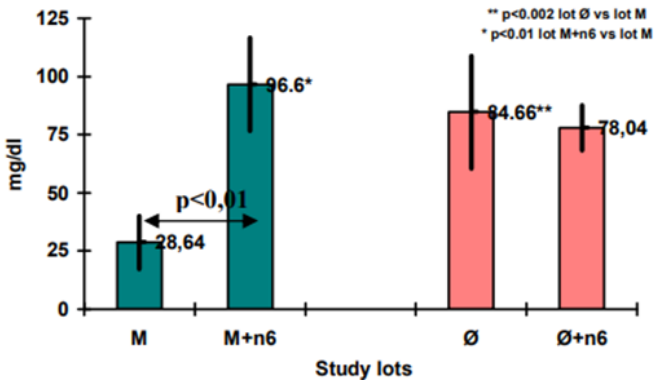


Figure I.3.6
Serum tryglicerides changes in hyperlipidic diet (n6 FA) in ovariectomised female rats (Ø), mean value

Suppression of endogenous estrogen by ovariectomy influenced liver lipid metabolism; when hyperlipidic diet was added the dyslipidemia together with increased oxidative stress and reduced antioxidant defense may contribute to the appearance and maintenance of endothelial dysfunction and to the functional impairment of hepatocytes.

Atherogenic index (AI), marker of endothelial dysfunction, showed statistically significant increase in ovariectomy group (Ø), compared with controls (M), under standard diet ($p < 0, 05$).

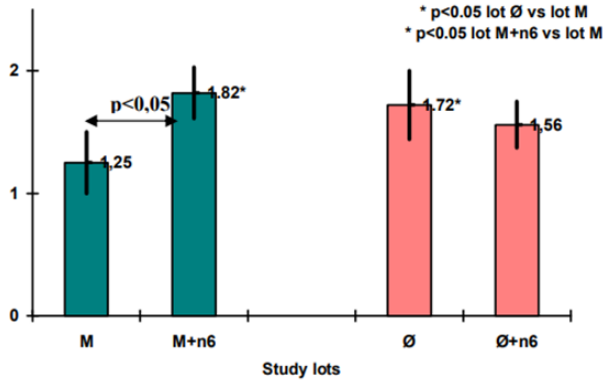


Figure I.3.7
AI changes in hyperlipidic diet (n6 FA) in ovariectomised female rats (Ø) (mean values)

After the administration of hyperlipidic regime we have not noticed significant changes in AI between these groups, suggesting that atherogenic effect of endogenous estrogen deficiency is stronger than the atherogenic effect of hyperlipidic diet - polyunsaturated fatty acids n6 in our experiment.

Our results regarding the association between cardiovascular risk factors, cell adhesion molecules (sVCAM-1), vWF, lipid metabolism parameters, and other parameters (CRP, insulinemia) are consistent with other studies in the literature (Socki, 2004, Ikeda, 1998).

Table I.3.1
Associations between increased levels of sVCAM-1 and cardiovascular risk factors or risk inducers adapted from Jager, 2000

Risk factors (independent variables)	Mean ± SD	RR (IC95%) for cardiovascular mortality	p for VCAM-1 (dependent variable)
vWF (IU/ml)	1,37 ± 0,7	1,95 (1,04-3,64)	**p<0,0005**
Chol. tot (mmol/l)	6,60 ± 1,2	1,23 (0,97-1,56)	**p<0,0005**
LDL-C (mmol/l)	4,50 ± 1,1	1,27 (0,97-1,65)	**p<0,0005**
HDL-C (mmol/l)	1,30 ± 0,4	2,74 (1,31-5,71)	**p = 0,03**
CRP (mg/l)	1,75	2,02 (1,08-3,80)	**p = 0,04**
Insulinemia (mg/dl)	84 ± 21	1,35 (0,73-2,48)	**p = 0,009**

Ultra-sensitive testing of C-reactive protein C could be an important indicator of the risk for endothelial dysfunction and early complications of atherosclerosis. Understanding the mechanisms by which triglyceride-rich lipoproteins (VLDL) activate certain transcription factors (PPARa) opens new perspectives in prevention and treatment of dyslipidemia and endothelial inflammation, major risk factors for endothelial dysfunction and thus for atherogenesis process. Molecular mechanisms responsible for the action of triglyceride-rich lipoproteins (VLDL) involve activation of transcription factors (PPARa) which can stimulate or inhibit inflammation. Anti-inflammatory actions of PPARa counteract the proinflammatory effects of transcriptions factors NFkB and AP-1. VLDL composition complexity and the various absorption ways explain many of their effects (Figure I.3.8).

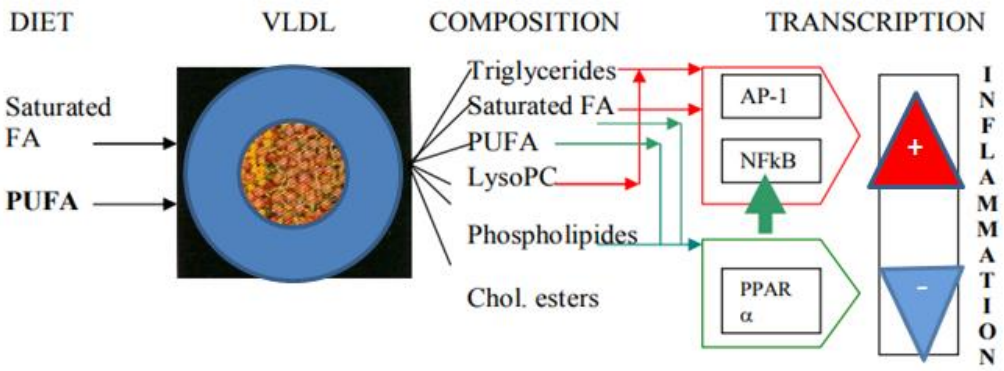


Figure I.3.8
Hyperlipidic diet effect on lipoproteins composition, adapted after Zouzenkova, 2005

Hyperlipidic diet modulates the various VLDL components ratio. Triglycerides, saturated fatty acids and lysophosphatide choline (LysoPC) are strong inducers of pro-inflammatory factors NFkB and AP-1 transcription. PUFA, certain phospholipids or saturated fatty acids released from VLDL under lipoprotein lipase (LPL) activate PPARa and inflammatory response. The way of VLDL delivery to the cells regulates transcription factors by inducing inflammation. Besides VLDL composition, their capture path can change transcription responses. LPL-mediate VLDL lipolysis or LDL fractions electronegativity activate PPARa. Capturing electronegative VLDL or LDL via receptors active pro-inflammatory transcription factors NFkB and AP-1. Other lipases, such as phospholipase A2 (PLA2) can activate in vitro transcription factors both pro-and anti-inflammatory. The significance of this transcriptional activation of inflammation in vivo is not clarified yet.

Platelet function parameters

Increased platelet adhesivity and aggregation are recognized as important factors that increase the risk of endothelial dysfunction and vascular thrombotic events. Hyperlipidemia, hyperglycemia, hyper-insulinemia and insulin-resistance may have a major role in triggering and development of atherosclerotic disease by promoting endothelial dysfunction, with damage to the arterial wall, changes involving oxidative stress and other factors. Endothelial activation, the result of various atherogenic risk factors aggression, the key response to aggression, is represented by endothelial dysfunction. Activated endothelial cells are more thrombogenic than normal endothelial cells due to low synthesis of PGI2, NO·, tPA, but also due to increased synthesis of plasminogen activator inhibitor and tissue factor. Therefore, determining the platelet adhesion and aggregation are essential for the evaluation of endothelial functions damage.

Platelets adhesion to the extracellular matrix is a complex phenomenon involving platelet membrane glycoproteins, plasmatic proteins and sub-endothelial tissue components. Constitutive, platelets have receptors for collagen, fibrinogen, fibronectine, vWF, laminine, thrombospondine, vitronectine etc. Physiological platelet agonists such as ADP, collagen or thrombin may increase the number and binding affinity of platelet glycoprotein receptors, receptors involved not only in adhesion but also in the transduction of the biological message due to platelet aggregation and clot formation. Two actors are involved in initiation of clot formation: platelet and vascular endothelium. Metabolic disturbances and leukocytes have a role in endothelial dysfunction and angiopathy development. Dyslipidemia, hyperglycemia and insulin resistance, separately or together, as happens in diabetes, induce a vicious circle of events in the vascular wall, which involves initiation and maintenance of the inflammatory process in the endothelium (endothelitis) and platelet hyperactivity.

During platelet activation, as a result of the action of phospholipase on platelet membrane result AA split and metabolized by cyclooxygenase to form platelet end peroxides - prostaglandin G_2 (PGG_2) and H_2 (PGH_2), which are transformed in TxA2, the most powerful vasoconstrictor and platelet aggregant. TxA2 production, determined by urinary levels of TxB2, the major metabolite of TXA_2, was positively correlated with increased platelet activation. The present study investigated platelet functions expressed by platelet adhesion to fibrinogen coating plates and platelet aggregation to ADP in female rats in surgical menopause by ovariectomy, who received a hyperlipidic diet rich in PUFA n6.

In our experimental study, the highest values of platelet adhesivity index were observed when ovariectomy was associated with hyperlipidic diet (Ø + n6), showing that platelets adhesion increases in multiple risk factors when compared to control animals. We noted statistically significant differences in the platelet aggregation rate between the control group with hyperlipidic diet (M + n6: 20.67 mOD/min) compared to the control group with standard diet (M: 8.20 mOD/min), ($p < 0,001$), see figure I.3.9.

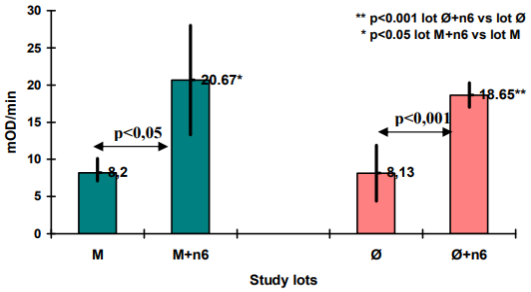


Figure I.3.9
Platelets aggregation rate changes in hyperlipidic diet (FA n6) in ovariectomised female rats (Ø), mean values

The results of our experimental study showed that platelet adhesivity and aggregation functions are modified in ovariectomised animals compared with controls, emphasizing the effects of estrogen deficiency, especially when it is associated with a diet rich in PUFA n6.

Morphological features

This study tried to identify early endothelial dysfunction changes and aimed also to detect possible relations between endogenous estrogen deficiency and excess PUFA n6 in inducing endothelial dysfunction by endothelium activation. Dyslipidemia and hypercholesterolemia are responsible for the generation of ROS and the development of oxidative stress, thus inducing LDL oxidation through excess free radicals (Yamada, 1998, Suttnar, 2006). Endothelial cells are the first directly affected by the impact of circulating oxidized lipids (oxidized LDL) with the vascular wall. Anatomo-pathological examination of aorta fragments revealed the following intimal morphological changes: in control healthy animals (Wistar rats), HE staining of aorta fragments showed a normal aspect, with small and rare endothelial disruptions.

Special attention was paid to endothelial changes that appeared under estrogen deficiency and increased intake of polyunsaturated essential fatty acids, known to have an increased susceptibility to lipid peroxidation. Early microscopic alterations of endothelial dysfunction were noted by examination of the vascular wall at the aortic arch and coronary branches level in female Wistar rats, ovariectomised, on hyperlipidic diet rich in PUFA n6. During the experiment we found endothelitis aspects, showing endothelial discontinuities, platelet adhesion, leukocytes margination, rare macrophages. Aspects of early endothelial dysfunction were constantly noted in animals exposed to two risk factors (ovariectomy + PUFA n6), especially in the aortic arch area.

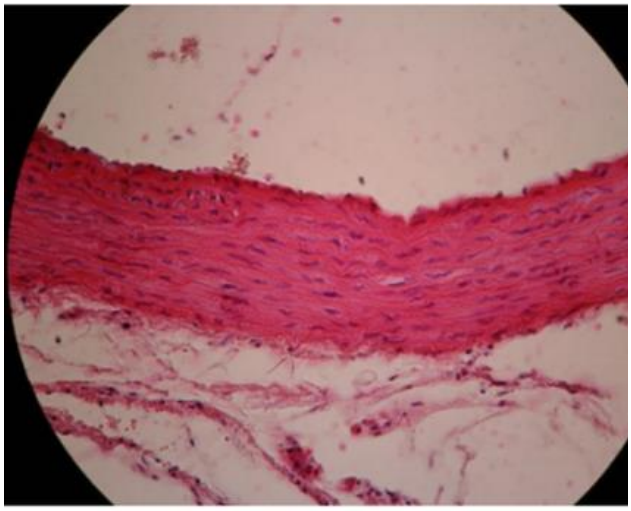


Figure I.3.10
Aorta – platelet adhesion to endothelium. Mucoid degeneration of smooth muscle fibers (HE x 40) Ø+n6 lot

These changes can be explained by the oxidative theory of atherogenesis, which claims that the initiation and advancement atherogenesis process originates in the endothelial cell. Therefore any aggression on the endothelium, especially those affecting the endothelial antioxidant defense, is of paramount importance; it cannot be ignored that endothelial cells are continuously exposed to the circulating regulating substances. The increased endothelial adhesion of leukocytes (monocytes), as shown by other studies (Eppihimer, 1998, Kevil, 2001) is characteristic of endothelial dysfunction in the prelesional stage of atherosclerosis.

Some monocytes that have entered the sub-endothelial space through the discontinuous endothelium, rare in incidence, become macrophages, but were not transformed into foamy cells, probably because there was not enough oxidized LDL available for phagocytosis. Oxidized low density lipoproteins (oxLDL) affect the endothelium through the production of superoxide anions, free radical that an estrogen seems to lower.

Recent data shows that estrogen atheroprotection is linked to arterial endothelial status and to NO synthesis, which is stimulated by estrogens (Strehlow, 2003). It is considered that these factors are involved in the paths by which the estrogens are delaying the atherogenesis, independently of plasmatic lipid modification (Hodgin, 2002). Endothelial activation and dysfunction sometimes involve an oxidative modulation of gene expression (Kaliora, 2006, Scioli, 2020). ROS excess are the major cause of injury to endothelial cells and sub endothelial extracellular matrix expressed through endothelitis, circulating blood cells adhesion to the endothelium, and increased synthesis/depression of glycosaminoglycan (GAG), (Gimbrone, 2000).

With ovariectomy, endogenous estrogen deficiency has favored lipoprotein metabolic disorders and such the oxidative stress, which has reduced the antioxidant capacity. All these changes have repercussions on the endothelium integrity and intima, as confirmed in other studies (Lucas, 2004, Jayachandran, 2003). Endothelial dysfunction syndrome present in our Ø+n6 study group, confirmed by biochemical and pathological investigation, has predicted typical pre-lesional atherosclerosis changes. Prudent restriction of atherogenic food is a preventive and also curative strategy, especially for women in physiological or surgical menopause.

I.3.4.b. Effects of dietary supplementation with integral flax seeds (PUFA n3+lignans) in experimental ovariectomy and hyperlipidic diet

The present study sought role of n3 fatty acids and lignans from flax seeds in modulating the oxidative stress, on markers of endothelial dysfunction, on platelet function and on lipid metabolism in endocrine imbalance conditions - estrogen deficiency associated with hyperlipidic diet. We haven't found studies in the literature that have analyzed the effects of flax seeds on the factors and mechanisms involved in endothelial dysfunction pathogen in this context. Some studies have analyzed the effects and role of integral flax seeds or their components, n3 FA, lignans and fibers on the risk factors of endothelial dysfunction and pro-thrombotic state.

Effect of diet supplementing with flax seeds on oxidative stress markers

There are many studies that have shown that the pathogenesis and progression of endothelial dysfunction involves increased oxidative stress. In our study, mean MDA values were determined in serum and liver homogenate; both in intact and ovariectomised animals with a diet rich in n6 polyunsaturated fatty acids (PUFA n6), lipid peroxidation quantified by MDA serum levels, was elevated compared with the standard diet. We found that the in the case of hyperlipidic diet (n6 FA) in animals with estrogen deficiency (ovariectomy), the beneficial antioxidant effects of PUFA n3 and lignans are reduced; the decrease in serum MDA was insignificant after dietary supplementation with flax seeds.

- control group - dietary supplementation with PUFA n3 and lignans decreased serum MDA levels (4.50 *vs.* 4.94 nmol/ml serum) compared with the hyperlipidic diet lot, but the differences are not significant ($p > 0.05$);
- ovariectomy group - dietary supplementation with PUFA n3 and lignans decreased serum MDA levels (4.96 *vs.* 5.48 nmol/ml serum) compared with the ovariectomy lot with hyperlipidic diet, without statistically significant differences ($p > 0,05$).

The fact that dietary supplementation with PUFA n3 and lignans was followed by slight decrease of serum MDA, in both lots, supports the hypothesis that hyperlipidic diet, especially in endogenous estrogen deficiency, increases lipid peroxidation, one of the most harmful effects of oxidative stress.

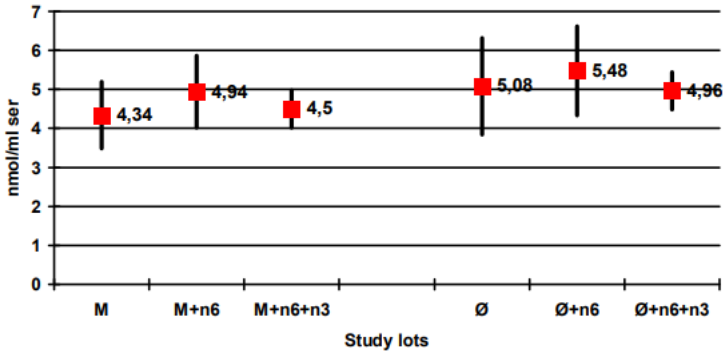


Figure I.3.11
Serum MDA changes in flax seed supplemented diet in ovariectomised female rats
(mean values and statistical significance)

MDA values in liver homogenate are also increased, with the same characteristics as those of serum MDA. Supplementing the diet with PUFA n3 and lignans, in case of hyperlipidic PUFA n6 diet, insignificantly decreased MDA values both in control and estrogen deficiency groups, compared to hyperlipidic diet group. The results confirmed that the increase in liver lipid peroxidation was reflected in serum as well.

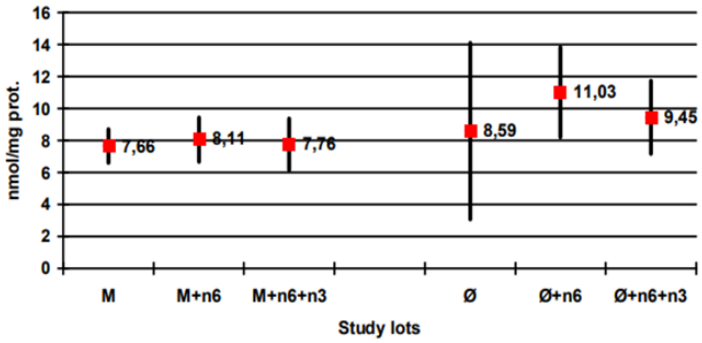


Figure I.3.12
Hepatic homogenate MDA changes in flax seed supplemented diet
in ovariectomised female rats (mean values and statistical significance)

Insignificant decrease of serum and liver homogenate MDA levels after dietary supplementation with PUFA n3 and lignans suggests that when there are two pro-oxidant risk factors, antioxidant effects of flax seed are reduced.

Hyperlipidic diet decreased the GSH levels both in healthy and ovariectomised animals. In hyperlipidic diet (PUFA n6) in ovariectomised animals, flax seeds determined:

- in the control group · insignificant increase in GSH in liver homogenate (9,10 µM/mg to 6,82 µM/mg;
- in the ovariectomy group · significant increase ($p < 0.05$).

Dietary supplementation with PUFA n3 and lignans in our study resulted in the restoration of hepatic GSH levels in both lots which have previously received a hyperlipidic diet. Although the differences between groups are not always significant, our results suggest

the beneficial antioxidant potential of flax seeds supplementation contain the largest amount of lignans.

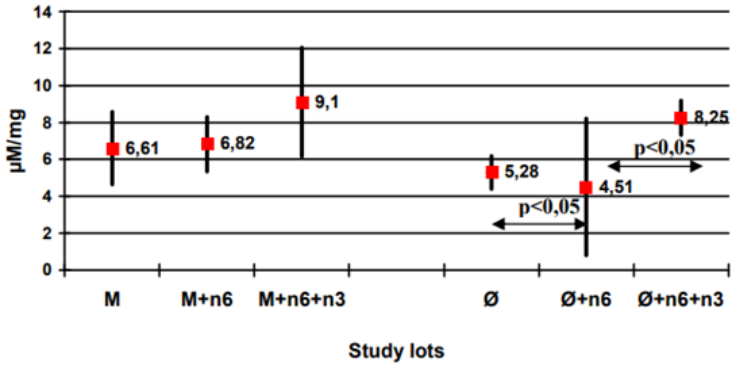


Figure I.3.13
Hepatic homogenate GSH changes in flax seed supplemented diet
in ovariectomised female rats (mean values and statistical significance)

Studies investigating the effects of n3 FA dietary supplements in normal or pathological conditions have reported not only mixed results but sometime contradictory. Thus, some human studies suggest a beneficial effect of diets rich in EPA in the prevention and therapy of atherosclerosis, as well as inflammatory diseases, autoimmune and tumors. The mechanisms are not fully elucidated, but several experimental studies suggest the involvement of a free radicals dependent mechanism.

Our results are in contradiction with some literature data showing that dietary supplementation with flax seeds healthy humans or animals is associated with increased oxidative stress. Jenkins (1999) found that patients with dyslipidemia after three months of dietary supplements with defatted flax seeds flour (50 g/day) had a decrease of proteic thiol groups, suggesting increased oxidative stress. Wiesenfeld (2003) found that food supplementation in pregnant female rats with 40% flax seeds produced in their offspring an increased oxidative stress, evidenced by the reduction of vitamin E in the liver. Cardiovascular risk factors can be significantly reduced with PUFA n3 from the plants/ seeds, while PUFA n3 from marine plants, has a lower impact (Arja, 2005). Trevor (2003) demonstrated that in patients with diabetes and hypertension, both EPA and DHA reduced the oxidative stress parameters in vivo without altering inflammation markers, other studies on animals fed an n3 FA rich diet, showed an increased lipid peroxidation in serum, liver and adipose tissue (Murphy, 1999).

Some studies have noted the ineffectiveness of flaxseed oil in the prevention of MDA increase in dietary hypercholesterolemia in rabbits, which confirms that n3 FA rich flaxseed oil has contradictory effects on the reduction of oxidative stress (Lee and Prasad, 2003). The effects of SDG on lipids, oxidative stress parameters and the development of atherosclerotic lesions in the aorta in rabbits fed a high cholesterol diet have been studied as well. These results suggest that SDG has beneficial effects by reducing hypercholesterolemia-induced atherosclerotic changes; these effects were associated with reduced lipid peroxidation products, total and LDL cholesterol, and increased antioxidant defense (Nestel, 2000). A reduced rate of lipid peroxidation may decrease the risk of endothelial dysfunction and atherogenesis (Chisolm, 2000).

As our study shows that dietary supplementation with flax seeds had moderate effects of increased antioxidant defense, which together with reduced lipid peroxidation products may help to reduce oxidative stress and its effects.

Effect on plasmatic biomarkers of endothelial dysfunction induced by flax seeds - supplement in diet

Endothelial dysfunction may be considered a marker of atherosclerosis debut strongly correlated with elevated chronic inflammation markers (sVCAM-1, ICAM-1, CRP) appears to confer it cardiovascular risk marker significance. Numerous experimental animal studies conducted in our laboratory have used the Wistar rat model to investigate possible mechanisms by which vascular endothelium and oxidative stress are involved with "*primum movens*" role in the pathogenesis of atherosclerosis, (Colev, 2000, 2003, Cimellaroa, 2016, Sitia, 2010, Mudau, 2012).

Lately we have been concerned about ways of recognizing, preventing and improving endothelial dysfunction by dietary supplements (functional food) containing antioxidants. Antioxidants from plants can improve endothelial dysfunction, but dietary supplementation with polyunsaturated fatty acids-rich, PUFA n3, vegetables and their beneficial effects are less known, which has attracted the researchers attention. The mechanisms by which antioxidants from plants (PUFA n3 and lignans) influence endothelial dysfunction are still studied; their quantification could be done by analysis of circulating soluble adhesion molecules expression, considered markers of endothelial dysfunction (Constans, 2006).

Some literature data show that soluble form of sVCAM-1 antigen was found increased in patients with atherogenic risk factors even in the initial stages of endothelial dysfunction. Therefore we considered useful to follow the effect of nutritional antioxidants of vegetable origin from flax seeds (PUFA n3 and lignans) on sVCAM-1 and vWF values in the presence of two risk factors: estrogen deficiency and increased lipid intake PUFA n6. Minamiyama (2007) introduced the term antioxidant bio factors for a fermented grain mixture containing wheat germ extract, soy bean, rice bran, wheat and lemon juice, green tea, green leaves extract. Other experimental studies have suggested that this antioxidant mixture can improve some disorders associated with endothelial dysfunction such as atherosclerosis, hypertension (Daiber, 2017).

Lately, many studies have investigated the cellular and molecular mechanisms involved in mediating the interaction between leukocytes and endothelial cells and showing its role in the endothelial dysfunction pathogenesis, and thus in the cardiovascular diseases pathogenesis (Mallick, 2022, Theofilis, 2021, Khan, 2020). The following factors contribute to leukocytes-endothelial cells adhesion:

- expression of adhesion molecules on the surface of leukocytes and/or endothelial cells;
- the release of inflammation mediators from leukocytes, endothelial cells, activated macrophages, etc.
- hemodynamic dispersal forces removing leukocytes from the micro-vascular wall.

Since leukocyte adhesion is partly regulated by the adhesion molecules of endothelial origin expression, some studies have followed their level in the atherosclerotic plaque in humans and animals showing increased expression of ICAM-1 (Haftcheshmeh, 2020, Taurone, 2021) and VCAM-1 (Spartalis, 2020). Other studies have shown that circulating levels of sVCAM-1 was strongly correlated with affected endothelium area (Lenasi, 2018). Distasio, 2021, stressed that the soluble VCAM-1 value changes may be considered an indicator for assessing the expression of this adhesion molecule in the affected endothelial territory, even in the pre-lesion stage.

The combination of "traditional" risk factors like hypertension, dyslipidaemia and hyper-glycaemia alone cannot explain the increased cardiovascular risk in the context of endothelial dysfunction. Many epidemiological studies have shown the relationship between inflammation markers and cardiovascular events risk (Hingorani, 2000, Jenkins, 1999, Jager, 2000). Later it was shown that exposure of endothelial cells to inflammatory cytokines in the

presence of endothelial dysfunction markers leads to adhesion molecules expression and impaired endothelium-dependent relaxation. Increased levels of these adhesion molecules are directly and independently related with cardiovascular disease mortality (RR = 1.10, IC95%: 1.05-1.15 for each increment of 100 ng/ml of VCAM-1 and vWF (Haliga, 2007, Vaverkovaa, 2013).

In our experimental conditions, the occurrence of endothelial dysfunction was favored by the suppression of endogenous estrogens with reduced antioxidant defense, especially when associated with increased PUFA n6 intake. The endothelial dysfunction was evidenced by increased levels of circulating soluble adhesion molecules sVCAM-1 and particularly vWF. PUFA n3 and lignans supplements led to moderate insignificant reduction of this parameter in ovariectomised lots.

vWF and fibrinogen studies showed a significant positive correlation of vWF and fibrinogen with increased urinary albumin excretion rate in diabetic patients. It was also found a correlation between plasma fibrinogen and serum triglycerides and total cholesterol values, suggesting a pathogenic role of increased endothelial dysfunction markers in cardiovascular diseases.

Studies of "nontraditional" risk factors showed that some factors are the acute/chronic inflammation phase markers, the cytokines and coagulation factors. They are the most important investigation subjects in relation to their role in the development of endothelial dysfunction. Patients with stable angina had higher vWF values than non-diabetics, reflecting the presence of endothelial dysfunction (Galbusera, 1997). Festa (2000) studies, have shown that inflammation markers (CRP, fibrinogen and leukocytes count) correlate with certain components of insulin resistance syndrome; strong correlation between CRP and body mass index, systolic pressure and insulin sensitivity index in multivariate linear regression model.

In this study, administration of hyperlipidic PUFA n6 diet, compared with standard diet, led to a significant increase in sVCAM-1 both in control and ovariectomised animals, confirming the presence of endothelial dysfunction.

- control group
  - PUFA n6 rich diet determined a significant increase in sVCAM-1 levels (821.7 *vs.* 320.55 ng/ml) compared to control group with standard diet ($p < 0,05$).
  - PUFA n3 and lignans supplements led to decrease in sVCAM-1 (732,22 *vs.* 821,70 ng/ml) compared to hyperlipidic PUFA n6 group;
- ovariectomy group ·
  - PUFA n6 diet determined a significant increase in sVCAM-1 levels (728.35 *vs.* 335.57 ng/m) compared to the ovariectomy group with standard diet ($p < 0,05$).
  - PUFA n3 and lignans supplements led to decrease in sVCAM-1 (821.7 vs. 320.55 ng/ml) compared to hyperlipidic diet group ($p > 0,05$).

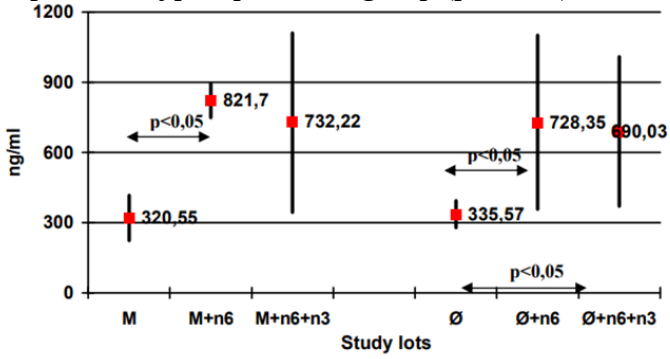


Figure I.3.14
sVCAM-1 changes in flax seeds supplemented diet
in ovariectomised female rats (mean values and statistical significance)

Hyperlipidic diet supplementation with flax seeds (n3 FA and lignans) in ovariectomy group (Ø+n6+n3) led to insignificant reductions in sVCAM-1 levels compared with ovariectomy group with hyperlipidic diet (Ø+n6). Control animals do not show significant differences in this parameter compared with animals subjected to PUFA n6-rich diet.

Our results are consistent with literature studies that have found the relation between endothelial dysfunction and plasma levels of adhesion molecules. Through their lignans content, the highest from all the plants, flax seeds have antioxidant effects (Mocanu, 2004). Thus flax seeds (exogenous antioxidants) and estrogens in physiological concentrations (endogenous antioxidants), have synergistic effects of endothelial dysfunction prevention and thus prevention of atherosclerosis, as found by Lucas et al. (2004).

Following the changes in vWF, marker of endothelial dysfunction in our experiment we observed that ovariectomy and hyperlipidic diet (PUFA n6) caused a significant increase in vWF levels compared with controls with the same diet ($p < 0,05$) which confirmed the presence of endothelial dysfunction.

Mean values of vWF decrease after PUFA n3 and lignans supplements in ovariectomy group (Ø+n6+n3) points out the beneficial effect of flax seeds on vascular endothelium, even in the presence of more than one risk factor.

In the ovariectomy group:

- PUFA n6 rich diet caused a slight increase in vWF levels (160.29% vs. 152.09% compared to standard sample) compared with the ovariectomised standard diet ($p>0,05$)
- PUFA n3 şi lignans supplements caused: a decrease in vWF (143.96% vs.160.29% compared to standard sample) compared with hyperlipidic diet group, without statistical significance ($p > 0,05$).

Since increased sVCAM-1 and vWF means endothelium activation, we believe that our results confirm the presence of endothelial dysfunction caused by hyperlipidic diet, both in control animals (M), and especially in those with endogenous estrogen deficiency (ovariectomy).

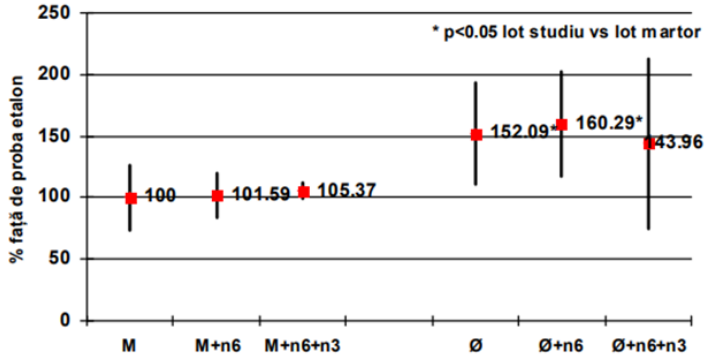


Figure I.3.15
vWF changes in flax seeds supplemented diet
in ovariectomised female rats (mean values and statistical significance)

Dietary supplements with flax seeds (PUFA n3 and lignans) reduced sVCAM-1 and especially vWF levels without strong statistical significance. Clinical studies have shown that the presence of major risk factors for endothelial dysfunction (hypercholesterolemia, diabetes mellitus, hypertension and smoking) is characterized by increased circulating vWF. Biological markers such as sVCAM-1, vWF and CRP are useful in monitoring the most important metabolic and hemodynamic risk factors. Some authors consider that the presence micro albuminuria is the best independent predictor of future cardiovascular events and

mortality both in diabetics and no diabetics because it reflects global and generalized vascular wall impairment (macro and microangiopathy). These researches show another "facet" of endothelial dysfunction represented by increased vWF levels or microalbuminuria (Serafinceanu, 2005, Velasquez, 2003).

Supplementing the diet with flax seeds - effect on lipid parameters

Normocaloric but hyperlipidic diet is one of the causes of hyperlipemia defined as an increase in serum cholesterol and/or triglycerides above the normal value. Since the lipids are transported as lipoproteins, dislipoproteinemia reflects the changes in circulating lipoproteins, especially the decrease of HDL-cholesterol below normal value (in humans under 35 mg/dl) which can be associated or not with hyperlipaemia. In animal studies it was shown that flax seed can have various effects on serum lipids (Velasquez, 2003, Lucas, 2004, Haliga, 2007, Cintra 2006, Bhathena, 2002 etc). Some authors have found that that dietary supplementation with 10% flax seeds did not alter serum lipid levels in rats, while 20% and 30% concentrations have reduced triglyceride levels, cholesterol and LDL-C (Ratnayake, 1992).

Normal diet containing saturated FA was associated with an increased risk of coronary artery disease, while dietary unsaturated FA (MUFA) and polyunsaturated FA (PUFA) appear to have cardiovascular protective effects. The lipid profile was investigated in animals (rats) fed high fat diet, which came from different sources: PUFA (flax seeds and trout), MUFA (peanuts) or saturated FA (chicken skin). It was found that the animals fed a diet supplemented with 10% flax seeds had the lowest serum cholesterol values compared with other types of dietary supplements. FA content in the diet could be associated with hypocolesterolemiant effect since it was shown that the degree of unsaturation is inversely correlated with serum cholesterol levels (Bhathena, 2002). Flax seeds, as already mentioned, are a very rich source of polyunsaturated fatty acids, especially ALA, PUFA n3, lignans and soluble fiber; all these components could reduce the influence of cardiovascular risk factors.

In our study we followed the effects of flax seeds dietary supplementation on serum and hepatic parameters of lipid metabolism in the absence of endogenous estrogens and hyperlipidic diet which separated/individually or together, could greatly contribute to the initiation and progression of endothelial dysfunction (pre-lesion stage of atherosclerosis), but also to the apparition of atheroma plaque (lesion stage of atherosclerosis). We supplemented the diet with 15g flax seeds/100 g food, representing the lowest dose with the most beneficial effects. Our results show that dietary supplementation with flax seeds had beneficial effects on serum lipids in the context of dyslipidemia caused by endogenous estrogens deficiency. We noted that flax seeds produced a slight decrease in total serum cholesterol, both in control and ovariectomy group, suggesting a slight protection:

- in control group - dietary supplements with PUFA n3 and lignans decreased total serum cholesterol values (72,13 vs. 91,18 mg/ml) compared with PUFA n6 group ($p>0,05$);
- in ovariectomy group · dietary supplements with PUFA n3 and lignans determined a slight decrease in total serum cholesterol as well (98,03 vs. 104,23 mg/ml), compared with hyperlipidic diet group ($p>0,05$).

The literature on the hypocholesterolemiant effect of flax seeds did not clarify yet which of their components are responsible for cholesterol lowering. Being a rich source of ALA it has been shown in experimental studies in animals and humans, a reduction in total cholesterol (Renaud, 2001). Ovariectomy was followed by significant increases in liver cholesterol ($p < 0,05$). We noticed that the dietary supplementation with flax seeds in hyperlipidic diet determined statistically insignificant decreases of hepatic cholesterol, both in ovariectomy and intact group.

Some clinical trials (Khalesi, 2015, Rodriguez-Leyva, 2013) have provided evidence supporting that an ALA rich diet, such as flaxseed oil may offer greater protection in cardiovascular diseases than oils rich in linoleic acid (LA), through multiple effects on lipid metabolism but also on platelet functions. Other flax seeds components may have a role in lipid metabolism; clinical studies of Jenkins, in 1999, claimed that cholesterol-lowering effects of flax seeds can be attributed to the fiber content. Besides the cholesterol-lowering effects, flax seeds may act directly on the vascular wall to prevent endothelial dysfunction (Prasad, 2000, 2005).

The experimental model of postmenopausal hypercholesterolemia and atherosclerosis developed by Lucas (2004) on ovariectomised female hamsters, investigated the dosedependent effects of integral flax seeds on lipid metabolism parameters. The study results showed that the concentration of flax seeds in the diet and cholesterol levels are in indirect correlation; cholesterol-lowering effects were inversely proportional to increasing the dose of flax seeds, which means that low doses of flax seeds may have cardioprotective effects (Lucas, 2004). Differences in response to supplementation with flax seeds can be explained partly by species, age and hormonal status of animals. Some clinical studies have concluded that the lignan SDG is the major active ingredient responsible for the hypolipemiant action. The lignans and the soluble fiber contained in flax seeds may help lowering cholesterol (Katare, 2012, Al-Sayeda, 2009). Previous studies in animals reported variable effects of flax seeds on serum triglycerides, some reporting increases in serum TG after dietary supplementation with flax seeds. These variable results may be explained by the difference between the flax seeds doses used, and by different animal models and sexes used (Morise, 2005, Penny, 2002, Djoussé, 2003).

In our research, ovariectomy caused the greatest serum triglyceride rise ($p < 0,05$):

- in control group · PUFA n6 hyperlipidic diet determined a significant increase in TG (96,60 *vs.* 28,64 mg/ml) compared to control group with standard diet (M+n6) ($p < 0.05$) - PUFA n3 and lignans supplementation led to a significant decrease in serum TG values (41,91 *vs.* 90,60 mg/ml) compared to PUFA n6 group ($p <0.05$);
- in ovariectomy group · PUFA n3 and lignans supplementation determined a significant decrease in serum TG values as well (57,79 vs. 78,04 mg/ml) compared to hyperlipidic diet group ($p < 0.05$).

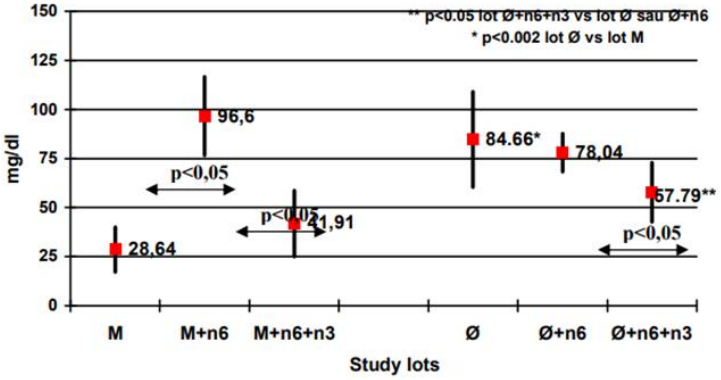


Figure I.3.16

Serum triglycerides changes in flax seeds supplemented diet in ovariectomised female rats (mean values and statistical significance)

In other studies, rats with a saturated fatty acids-rich diet had significantly higher levels of TG compared to PUFA n3 from flax seeds supplementation. These differences can be attributed to the fatty acid composition of their diet, as it was found that n3 FA decreased TG levels in subjects with hyperlipemia and in the Eskimos who eat a low-saturated fat diet (Connor, 2000). According to the researches of Cintra, 2006, the diet supplemented with flax

seeds was the most effective in reducing serum cholesterol and triglycerides, and protecting the liver; these effects may be due to the rich content in n3 FA, lignans, and soluble fiber of the flax seeds. Flax seeds have a high-n3 FA, ALA content; many studies have shown that PUFA n3 EPA and DHA (long chain polyunsaturated FA - LC PUFA) lower plasma TG levels, by decreasing hepatic TG synthesis and reducing the release of TG-rich VLDL in the blood (Mocanu, 2004, 2007, Archer 1998, De Caterina, 2000 etc).

According to the results of our study, flax seeds dietary supplementation resulted in a significant reduction in serum triglyceride levels compared with the hyperlipidic diet ($p < 0.05$) showing the beneficial effect of PUFA n3 and lignans in lowering serum triglycerides, giving them a positive role in endothelial dysfunction prognosis. High fat diet (n6 FA) increased liver triglyceride levels in both study groups, but especially in ovariectomised group (151.08 mg/dl).

Supplementing the diet with PUFA n3 and lignans insignificantly decreased the mean values of hepatic triglycerides in both the control group (121.0 vs. 123.81 mg/dl) and in ovariectomised group (134.72 *vs.*151.08 mg/dl), compared with the hyperlipidic diet group ($p > 0.05$).

Statistical processing of liver triglyceride levels after dietary supplementation with flax seeds reveals that in ovariectomised group with hyperlipidic diet (n6 FA) it produced a decrease in hepatic TG compared with the group without supplements (134.72 mg/dl vs. 151.08 mg/dl) but differences are not statistically significant.

HDL-Chol fraction participates in two processes: reverse cholesterol transport (native HDL), from peripheral tissues to the liver, followed by lipolysis and inhibition of LDL oxidation; HDL delivers Apo C II cofactor to triglyceride-rich lipoproteins (VLDL and chylomicrons) participating in their catabolism in the liver. These features give HDL-cholesterol a protective, antiatherogen role; any decrease in HDL2-chol has to be interpreted differently in relation to risk factors or pathogenetic mechanisms that have contributed to this change. Besides apoproteins, in lipoproteins structure there are included PUFA as well, with an important role in cholesterol esterification and transport to the cells.

The lipoprotein abnormalities favoring dyslipidemia are: quantitative, consisting of increased triglycerides, based on VLDL and LDL, and decreased HDL-cholesterol based on HDL_2 fraction, increased plasma VLDL due to increased hepatic synthesis and decreased extrahepatic clearance, decreased HDL-cholesterol due to increased catabolism, and qualitative, including LP size changes (large VLDL, small dense LDL), increased triglycerides content of LDL and HDL, apolipoproteines and lipids glication, and increased LDL susceptibility to oxidation.

Serum HDL cholesterol increased slightly both in intact and ovariectomised animals, with flax seeds supplemented diet.

- in control group · PUFA n3 and lignans supplements determined a slight increase in HDL-C levels (57,06 vs. 49,96 mg/ml) compared to hyperlipidic diet group ($p>0,05$);
- in ovariectomy group · PUFA n3 and lignans supplements determined a slight increase in HDL-C levels (64,58 vs. 63,05 mg/ml) compared to hyperlipidic diet group ($p > 0,05$);

Flax seeds (*Linum ussitatissimum* supplements in ovariectomy and n6-rich diet resulted in a slight increase, statistically insignificant, of serum HDL cholesterol values, similar with intact animals. Although the differences are not significant, it confirms the beneficial effects of flax seeds, even when two risk factors are associated. Lignans from flax seeds may play an important role in lipid metabolism modulation; synthetic lignans significantly reduce serum cholesterol and LDL cholesterol while increasing HDL cholesterol; it is considered that lignans modulate 7α-hydroxylase and acyl-CoA cholesterol transferase activity, two key enzymes involved in cholesterol metabolism (Kuroda, 1997). Our results are consistent with other experimental studies that have sought changes in HDLcholesterol in diets with various

fat contents. Ovariectomy and n6-rich diet, caused the highest values of serum non HDL-C compared to control (p<0,05). Diet supplementation with flax seeds (PUFA n3 and lignans), determined:

- in control group · significant reduction of serum nonHDL-C (15,08 vs. 41,22 mg/ml) compared to hyperlipidic diet p< 0,05)
- in ovariectomy group · PUFA n3 and lignans supplements did not determined significant decrease in serum nonHDL-C (33,45 vs. 33,77 mg/ml) compared to ovariectomy group

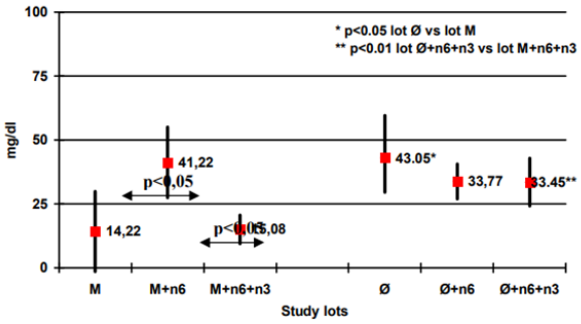


Figure I.3.17
Serum nonHDL-cholesterol changes in flax seeds supplemented diet in ovariectomised female rats (mean values and statistical significance)

Dietary supplementation with n3 fatty acids and lignans in intact animals with hyperlipidic diet resulted in significantly reduced serum nonHDL-C; in animals with estrogen deficiency with the same diet, flax seeds determined slightly reduced values. Thus, when two risk factors are associated, the flax seeds effect of lowering serum nonHDL-C (LDL + VLDL), is diminished.

Atherogenic index (AI) had significantly higher values in ovariectomy group compared to intact group (p < 0,05). While in intact animals flax seeds determined a statistically significant reduction of AI values, in ovariectomy groups with hyperlipidic diet, supplementation with n3 fatty acids and lignans resulted in a slight decrease, statistically insignificant AI. These differences show that beneficial antiatherogenic effects are stronger when a single risk factor is present (hyperlipidic diet).

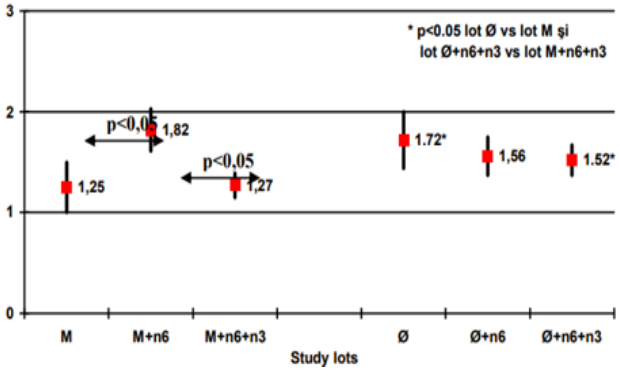


Figure I.3.18
Atherogenic index changes in flax seeds supplemented diet in ovariectomised female rats (mean values and statistical significance)

The lignan complex isolated from integral flax seeds contains SDG, with antioxidant properties, and the hypocholesterolemiant agent 3-hydroxy-3metilglutaric acid (HMGA). Results of studies have suggested that the lignan complex from flax seeds reduces the extension of hypercholesterolemic atherosclerosis; this effect is associated with marked

reduction in oxidative stress markers, serum cholesterol, LDL cholesterol, AI, but also with increased serum HDL cholesterol. These results lead to the conclusion that the lignan complex may be beneficial in atherosclerosis prevention and in risk factors for coronary heart disease and stroke reduction (Yang Hu, 2021, Flight & P Clifton, 2006). Although linseed n3 FA are different from those in fish oil, known for cardiovascular diseases prevention, linseed oil may be also considered a healthy food, and more easily accessible.

Therefore, some experimental studies have investigated the effects of linseed oil on oxidative stress markers, lipid profile, in hyperlipidic diet in rabbits. The results of this study showed that flaxseed oil does not affect serum lipids or hypercholesterolemic atherosclerosis extension and did not reduce the oxidative stress markers (Harper, 2006, Kontogianni, 2013). These differences between the flaxseed oil effects and flax seeds supplemented diet effects could be explained by the fact that flaxseed oil, although rich in n3 FA, does not contain lignans and fibers. Hypotriglyceridemiant and hyoocholesterolemiant effects of flax seeds could be useful in patients with hypertriglyceridaemia and hypercholesterolaemia, but further studies on human models are needed. In conclusion, the results of this study suggest that dietary supplementation with integral flax seeds by reducing total serum cholesterol, serum and liver triglycerides, atherogenic index, and by increasing HDL-cholesterol are effective to decrease the cumulative effects of risk factors in development and progression of endothelial dysfunction.

Supplementing the diet with flax seeds PUFA n3 and lignans - effect on platelets function

Our study investigated platelet function expressed by adhesivity and by ADP platelet aggregation in ovariectomised Wistar female rats, whose PUFA n6 rich diet was supplemented with integral flax seeds (PUFA n3 and lignans). High n6 FA diet increased the average values of platelets adhesivity in ovariectomy group (Ø+n6), compared with intact animals (M + n6) (38.0% vs. 33.3%). Supplementing the diet with flax seeds (n3 FA and lignans), decreased platelet adhesivity without statistical significance, compared to the groups without dietary supplements (35.71% *vs.* 38.0%):

- in control group - PUFA n3 and lignans supplemented diet determined a statistical insignificant decrease of platelets adhesivity values (32% *vs*. 33,3%) compared to the ovariectomy group with hyperlipidic diet (p>0,05);
- in ovariectomy group - PUFA n3 and lignans supplemented diet determined also a statistical insignificant decrease of hepatic triglycerides (35,71% *vs.*38%) compared to the ovariectomy group with hyperlipidic diet (p>0,05);

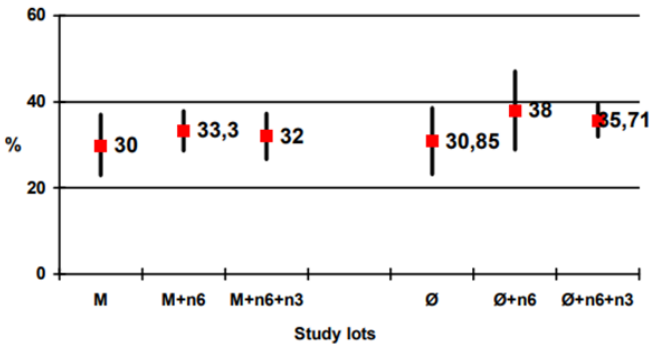


Figure I.3.19
Platelet adhesion index changes in flax seeds supplemented diet in ovariectomised female rats (mean values and statistical significance)

n6 FA rich diet was followed by an increased platelet aggregation rate both in intact and ovariectomised animals. Supplementing the diet with n3 FA and lignans from flax seed on female rats with estrogen deficiency (ovariectomised) compared with ovariectomised animals with unsupplemented n6 FA-rich diet, did not induce statistically significant decreases.

- in control groups - PUFA n6 rich diet determined a statistically significant increase of platelets aggregation (20,67 vs. 8,2 mOD/min) compared to the control group with standard diet ($p<0,05$);
- in ovariectomy groups - PUFA n6 rich diet determined a statistically significant increase of platelets aggregation (18,65 vs. 8,13 mOD/min), compared to the ovariectomy group with standard ($p > 0,05$);

PUFA n3 and lignans (flax seeds) supplemented diet in PUFA n6 ovariectomised group determined a statistically significant increase of platelets aggregation ($p< 0,001$) and an insignificant decrease compared to the hyperlipidic diet group ($p > 0,05$).

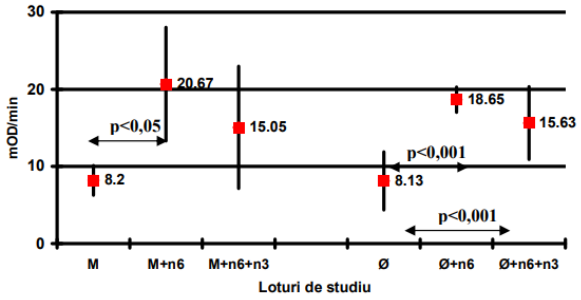


Figure I.3.20
Platelets aggregation rate changes in flax seeds supplemented diet
in ovariectomised female rats (mean values and statistical significance)

Supplementing the diet with n3 FA and lignans from flax seed in female rats with estrogendeficiency compared with unsupplemented n6 FA-rich diet ovariectomised animals, did not induce statistically significant decreases of platelet aggregation rate (15.65 to 18.65 mOD / min).

Ceriello (2005) researches showed the role of oxidative stress by increasing superoxide anion production in hyperglycaemia conditions on endothelial NO synthase (NOS); by blocking NOS activation, with protein kinase C (PKC) and nuclear factor kB (NF-kB) activation, increased SRO level that alter DNA is favored. Subsequently, NF-kB and other transcription factors induce the expression of inflammatory genes by increasing the production of leukocytes chemotactic factor, of inflammatory cytokines and augmentation of adhesion molecules expression. These changes in endothelial cells and monocytes will increase the platelet activation and aggregation with the altering of coagulation-fibrinolysis balance (Beckman, 2002).

Various studies have shown that PUFA n3 is the main FA that decreases platelet aggregation, provided that the aggregation is increased (Ristic-Medic et al., 2003). EPA, one long-chain polyunsaturated FA, is acting on platelet aggregation mainly through its involvement in the eicosanoids synthesis. Eicosanoids vary in structure and bioactivity based on their PUFA precursor (dihomo-gamma-linolenic acid) or arachidonic acid (AA) from omega-6 family or EPA from omega-3 family.

Modulation of platelet activity in our study was performed by supplementing the diet with flax seeds containing PUFA n3 and lignans. n3pPUFA, especially EPA, long chain essential FA derivative, influence platelet aggregation through eicosanoids synthesis (TxA2 and PG I2) whose balance regulates vasomotricity and primary hemostasis. EPA-rich diet

reduces the membrane arachidonic acid, including at platelet membrane level and of derivate end peroxides (TxA2 and PGI2). Thromboxane A3 (TX A3) is synthesized in compensation, a weaker proagregant and vasoconstrictor than TxA2, respectively PGI3 inhibitor of platelet function and vasodilator (Weber, 1987). PUFA n3-rich diet was not sufficient to restore TxA2/PGI2 balance suggesting that other factors contributing to increased oxidative stress may play a role in platelet function alteration.

Thus, in chronic hyperglycemia it was found an increased activity of specific platelet receptors, surface glycoproteins, which may explain the increased platelet adhesivity and aggregation:

- increased GP IIb/IIIa –fibrinogen receptors (Tshoepe, 1995);
- increased GP Ib-IX – vWF receptors (Tshoepe, 1990);
- increased CD62 –P-selectine receptors (Neubauer, 2010).

Our study showed that dietary supplementation with PUFA n3 and lignans in ovariectomy and hyperlipidic diet led to slight decreases in the rate of platelet aggregation and adhesivity. We note that the beneficial effects of flax seeds supplements on platelet aggregation function are more visible in intact animals than those with endocrine imbalance (estrogen deficiency), which indirectly shows the endogenous estrogens contribution to a normal platelet function. All flax seeds components (PUFA n3, lignans and fibers) may be responsible for the observed beneficial effects and future clinical studies are needed to identify the individual effects of each component.

Supplementing the diet with flax seeds PUFA n3 and lignans – morphological features

In endothelial dysfunction, prelesional stage of atherosclerosis, morphological changes were noted in the arterial intima, interesting especially the endothelial cells, and sometimes equally the elastic tissue (internal elastic membrane).

Microscopic examination of the aortic wall (aortic arch) and the coronary artery branches, reported in both groups of animals, minimal change in the aortic wall intima of aortic arch, an area subject to constant hemodynamic stress (Socki, 2004).

Were also noted discontinuities of internal elastic membrane and disruptions of elastic lamina. Elastic tissue was strongly and consistently affected in the ovariectomy the group, in which we observed changes in the coronary branches; in those areas moderate interstitial and perivascular inflammatory infiltrations coexisted.

Endothelium involvement occurs after the endocytosis of circulating lipoprotein with affinity for proteoglycans (LDL and VLDL), reaching the sub endothelial space where they are phagocytized by macrophages. These phenomena were reduced in groups whose diet was supplemented with flax seeds (PUFA n3 and lignans); both endogenous estrogens and flax seeds are complementary and synergistic antiatherogenic.

Involvement of internal elastic membrane implies that a protective structure is affected, a barrier preventing cells and macromolecules migration between the intima and the media, which favors disruption of elastic tissue with repercussions on the vasomotor behavior in general. Loss of elasticity increases hemodynamic stress on the arterial wall, with the possibility of endothelial activation and increased propensity for endothelial dysfunction installation.

Our experimental model showed early changes of intima interesting the endothelium and the subendothelial layer in ovariectomised rats with PUFA n6 rich diet. The absence of changes in control animals, with the same type of hyperlipidic diet, but supplemented with PUFA n3 showed its protective action independent or associated with that of endogenous estrogens. However, it is important to remind that neither endogenous estrogens nor PUFA

n3 are perfect antioxidants; according to data from the literature (Dejica, 2001, Lucas, 2004) they may become pro-oxidant factors in certain circumstances.

The results noted after dietary supplementation with flax seeds emphasizes the endothelial protective role of endogenous estrogens separately, but especially in combination with PUFA n3 and lignans.

Flax seeds, exogenous antioxidants rich in lignans, and estrogens in physiological amounts, endogenous antioxidants, showed a synergistic effect of vascular endothelium protection, delaying the endothelial dysfunction and thus having an antiatherogenic effect. It is considered that the PUFA n6 promote oxidative processes rather than PUFA n3.

The most important thing remains the relationship between the two types of polyunsaturated fatty acids: the more decreased the proportion of PUFA n6 and increased of PUFA n3 in the diet, the better the health prognosis by reducing the risk factor of dysfunction.

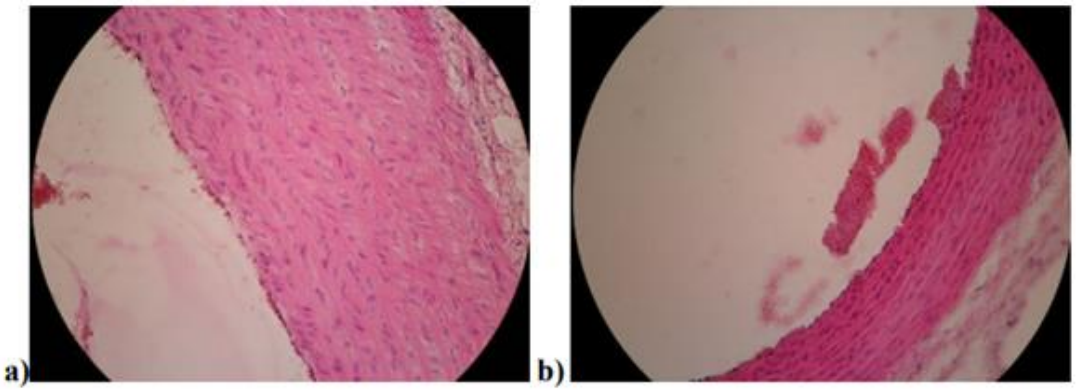


Figure I.3.21
Aorta – a. isolated b. discrete platelets adhesions to the endothelium
(visible elastic network) (HE x 20) Ø+n6+n3 lot

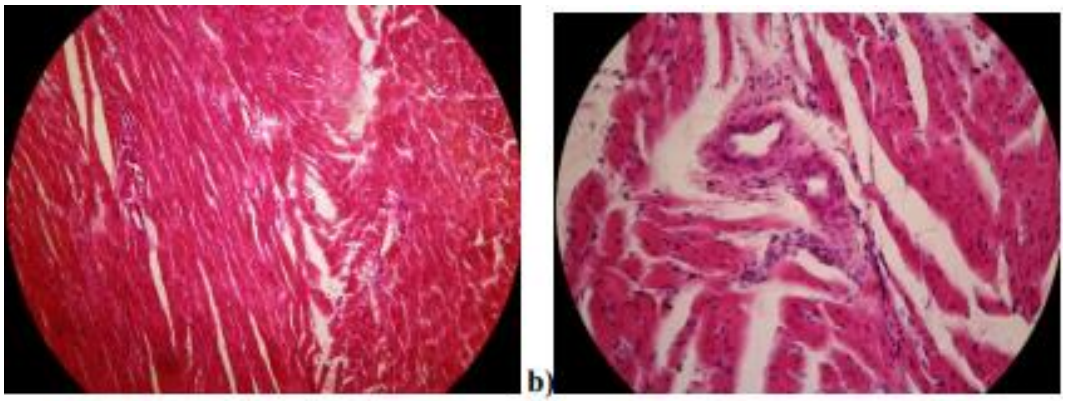


Figure I.3.22
Myocardial tissue a. overview–minor perivascular infiltrate (HE x 20) b. focal interstitial adjacent to the vessel (HE x 40) Ø+n6+n3 lot

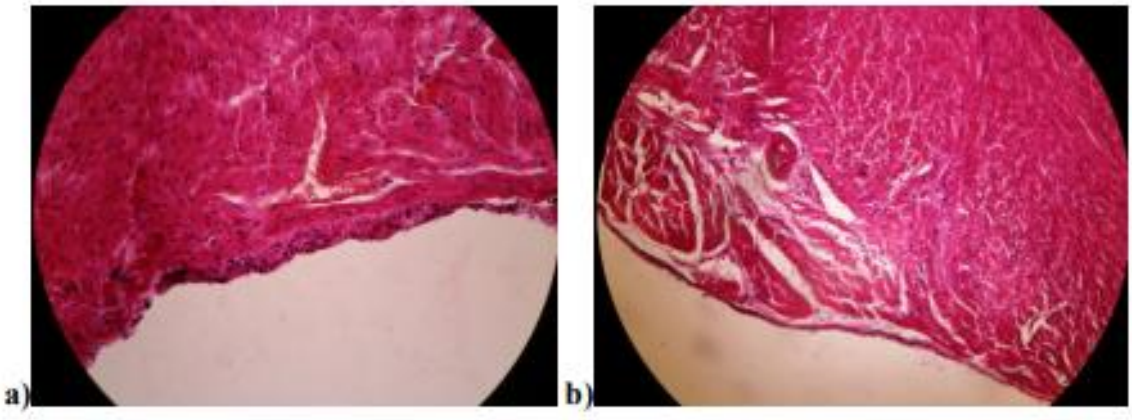


Figure I.3.23
Myocardial tissue a. focal subendocardic lymphocytic inflammation b. perivascular inflammatory infiltrate (HE x 20) M+n6+n3 lot

This study has provided evidence that coincides with the literature, supporting the beneficial effects of flax seeds in cardiovascular disease and supports the usefulness of increased consumption of integral flax seeds in the diet either alone or as adjuvant with other therapeutic means, being an important sanogenesis factor (McKevith, 2005, Carraro, 2012, Patel, 2022, etc).

I.3.5. Conclusions

1. Ovariectomy - model of atherosclerosis induced by estrogen deficiency - was characterized by increased oxidative stress, especially when associated with hyperlipidic diet; increased oxidative stress was evidenced by high levels of serum and hepatic MDA and reduced GSH in liver homogenate, which confirmed the reduction of non-enzymatic antioxidant defense in "surgical menopause".
2. Supplementing the diet with integral flax seeds in hyperlipidic diet, although having a slight effect of reducing oxidative stress and lipid peroxidation, was accompanied by significant increase in liver GSH levels and thereby contributed to increased antioxidant defense.
3. Investigation of soluble circulating biomarkers of endothelial dysfunction, sVCAM-1 characteristic for inflammation and vWF, confirmed the presence of endothelial dysfunction by high values of these parameters, statistically significant increases compared with control animals, when two risk factors are present: lack of endogenous estrogen hormones and hyperlipidic diet. Inflammation may represent an important link in the endothelial dysfunction - atherogenesis relation, clinically known link, but still incompletely studied and understood.
4. Dietary supplementation with n3 FA and lignans resulted in slight insignificant decrease of soluble adhesion molecules and of vWF. These biological markers and CRP investigation are useful to confirm the presence of risk factors with predictor role for future cardiovascular events, because rising values reflect global and generalized impairment of the vascular wall.
5. In animals with hormonal deficiency (ovariectomy) we found dyslipidemia: hypercholesterolemia and/or hypertriglyceridemia; hepatic homogenate showed similar findings. High fat diet decreased serum HDL-C associated with increased non-HDL-C and AI, showing an increased risk of endothelial dysfunction in this context.

6. Supplementing the diet with integral flax seeds in hyperlipidic diet group, led to lower total serum cholesterol ($p>0.05$) and serum triglycerides ($p < 0,05$), effects observed in liver homogenate as well. Non HDL-C and AI values decreased with significant differences ($p < 0,05$) compared with animals receiving a diet rich in PUFA n6, except the ovariectomy group. Decreased AI values shows that antiatherogen benefits are stronger when a single risk factor exists.
7. Serum HDL-C was slightly increased ($p > 0.05$) after dietary supplementation with integral flax seeds, suggesting endothelial protection, with diminishing risk of endothelial dysfunction; these imply restoring reverse cholesterol transport, recovery of antioxidant capacity and thereby decreased LDL oxidation and improved endothelium-dependent vasodilatation.
8. Platelets activity was investigated by determining the rate of platelet aggregation and platelet adhesivity, both with significantly higher values ($p<0,001$) in both ovariectomy and normal groups, with hyperlipidic diet. These results confirm that the combination of two traditional risk factors, favored platelets activation and endothelium involvement.
9. Supplementing the diet with PUFA n3 and lignans in ovariectomy and hyperlipidic diet decreased the platelets aggregation rate and adhesivity. The beneficial effects of dietary supplementation with integral flax seeds on platelets aggregation are more obvious in healthy animals, compared with those with endocrine imbalance which indirectly points out the importance of endogenous estrogen hormones in maintaining platelets function within normal limits.
10. Anatomo-pathologic examination of the aorta showed in ovariectomised animals intimal changes, i.e. endothelitis aspects, with endothelial discontinuities, platelets adhesion and leukocytes margination; we also found disruption of elastic fibers, sometimes absent, accumulation of plasma molecules (lipid micro vesicles) and rare macrophages infiltration, especially in the hyperlipidic diet group (PUFA n6). These changes show the negative effects on the endothelium and intima produced by two risk factors, estrogen deficiency and hyperlipidic diet.
11. Supplementing the diet with integral flax seeds (15%) had a beneficial protective effect, by reducing the vascular wall changes, in correlation with the endothelial dysfunction markers changes, some parameters of lipid metabolism and platelets functions.
12. This research is the first in our country, so far, that studied the effects of supplementing the diet with integral flax seeds on circulating soluble molecules, markers of endothelial dysfunction in correlation with oxidative stress, dyslipidaemia and platelets activation in ovariectomised animals with hyperlipidic diet.
13. According to our results, integral flax seeds have a protective effect, lowering the risk of endothelial dysfunction (pre-lesional stage of atherosclerosis) and can be considered actual nutritional supplements, without neglecting the possible interaction of PUFA n3 with antithrombotic medication (aspirin, warfarin), cholesterol-lowering (statins), kidney transplant patients treatment (cyclosporine) or non-steroidal anti-inflammatory drugs.
14. The promising results of this experimental research on the effects of adjuvant therapy, in endothelial dysfunction prevention through dietary supplements, requires further studies in humans due to the multiple benefits of using flax seeds (hypocholesterolemiant, antiaggregant and reducing insulin resistance) in current medical practice applications.
15. At the same time, this study points out the need of better understanding of the first endothelial changes, less known, and of non-pharmacological interventions that may delay the developing of endothelial dysfunction, especially in this reversible prelesional stage of atherosclerosis.
16. In light of these recent data it is very important to define multifactorial therapeutic approaches, for the prevention and amelioration of cardiovascular diseases. New

therapeutic strategies in this direction will have to include among the essential components: antiinflammatory drugs, antioxidants (preferably nutritional supplements), renin-angiotensin blockers and lipid-lowering.

I.4. Platelet adhesion to endothelial cells in experimental atherosclerosis; the role of serum molecules sVCAM-1 and vWF

I.4.1. Introduction

The adhesion of both leukocytes and platelets to endothelial cells has been implicated in the progression of atherosclerosis and thrombus formation (P. Libby, 2008, 2012). In recent years, the use of foods with health protective effects, named functional foods, has received considerable attention for reducing cardiovascular disease risk, one of these being flaxseed (Lucas, 2004). Flaxseeds are known to contain 35–40% fat, of which 55% is represented by α-linolenic acid (n3 PUFA) and 15–18% linoleic acid (n6 PUFA) and their metabolites. In addition to being the richest plant source of α-linolenic acid (50–62% of flaxseed oil or ≈22% of whole flaxseed) and lignans (mainly SDG)), which have phytoestrogen properties, flaxseed is an essential source of dietary fiber (28% by weight), of which 25% is in the soluble form (Babu et al., 2000). All of these components could positively influence women's cardiovascular disease risk profile (Mocanu 2011, Martinchik, 2012).

In our current diet, the ratio n6 PUFA/n3 PUFA was 20–30/1, comparing to a ratio of 1–4/1 in the period when the human genetic code was established in relation to the type of diet. Previous studies (Lucas, 2004, Mocanu, 2011) have demonstrated that flaxseed is beneficial in reducing hypercholesterolemia and progression of atherosclerotic lesions in ovarian hormone deficiency. The available data sustain that the cardio protective properties of flaxseed are due not only to hypocholesterolemic effects but also to other potential mechanisms, such as antioxidative, anti-inflammatory, and antithrombotic effects. Few studies have been conducted in order to assess the effects of flaxseed components on platelet and endothelial dysfunction (Wang, 2012).

Endothelial cells express adhesion molecules such as P-selectin, E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) on the cell surface that are involved in leukocyte recruitment and platelet adhesion during thrombosis and inflammation (Libby, 2012). In addition, endothelial cells synthesize plasma proteins such as von Willebrand factor (vWF) for platelet adhesion in thrombosis and soluble molecules such as E-selectin and thrombomodulin (Prasad, 1999). When the vascular endothelium encounters inflammatory stimuli, it undergoes several changes, including the up regulation of surface and soluble cell adhesion molecules and the release of cytokines. This process, termed endothelial activation, can be triggered by a variety of inflammatory stimuli encountered in the blood, including oxidized LDL, free radical species, lipopolysaccharide (LPS), and cytokines, such as tumor necrosis factor α (TNFα). The activated endothelium plays an integral role in the development of atherosclerosis. Circulating monocytes are attracted to the endothelium by chemokine, bind to the adhesion molecules, adhere, and transmigrate to the sub endothelial space, where they become macrophages, scavenge oxidized LDL, become foam cells, and contribute to the development of the fatty streak in the early stage of atherosclerosis (Ross, 1999).

The purpose of this study was to determine the possible effect of flaxseed to prevent leukocytes and platelets adhesion to endothelial cells and to reduce soluble adhesion molecules (sVCAM-1) and endothelial integrity markers (vWF) in ovariectomized rats fed a high-fat diet.

I.4.2. Materials and methods

Forty-two female Wistar rats (14 weeks old, weight 200 ± 20 g) were used in the experiment. The rats were purchased from the animal farm of the discipline of pathophysiology, Grigore T Popa University of Medicine and Pharmacy, Iasi, Romania.

All experimental procedures used in this study were in strict accordance with international ethical regulations and were approved by the medical ethics committee of the Grigore T. Popa University of Medicine and Pharmacy. The experiment respected as well the instructions of the guidelines on the care and use of animals for scientific purposes, national advisory committal for laboratory animal research.

The animals were anaesthetized with an intraperitoneal injection of a mixture of ketamine, doses of 100 mg/kg bodyweight and xylazine, doses of 10 mg/kg bodyweight. Half of the rats (n = 21) were subjected to bilateral ovariectomy (ovx using the dorsolateral approach) the remaining animals (n = 21) were subjected to sham surgery (sham), during which the ovaries were exteriorized but replaced intact.

Diets

The rats were kept in standard laboratory conditions, with a controlled temperature (20 ± 2^0C) and a 12 h light/12 h dark cycle. The rats were provided with laboratory chow 20 g food/rat/day and tap water ad libitum. Each of the two groups (ovx and sham) was randomly assigned for 36 weeks to three different diets:

(1) low-fat diet (8% energy as fat, deficient in ALA, control;

(2) high-fat diet (40% energy as fat, lard based, lard group);

(3) high-fat diet enriched with ground flaxseed 15 g/100 g of food, rich in ALA (lard + flaxseed group).

Diets had similar carbohydrate, total fiber, protein, and fat content (Table I.4.1).

Table I.4.1
Composition of experimental diets (%; w/w)

Experimental diets	8% fat	40% fat Lard	40% fat Lard + flaxseed
Proteins	20.00	20.00	20.00
Corn starch	62.00	32.00	32.00
Cellulose powder	5.00	3.00	3.00
L-cysteine	0.25	0.25	0.25
Vitamin mix	1.00	1.00	1.00
Mineral mix	3.50	3.50	3.50
Choline	0.25	0.25	0.25
Fat	8.00	40.00	40.00
Sunflower oil	8.00	15.00	7.00
Lard	—	25.00	25.00
Flaxseeds*	—	—	8.00

*Flaxseeds (*Linum usitatissimum)* belonged to the Olin variety and were provided by the Department of Phytotechny, Faculty of Agronomy Iasi. The composition of flaxseeds was: 40.2% oil (55.6% linolenic acid) and 19.5% proteins.

Animal Necropsy and Processing of Samples

After 36 weeks, the animals were sacrificed, by thiopental anesthesia (1 mL/100 g body weight from 0.01% solution), followed by opening the chest and collecting the blood by cardiac puncture. Blood samples were collected using sodium citrate as anticoagulant buffer, blood/citrate ratio of 9:1, or without anticoagulant. The anticoagulated blood was centrifuged (200 ×g) for 10 min and the PRP was removed and kept at room temperature for use within 4 h. Aliquots of serum were frozen and kept at −80∘ C for later analysis.

Parameters of Endothelial Dysfunction

Serum VCAM-1 was measured by ELISA method for quantitative evaluation of human sVCAM-1 (Bender Medical System) (Hession et al., 1992). Serum vWF was measured by an immunoenzymatic "sandwich" method for vWF antigen (Life Therapeutics), (Bartlett, 1976).

Platelet Functions Aggregation and Adhesion

Platelet aggregation to ADP 10 μM final concentration was performed using a kinetic microplate reader (Tecan Sunrise, Switzerland), according to Bednar's method (1995), changed by Chadderdon and Cappello (1999), and was expressed in absolute value (mOD/min), which represent the mean decreasing of the optical density as a consequence of platelet aggregation induced by ADP. The platelet adhesion to fibrinogen was performed also with the microplate reader TECAN, according to Bellavite's method (1994). The results are expressed as percentage to the total number of platelets. The percentage of adherent cells was calculated on the basis of a standard curve.

Parameters of Lipid Profile

Serum total cholesterol, HDL-cholesterol, and triglycerides (TG) were measured by enzymatic colorimetric methods on a TECAN microplate reader by commercially available kits (Audit Diagnostics Ireland). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

Morphological Study of Aorta For light microscopy evaluation of aortic atherosclerotic lesions, we used HE staining.

Statistical Analysis

Data were expressed as mean ± standard deviation SD. Univariate statistical analysis was performed using the Student's t-test and Bonferroni's Multiple Comparison Test (Statistical Software Package SPSS, version 13, SPSS Incorporation, Chicago, IL, USA).

I.4.3. Results and Discussions

The literature's data supports the participation of the vascular wall in the atherosclerosis process, involving an inflammatory process, endothelial dysfunction, and platelets activation with the consequences arising from this process Platelets receptors activation, platelet membrane fluidity alteration and the membrane lipids composition change contribute to platelets activation. It has been shown that platelet activation is characterized by increased platelet adhesion and increased platelet aggregation, particularly to ADP, but also to thrombin or collagen (Ribeiro, 2009).

In our research, the antiatherogenic mechanism of flaxseed enriched diet was investigated in ovariectomized female rats, a model of experimental atherosclerosis (E. A. Lucas, 2004). The absence of endogenous estrogens disturbs the lipid metabolism, decreases the antioxidant capacity, and alters the expression of adhesion molecules and platelet adhesion to endothelial cells (Cossette, 2012, Cutini, 2012). Moreover, the excess of saturated fatty acids determines hypercholesterolemia and could increase the atherogenic potential (Cutini, 2012).

In our study, platelet aggregation significantly increased in lard-fed groups as compared to low-fat diet groups, while supplementing the diet with flaxseeds significantly decreased platelet aggregation only in Sham group. Lard diet resulted in significantly increased platelet adhesion in Ovx group and the addition of flaxseeds significantly decreased it.

Table I.4.2 shows the changes in platelet and endothelial markers by addition of lard or lard + flaxseed in Sham and Ovx groups.

Table I.4.2
Mean ± SD values for platelet functions and endothelial markers in studied groups

Measures	Sham	Sham + lard	Sham + lard + flaxseed	Ovx	Ovx + lard	Ovx + lard + flaxseed
Platelet functions						
Aggregation (mOD/min)	7.9 ± 1.2	21.0 ± 1.3[b]	14.6 ± 1.3[bc]	8.1 ± 1.2	18.8 ± 1.3[b]	15.6 ± 1.2[b]
Adhesion (%)	29 ± 6	34 ± 3	31 ± 4	31 ± 7	40 ± 9[b]	34 ± 4[c]
Endothelial markers						
sVCAM (ng/mL)	175 ± 64	286 ± 26[b]	294 ± 11[b]	252 ± 53[a]	539 ± 162[ab]	404 ± 10[ac]
vWF (%)	111 ± 6	118 ± 6	104 ± 10	178 ± 13[a]	193 ± 16[ab]	155 ± 10[ac]

Values are means ± SD, $n = 7$ in each group.
[a] $P < 0.05$ as compared to corresponding Sham groups.
[b] $P < 0.05$ as compared to groups fed with low-fat diet.
[c] $P < 0.05$ between lard and lard + flaxseed fed groups.

Serum sVCAM-1 increased in lard groups as compared to low-fat diet groups and the addition of flaxseeds significantly decreased this endothelial marker in Sham and Ovx groups. Serum vWF increased in Ovx groups as compared to Sham groups. The ovariectomized rats fed with lard + flaxseeds had significantly lower serum concentrations of vWF as compared to Ovx + lard group.

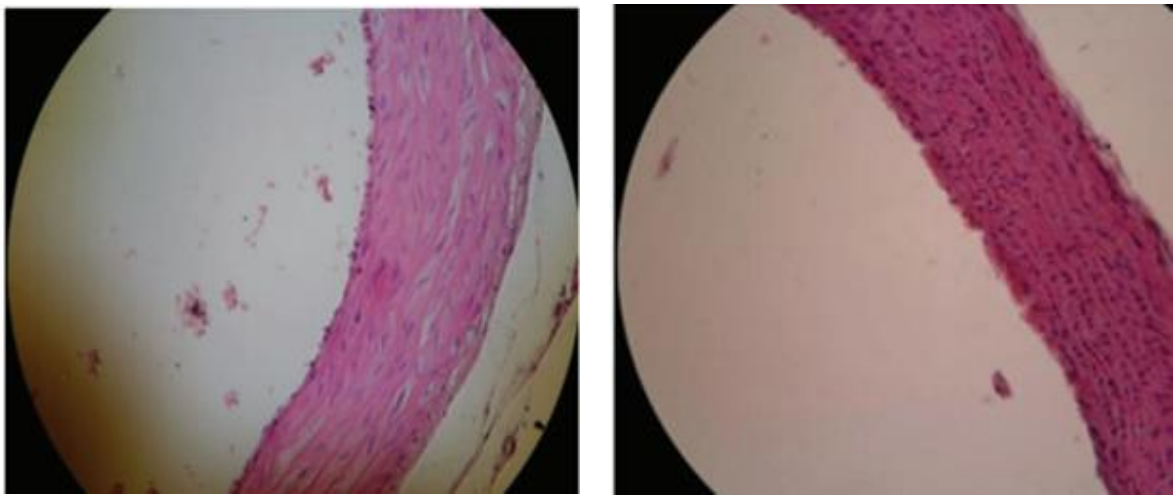
Ovariectomy significantly increased serum total cholesterol, non-HDL cholesterol, and TG. High-fat diet resulted in increased serum total cholesterol, non-HDL cholesterol, and TG as compared to low-fat diet in Sham groups. The flaxseed addition to the high-fat diet led to significant reduction of TG in Sham and Ovx groups. The supplementation of diet with flaxseed significantly decreased non-HDL cholesterol in Sham animals and no significantly decreased total cholesterol and non-HDL cholesterol in ovariectomized female rats. Table VII shows the changes in serum lipid parameters by addition of lard or lard + flaxseed in Sham and Ovx groups.

Table I.4.3
Mean ± SD values for lipid profile in studied groups

Measures	Sham	Sham + lard	Sham + lard + flaxseed	Ovx	Ovx + lard	Ovx + lard +
Serum						
Total cholesterol (mg/dL)	71 ± 4	92 ± 4[b]	72 ± 4[c]	104 ± 4[a]	104 ± 4	98 ± 4
Triglycerides (mg/dL)	28 ± 9	94 ± 16[b]	40 ± 14[c]	80 ± 14[a]	81 ± 12	57 ± 15
HDL-cholesterol (mg/dL)	51 ± 10	52 ± 11	55 ± 11	61 ± 11	61 ± 12	65 ±
Non-HDL cholesterol (mg/dL)	20 ± 9	41 ± 12[b]	16 ± 3[c]	43 ± 16[a]	43 ± 18	33 ±

Values are means ± SD, $n = 7$ in each group.
[a] $P < 0.05$ as compared to corresponding Sham groups.
[b] $P < 0.05$ as compared to groups fed with low-fat diet.
[c] $P < 0.05$ between lard and lard + flaxseed fed groups.

Examination of the aorta under a light microscope in lard-fed Ovx animals revealed signs of incipient atherosclerosis (endothelitis, leukocyte, and platelet adhesiveness and leukocyte margination, macrophage loaded with lipids in intima) (Figure I.4.1).



a. b.

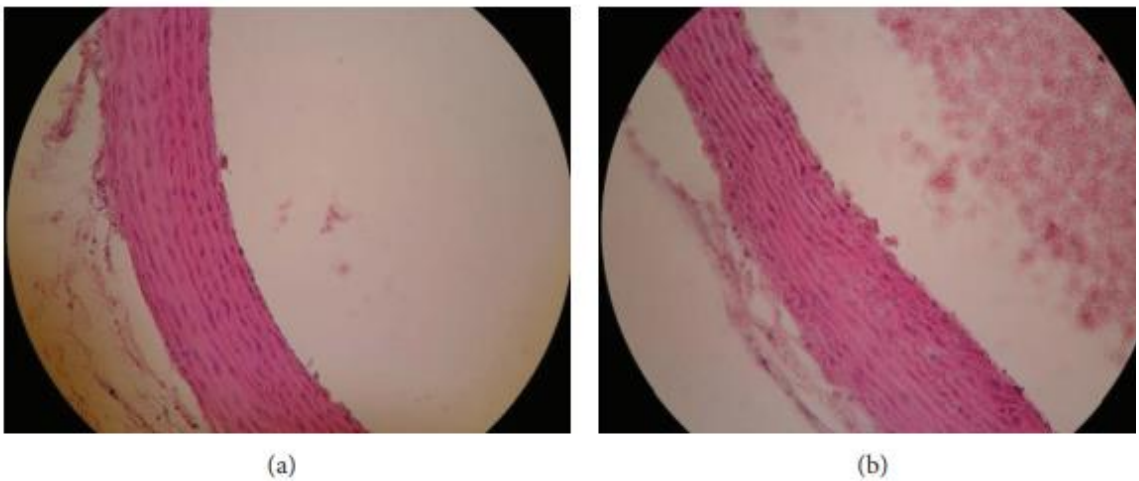
Figure I.4.1
Aorta—Ovx + lard group (HE staining ×20).
a. Endothelitis. Adherent platelets. Leukocyte margination. b. Isolated macrophages loaded with lipids in the internal third of the intima.

The histological evaluation of the aorta in ovariectomized group fed with lard + flaxseed diet showed an ower extent of platelet and leukocyte adherence to endothelium (Figure I.4.2) similar to Sham group (Figure I.4.3).

In the present study, ovariectomy and lard-based diet increased serum concentrations of total and non-HDL cholesterol, platelet aggregation and adhesion, and endothelial dysfunction markers (sVCAM-1 and vFW) and led to incipient atherosclerotic lesions in female rats fed on high fat diet.

The addition of ground flaxseed (15 g *Linum usitatissimum*/100 g food) to lard-based diet significantly reduced platelet adhesion and serum concentrations of endothelial integrity markers (vWF) and prevented the progression of atherosclerotic lesions in estrogen deficiency states.

Our results clearly demonstrated that the flaxseed diet may protect against atherosclerotic lesions by decreasing platelet reactivity, without lowering effect on serum cholesterol.



(a) (b)

Figure I.4.2
Aorta—Ovx + lard + flaxseed group (HE staining ×20).
a. Isolated adherent platelets b. Rare adherent platelets

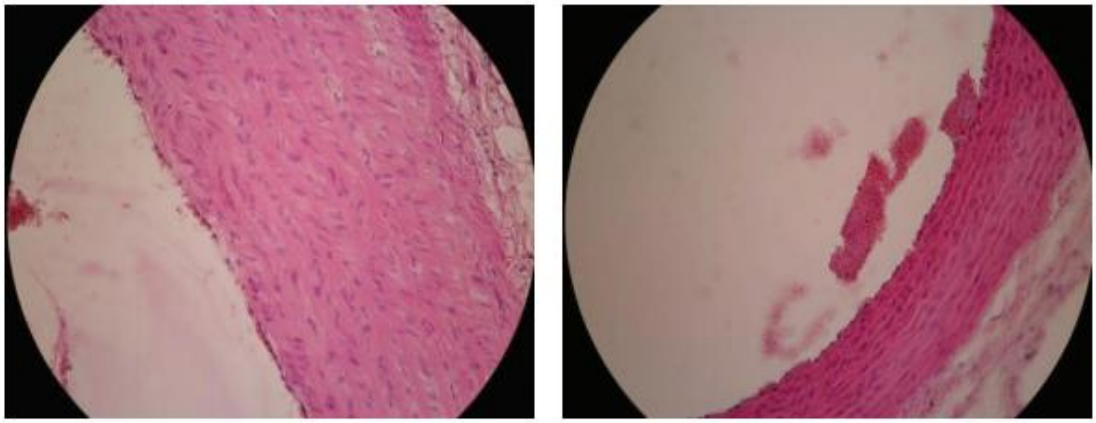


Figure I.4.3
Aorta—Sham + lard + flaxseed group (HE staining ×20).
a. Rare adherent platelets. b. No platelets adhered to the endothelium

The literature data on the hypocholesterolemic effect of flaxseeds are controversial. The experimental model of postmenopausal hypercholesterolemia and atherosclerosis, developed by Lucas et al. (2004), on ovariectomized female hamsters, demonstrated that flaxseed was beneficial in reducing hypercholesterolemia and the progression of atherosclerotic lesions in ovarian hormone deficiency.

Other studies (Prasad, 1999), made on rabbits, and showed that the formation of atherosclerotic lesions was decreased without a noticeable cholesterol lowering effect. Differences in response to supplementation with flaxseeds can be explained partly by species, age, and hormonal status of animals. The hypocholesterolemic effect of flaxseed could be attributed to flaxseed gum (Jenkins, 1999, Bierenbaum, 1993), or to the lignan precursor present in flaxseed, SDG (Prasad, 2005). The lignans and the soluble fiber contained in flaxseeds may help lowering cholesterol (Brown, 1999).

These results suggested that the cardioprotective property of flaxseed could be due to its hypocholesterolemic effect but also to other potential mechanisms such as being antioxidative, anti-inflammatory, and/or antithrombotic. Serum HDL-cholesterol changes in flaxseed-fed sham operated and ovariectomized animals revealed that ALA and lignans supplements had beneficial effects by slight and nonsignificant increase in HDL-cholesterol as compared to lard-fed animals. Although the differences are not significant, it confirms the beneficial effects of flaxseeds, even when two risk factors are associated. Lignans from flaxseeds may play an important role in lipid metabolism modulation; synthetic lignans significantly reduce serum cholesterol and LDL cholesterol, while increasing HDL cholesterol; it is considered that lignans modulate 7α-hydroxylase and acyl-CoA cholesterol transferase activity, two key enzymes involved in cholesterol metabolism (Kuroda, 1997).

The results of our study suggested that in a condition associated with two cardiovascular risk factors, estrogen deficiency and increased saturated fatty acids intake, the endothelial markers and platelet functions are significantly changed and the diet supplementation with flaxseed had a beneficial effect.

The lack of estrogen atheroprotection in our animal model of atherosclerosis could be connected to the state of the NO endothelial production (Kevil, 1999, Lucas, 2004). The NO production would be involved in the mechanisms by which estrogens inhibit atherosclerosis and estrogen deficiency that are responsible for atherogenic changes in lipids, endothelial cells, smooth muscle cells, and platelets (Mocanu, 2004).

Moreover, in estrogen deficiency, the endothelial synthesis of NO is low, while superoxide anion concentrations are increased, destroying NO (Kitahara, 2010). Since recent studies revealed that fish oil increased NO production and endothelial NO synthase expression (Casos, 2010), the diet enriched in flaxseed may protect against increased platelet reactivity and endothelial dysfunction induced by estrogen deficiency (Mocanu, 2011). High dose of flaxseed used in our study significantly reduced platelet adhesion in ovariectomized female rats.

We have previously demonstrated that the addition of ground flaxseed (15 g *Linum usitatissimum*/100 g food) improved platelet functions and had antioxidative effect in ovariectomized hamsters (Haliga, 2007). The high flaxseed dose led to the enrichment of platelet membrane phospholipids with large amounts of EPA and/or DHA (Lucas, 2004, Mocanu 2004), resulting in decrease platelet reactivity or endothelial activation, by reducing sP-selectin concentrations (Nomura, 2003). On the other hand, SDG, isolated from flaxseed, has oxygen radical scavenging properties (Prasad, 2000, Lee, 2008) and inhibits lipid peroxidation (Szuwart, 2000), which could reduce oxidative changes of plasma lipoproteins and therefore decrease platelet adhesion to oxidized LDLs (Szuwart, 2000).

I.4.4. Conclusions

In our study, high dose of ground flaxseed incorporated to lard-based diet prevented the progression of atherosclerotic lesions in estrogen deficiency rats by decreasing platelet and endothelium reactivity without serum cholesterol lowering effect.

Assessment of platelet adhesion, serum soluble adhesion molecule s-VCAM, and endothelium integrity molecule vWF could be useful to detect the risk for atherosclerotic lesions in estrogen deficiency states and to estimate the effect of flaxseed supplementation.

Supplementing the diet with high doses of ground flaxseed may lower the atheroslerotic risk in postmenopausal women by increasing the vascular wall protection, reducing the thrombotic risk and improving the lipid metabolism.

I.5. Antioxidant and antithrombotic effect of whole grain and vitamin E in experimental atherosclerosis

I.5.1. Introduction

The cardiovascular protective effects of ALA could be explained through a variety of biological mechanisms, including platelet function, endothelial cell function (Dupasquier, 2006), anti-inflammatory effects (Zhao, 2007), antiarrhythmic effect (Ander, 2004) and lipid profile improvement (Pan, 2009). Whole grains of flaxseed are the richest source of n-3 polyunsaturated fatty acids, alpha-linolenic acid and lignans mainly, secoisolariciresinoldiglucoside.

Because circulating platelet are known to play a crucial role in the regulation of hemostasis and thrombosis and consequently in the major cardiovascular complications and because activated platelets release many pro-inflammatory mediators contributing to a chronic inflammatory state and atherosclerosis, therefore high-dose supplementation with n-3 PUFA may protect against atherosclerotic lesions by decreasing platelet reactivity. Animal testing has demonstrated that flaxseed oil reduced platelet aggregation, an important step in thrombosis but increase oxidative stress (Ramaprasad, 2005). The use of whole grain flaxseed (high contents of n-3 PUFA and lignans) seemed to significantly reduce reactive oxygen species generation (Prasad, 1997, Lee, 2008) but this effect is still under debate.

Some concerns were raised by the observation that increased supplementation with flaxseed could decrease plasma vitamin E (gamma-tocopherol) in rats (Yamashita, 2003). A diet high in cholesterol and deficient in vitamin E induced lipid peroxidation and

hypercholesterolemia in Syrian hamster and dietaryinduced hyperlipidemia could be inhibited by high vitamin E intake (Kubow, 1996). Few studies have been conducted to measure the impact of vitamin E on lipid oxidation when increased amounts of flaxseed are included in the diet (Muggli, 1994) and limited information is available to suggest the effect of vitamin E added to flaxseed diet on coronary heart disease risk.

Adding vitamin E to flaxseed enriched diet could enhance antioxidative effects of lignans and inhibit susceptibility to lipid peroxidation related to high levels of n-3 polyunsaturated fatty acid (Muggli, 1994, Poirier, 2002). To our knowledge, the impact of flaxseed and vitamin E supplementation on platelet functions in an atherosclerosis animal model has not been fully documented.

Therefore we hypothesized that vitamin E supplementation could prevent the lipid oxidation when high doses of flaxseed are included in the diet and this could amplify the antiplatelet effect of flaxseeds. To test this hypothesis, we studied the comparative effect of ground flaxseed (15 g/100 g of food) or/and alpha-tocopherol (40 mg/100 g of food) added to high-fat diet (40 % energy as fat) on platelet function in an animal model of atherosclerosis (ovariectomized hamsters fed on high-fat diet), Lucas, 2004.

I.5.2. Materials and Methods

Animals

Eighty six months-old Golden Syrian hamsters weighing 145.5 ± 26.5 g were used in the experiment. The hamsters were anaesthetized with an intraperitoneal injection of a mixture of Ketamine, doses of 100 mg/kg bodyweight and Xylazine, doses of 10 mg/kg bodyweight. Half of the hamsters (n = 40) were subjected to bilateral ovariectomy (Ovx) using the dorsolateral approach. The remaining animals (n = 40) were subjected to sham surgery, during which the ovaries were exteriorized but replaced intact.

This study was approved by the Laboratory Animal Care Committee of "Gr. T. Popa" University of Medicine and Pharmacy and the hamsters were maintained in accordance with the general guidelines for the care and use of laboratory animals recommended by the Council of European Communities.

Diets

The hamsters were kept in standard laboratory conditions with a controlled temperature (20 ± 2°C) and a 12h light/12h dark cycle. The hamsters were provided with laboratory chow 15 g food/hamster/day and tap water ad libitum.

Each of the two groups (Ovx and Sham) was randomly assigned four different diets for an 8-week period. All four diets had similar carbohydrate, total fiber, protein, and fat content (Table I.5.1).

1) high-fat diet (40 % energy as fat), deficient in ALA;

2) high-fat diet enriched with ground flaxseed 15 g/100 g of food, rich in ALA (Linum group);

3) high-fat diet enriched with vitamin E, 40 mg alpha-tocopherol/100g of food (E group);

4) high-fat diet enriched with flaxseed and vitamin E (Linum+E group).

Table I.5.1
Composition of experimental diets

Ingredients (g/100 g diet)	High-fat diet (40% of energy as fat)	High-fat diet supplemented with flaxseed	High-fat diet supplemented with vitamin E	High-fat diet supplemented with flaxseed and vitamin E
Carbohydrate	33	33	33	33
Fiber	11	11	11	11
Protein	24	24	24	24
Fat	16	16	16	16
Saturated*	9.6	9.6	9.6	9.6
Monounsaturated	0.7	0.7	0.7	0.7
Polyunsaturated	5.7	5.7	5.7	5.7
n-6 PUFA (sunflower oil)	5.7	2.3	5.7	2.3
n-3 PUFA (flaxseeds**)	0	3.4	0	3.4
Choline	0.3	0.3	0.3	0.3
Vitamin A	20,000 IU	20,000 IU	20,000 IU	20,000 IU
Vitamin C	0.1	0.1	0.1	0.1
Vitamin D	1000 IU	1000 IU	1000 IU	1000 IU
Vitamin E	0.5	0.5	4	4
Calcium	0.6	0.6	0.6	0.6
Phosphate	0.4	0.4	0.4	0.4
Potassium bicarbonate	2	2	2	2

* Hydrogenated coconut oil
** Flaxseeds (*Linum usitatissimum)* belonged to the Olin variety and were provided by the Department of Phytotechny, Faculty of Agronomy Iasi. The composition of flax seeds was: 40.2 % oil (55.6 % linolenic acid) and 19.5 % proteins

Animal necropsy and processing of samples. At the end of the experiment, the animals were sacrificed at 8 weeks after ovariectomy, by cardiac puncture under ketamine anesthesia (100 mg/kg body weight). Blood samples were collected using sodium citrate as anticoagulant buffer blood/citrate ratio of 9:1, or without anticoagulant. The anticoagulated blood was centrifuged (200 x g) for 10 min and the platelet-rich plasma (PRP) was removed and kept at room temperature for use within 4 h. Aliquots of serum were frozen and kept at -80°C for later analysis. The liver was immediately removed, rinsed with ice cold saline, weighed, placed in a sealed container and stored at −20°C until analyzed. Uterus and small intestine were collected, blotted, and weighed. The aortas were dissected, rinsed with cold saline and preserved in a phosphate buffer (pH 7.2).

Platelet functions: aggregation and adhesion

Platelet aggregation assay was performed according to Bednar's method (1995). In brief, 100 mL of PRP was added at 25°C to individual wells of a microtiter plate containing 10 µL of ADP (100 µM). The change in light absorbance in each well (decrease optical density (OD)/min at 630 nm) was subsequently measured by use of a kinetic microplate reader Tecan Sunrise, Switzerland.

Platelet adhesion was assessed according to Bellavite's method (1994). In short, the 96-well microtiter plates were coated (overnight at 4°C) by adding 50 µl/ well of a fibrinogen solution (50µg/ml). After cleansing, the non-specific adhesion was blocked by incubation of wells with 1% BSA for 1h at 37°C. At the end of incubation the plates were washed again. Platelets (50 µl PRP/well) were stimulated with ADP (50 µM), and allowed to adhere to the wells for 30 min at 37°C. Thereafter, plates were carefully washed twice with 200 µl/well of Krebs solution in order to remove unattached platelets. Adherent platelets were quantified by

measuring acid phosphatase activity. The p-nitrophenol produced by the reaction with acid phosphatase substrate solution was measured with the microplate reader at 405 nm. The percentage of adherent cells was calculated on the basis of a standard curve.

Parameters of lipid profile

Serum total cholesterol, HDL-cholesterol and triglycerides were measured by enzymatic colorimetric methods on a TECAN microplate reader by commercially available kits (Audit Diagnostics Ireland). Non-HDL cholesterol was calculated by subtracting HDL cholesterol from total cholesterol.

Parameters of oxidative stress

Serum and liver thiobarbituric acid reactive substances (TBARS) are an index of lipid peroxidation and oxidative stress. TBARS were determined by an adapted method from Phelps, (1993). The quantity of the TBARS was measured using a TECAN microplate reader at a wavelength of 540 nm. Liver reduced glutathione was determined by an enzymatic reaction, based on the oxidation of reduced glutathione by 5.5'- ditiobis (acid 2-nitrobenzoic) in the presence of glutathione reductase and $NADPH_2$, monitored at a wavelength of 405 nm (Tietze, 1969). Liver superoxide dismutase was determined by an adapted method from Minami and Yoshikawa (1979).

Determination of cholesterol contents in aorta

The aortas of the hamsters in each group were removed. A section of the aorta arch of each animal was soaked in a 10 % (v/v) formal saline solution for hematoxylin-eosine and orcein staining and another section was stored on ice before being stained and fixed. The remaining aorta was soaked in PBS and homogenized for biochemical analysis. The lipid content was extracted by treatment with chloroform and methanol followed by centrifugation (Folch, 1957). The extracted lipid was dissolved in ethanol and measured via a cholesterol assay kit Audit Diagnostics Ireland utilizing absorbance spectrophotometry, and the amount quantified as μg/mg wet tissue.

Morphological study of aorta

For light microscopy evaluation of aortic atherosclerotic lesions we used ice and paraffin sections, with oil red, orcein and HE staining.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical significance was determined by variance analysis and one-way ANOVA followed by a post hoc Tukey's test using Statistical Software Package SPSS®, version 13 (SPSS Incorporation, Chicago, IL, USA). Unpaired Student's t-tests were performed to determine whether there were significant ($p< 0.05$) differences between groups.

I.5.3 Results

Body and uterus weight

After 8 weeks of high-fat diet (animals receiving 15 g of food/day), the final weights increased similarly with no differences in mean final body weights in all groups. As expected, the relative mean uterine weight (g/100 g body weight) of the ovariectomized group was significantly ($p < 0.05$) lower than the sham operated animals, confirming the effect of ovariectomy (Table I.5.2).

Table I.5.2
Mean ± SD values for body and uterus weights in studied groups

Measures	Sham	Sham +Linum	Sham +E	Sham +Linum+E	Ovx	Ovx +Linum	Ovx +E	Ovx +Linum+E
Body weight (g)								
Initial	164±28	168±14	161±18	162±14	173±11	174±12	168±16	171±15
Final	177±14§	178±17	168±6	169±8	181±9	187±16§	177±16	182±15§
Uterus weight (g)	100±6	83±6	78±14	62±9	24±6*	25±3*	19±2*	17±3*

Values are means ± S.D., $n = 10$ in each group
* $p < 0.05$ as compared to sham groups
§ $p < 0.05$ as compared to initial data

Platelet function

In Sham groups, the addition of flaxseed or combined diet (flaxseed and vitamin E) slightly decreased platelet aggregation as compared to unsupplemented high fat diet animals (Figure 30). In Ovx hamsters, the supplementation with flaxseed slightly increased platelet aggregation. In Ovx groups, the adhesion was significantly decreased by the addition of flaxseed, vitamin E or combined diet as compared to non-supplemented groups in ovariectomized hamsters (Figure I.5.1).

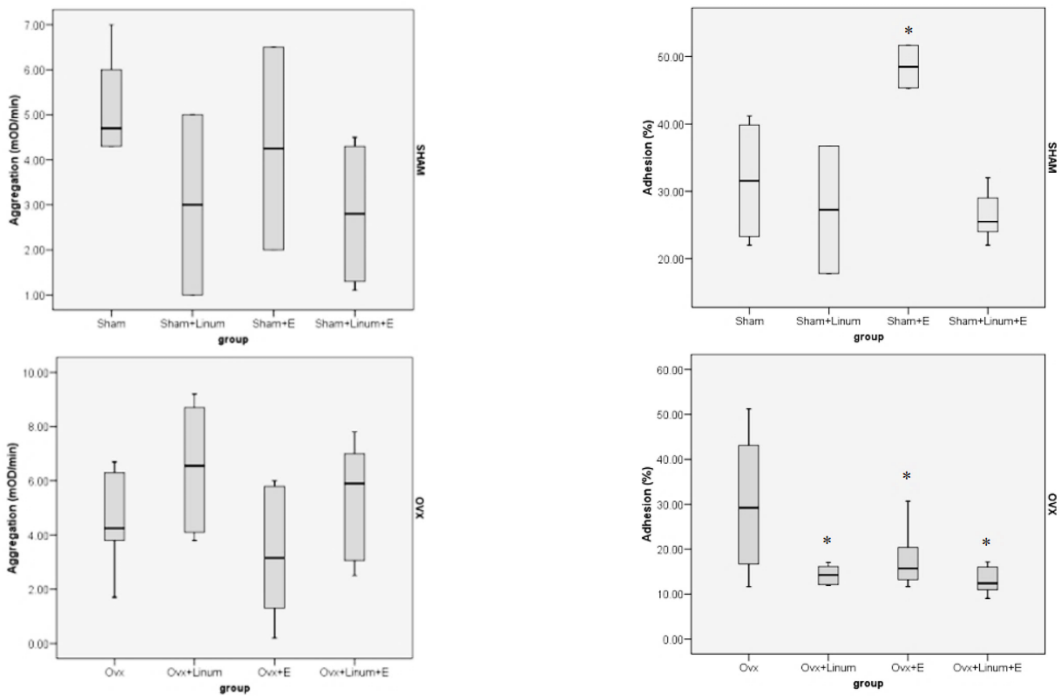


Figure I.5.1
Platelet aggregation and platelet adhesion in sham and ovariectomized hamsters fed with high-fat diet. Results are median, maximum and minimum values. * $p < 0.05$

Parameters of oxidative stress

Our main findings are summarized in Table I.5.3. In Ovx groups, serum and liver TBARS were significantly decreased by the addition of flaxseed or vitamin E as compared to unsupplemented groups. Combined diet (flaxseed and vitamin E) further lowered levels of serum and liver TBARS.

Table I.5.3
Mean ± SD values for oxidative stress parameters in studied groups

Measures	Sham	Sham +Linum	Sham +E	Sham +Linum+E	Ovx	Ovx +Linum	Ovx +E	Ovx +Linum+E
Serum TBARS (nmol/ml)	6.9±0.6	4.7±0.6*	8.9±2.1*	12.7±1.1*	2.2±2.5§	6.8±2.0§*	8.9±1.1*	6.5±3.1§*
Liver TBARS (nmol/mg protein)	13.4±1.0	8.8±2.1*	13.1±2.1*	15.2±3.7*	14.2±2.8	8.4±2.9*	9.2±3.5*	7.9.8±2.7*
Liver SOD (%/mg protein)	1.9±0.1	0.6±0.4*	1.6±0.1*	1.4±0.1*	1.3±0.3§	1.0±0.3§	1.5±0.4	2.1±0.2§*
Liver GSH (µmol/mg protein)	16.6±0.1	29.4±0.1*	14.6±1.8	8.6±2.5*	18.2±9	27±5.2	31±10§*	55.5±3.6§*

Values are means±S.D., n = 10 in each group
§ p< 0.05 as compared to corresponding Sham groups
*p< 0.05 as compared to groups fed with unsupplemented high-fat diet

Parameters of lipid profile

Ovariectomy increased serum levels of total cholesterol, non-HDL-cholesterol and triglycerides as compared to Sham groups. The supplementation of diet with flaxseed significantly decreased serum triglycerides and non-significantly decreased total cholesterol and non-HDL-cholesterol in ovariectomized hamsters (Table XI). Total cholesterol contents in the aorta Aortic cholesterol contents were lower in the Sham group as compared with the Ovx one. Aortic cholesterol contents were almost the same for both the Sham and the Ovx groups (Table I.5.4).

Table I.5.4
Mean ± SD values for serum lipid profile and aorta cholesterol in studied groups

Measures	Sham	Sham +Linum	Sham +E	Sham +Linum+E	Ovx	Ovx +Linum	Ovx +E	Ovx +Linum+E
Serum								
Total cholesterol (mg/dl)	16±9	119±17	123±11	127±16	131±21§	119±40	140±21§	142±16
Triglycerides (mg/dl)	149±56	94±35*	184±70	91±37*	196±79§	123±50*§	168±82	171±38§
HDL-cholesterol (mg/dl)	44±8	34±3*	35±2*	42±14	42±11	42±17§	49±13§	41±16
Non-HDL chol (mg/dl)[1]	72±9	86±15*	89±9*	86±14*	89±17§	71±38	92±12	101±12§
Aorta								
Total cholesterol) (µg/mg wet tissue	3.8±1.4	3.6±1.8	3.9±0.6	3.5±1.9	4.5±1.7§	4.0±1.9	3.9±0.9	4.1±1.5

Values are means±S.D., n = 10 in each group
§ p< 0.05 as compared to corresponding Sham groups
*p< 0.05 as compared to groups fed with unsupplemented high-fat diet

Morphological alterations in aortic tissues

Sham groups. The percentages of hamsters with early atherosclerosis or without vascular lesions (normal) are represented in Figure I.5.2.

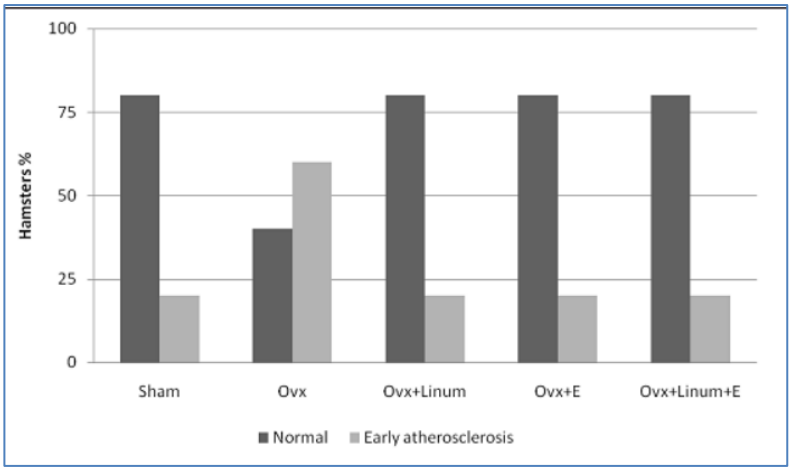


Figure I.5.2
Effects of ovariectomy (Ovx), flaxseed,vitamin E and combined diet (Linum+vitamin E) on the progression of atherosclerotic lesions in hamsters. Bars represent the distribution of hamsters (%) with incipient atherosclerotic lesions (early atherosclerosis) or without vascular lesions (normal)

Within the Sham group fed with high fat diet, histological evaluation of the aorta arch revealed early atherosclerosis. In the H-E staining, rare endothelial discontinuities and moderate thickening of the aortic intima were noticed; in the O-Rd staining, lipid droplets adherent to the endothelium were observed (Figure I.5.3).

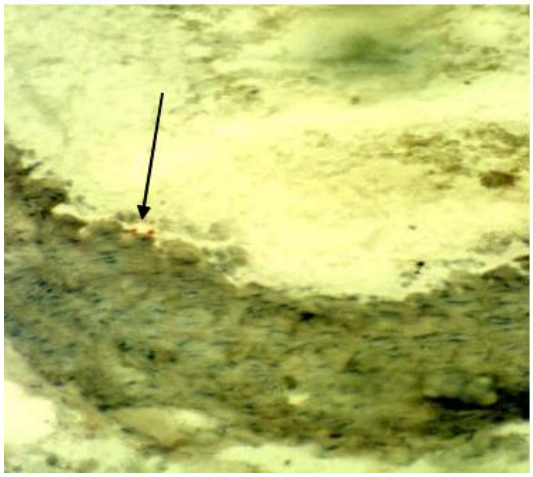


Figure I.5.3
Ovariectomized hamsters fed with high fat diet
Aorta – control (Sham) with high-fat diet (oil-red staining x 2). Moderate thickening of the aortic intima. Lipid droplet adherent to the endothelium arrow)

There were more hamsters in the Ovx group with atherosclerotic lesions compared to the sham and flaxseed groups. Examination of the aorta under a light microscope revealed signs of incipient atherosclerosis. Endothelitis, red blood cell and platelet adhesiveness and leukocyte margination were identified, together with endothelial disruptions, moderate intimal thickening and macrophage intimal infiltration as well as intimal lipid microvesicles (Figure I.5.4). Irregular elastic lamina and smooth muscle cells and lipid-like vacuoles were increased in media tunica (Figure I.5.5).

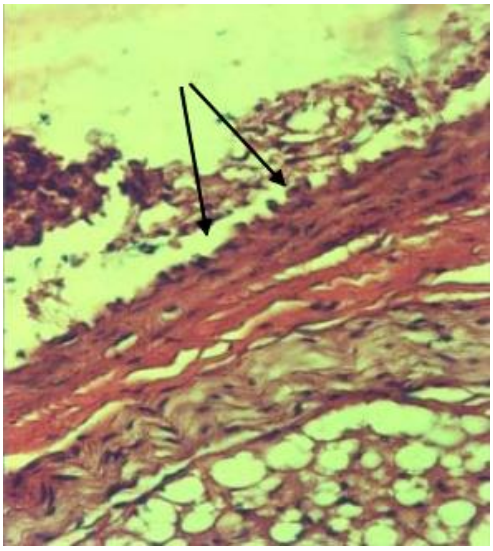


Figure I.5.4
Aorta –OVX with high-fat diet (H-E x 40)
Endothelitis. Platelet adhesiveness.
Leukocyte margination.

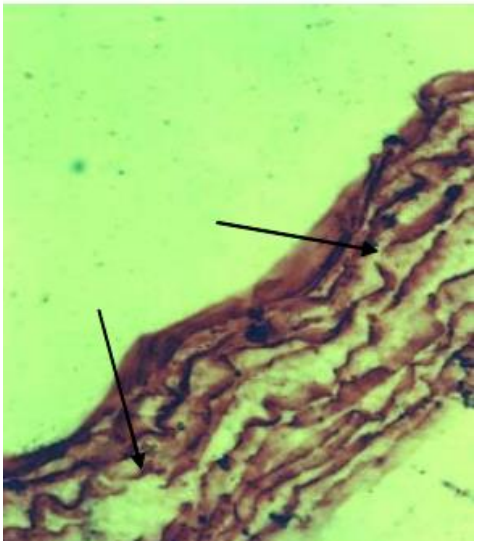


Figure I.5.5
Aorta – OVX with high-fat diet (Orcein stain x 40) Reduced intimal elastic tissue.
Disruption and fragmentation of medial elastic lamellae (arrows).

Ovariectomized hamsters fed with Linum/Vitamin E enriched diet

The percentage of hamsters with atherosclerotic lesions was reduced by 40 % in groups that received supplementation with flaxseed or/and vitamin E as compared to unsupplemented group. Comparison of the light microscopic view with the ovariectomized group fed high-fat diet, the ovariectomized groups fed with Linum/Vitamin E enriched diet showed reduced red blood cell and platelet adhesiveness and a lower extent of degeneration in tunica intima, with moderate intimal thickening, rare macrophages in the subintimal area and reduced intimal fatty streaks (Figure 34). There were also minor changes of elastic laminae in the media (Figure I.5.6).

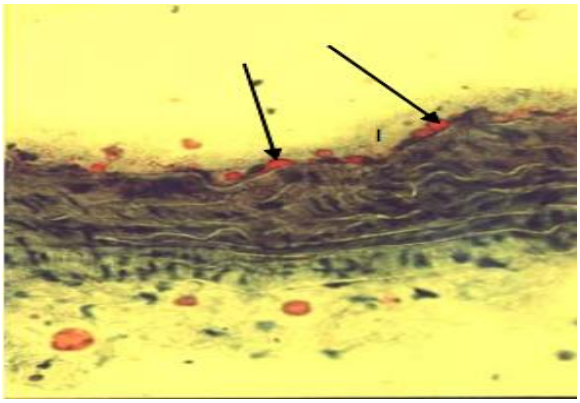


Figure I.5.6
Aorta – OVX with Linum enriched diet (oil-red staining x 40)
Intimal lipid droplets attached to the internal elastic lamina (arrows)

I.5.4. Discussion

In our research the mechanism for the antiplatelet action of flaxseed enriched diet (15 g *Linum usitatissimum*/100 g of food) was investigated in an animal model that replicates a

closer representation of the human atherosclerotic condition. Golden Syrian hamsters seemed more prone to develop atherosclerotic lesions than rat (Alexaki, 2004).

Our research confirmed previous studies (Lucas, 2004) upholding that ovariectomy increased serum total and non-HDL cholesterol concentrations and led to incipient atherosclerotic lesions in Golden Syrian hamsters fed on high-fat diet. In the present study, the hamsters had minimal aortic lesion surface area. Lesions were characterized by increased endothelial permeability to lipids and other plasma components. Intimal lipid micro vesicles have been noticed on our slides, but equally red blood cell and platelet adhesiveness and leukocyte binding to the endothelium suggesting atherosclerosic lesions in ovariectomized hamsters. Moreover, high saturated fat feeding resulted in more aortic cholesterol accumulation in Ovx hamsters than in Sham ones.

Recent findings emphasize the fact that estrogen atheroprotection is vitally connected to the state of the arterial endothelium and its subsequent NO production, which is stimulated by estrogens (Kevil, 1999). The NO production would be involved in the mechanisms by which estrogens inhibit atherosclerosis independently of plasma lipid changes.

The decreased e-NOS activity (Kitahara, 2010) led to atherosclerosis and its complications, causing vasoconstriction and enabling platelet aggregation, enhanced leukocyte adhesion and invasion to the endothelium (Kevil, 1999). Since n-3 PUFA increased NO production and endothelial NOS expression (Casos, 2010), the diet enriched with n-3 PUFA may protect against atherosclerosis induced by estrogen deficiency.

In our study, a high amount of ground flaxseed was incorporated into the diet in order to ensure significant increase in ALA to the body, to reduce hypercholesterolemia (Pellizzon, 2010), to inhibit platelet reactiveness (Allman, 1995) and to prevent the progression of atherosclerotic lesions in ovarian hormone deficiency (Dupasquier, 2007; Lucas, 2004; Winnik, 2011). Among n-3 PUFA, DHA contributes to the inhibition of platelet functions when it is incorporated into membrane phospholipids, acting as a potent inhibitor of TxA2 - induced aggregation (Lagarde, 2003).

Moreover, higher cellular levels of DHA support a higher degree of platelet membrane fluidity in the presence of a membrane rigidified such as cholesterol (Hashimoto, 2006). ALA enriched sources could have similar effects to DHA-enriched sources on platelet activity but high doses are required since the average efficiency of conversion of ALA to total EPA and DHA is less than 0.5 %, and that of ALA to DHA alone is less than 0.1 % 21. In humans, high doses of 40-50 g flaxseed/day, which corresponds to about 10 % of total energy intake, have been used in the trials (Cunnane, 1993, Dodin, 2008). In hamsters, a 15 % flaxseed supplemented diet is similar in energetic load to the 40 g/day dosage used in human trials. The addition of 15 % flaxseed to diets had similar effects to estrogen injections in decreasing total cholesterol in ovariectomized hamsters (Lucas, 2004). A side effect of using high concentrations of n-3 fatty acids is the increased lipid peroxidation both *in vivo* (Polette, 1996, Allard, 1997) and in vitro (Liu, 2003, Polette, 1996). Since only high flaxseed dose (Lucas, 2004, Dupasquier, 2007) led to the enrichment of platelet membrane phospholipids with large amounts of EPA and DHA, in our study we have added vitamin E (alpha-tocopherol) to high flaxseed diet (15 g/100 g of food) to prevent lipid peroxidation and vitamin E decrease associated with high concentrations of n-3 PUFA (Lemaitre, 1997, Wiesenfeld, 2003, 1998).

Similar to what others have reported (Lucas, 2004), the ovariectomized hamsters have elevated total cholesterol and non-HDL cholesterol levels relative to sham operated control in females. The addition of 15 % flaxseed to diets reduced plasma total cholesterol levels of ovariectomized female hamsters back to those of sham operated animals.

The hypocholesterolemic effect of flaxseed could be attributed to flaxseed gum 23, to ALA (Lucas, 2004; Bierenbaum, 1993) or to the lignan precursor present in flaxseed-SDG

(Prasad, 2005). There were no significant differences in plasma non-HDL-cholesterol among the ovariectomized groups. By contrast, the sham female hamsters fed the flaxseed-enriched diets had higher concentrations of non-HDL cholesterol than those fed control diet. Some previous studies have reported hyperlipidaemic effect of fish oil in Golden Syrian hamsters, which was more obvious in hamsters fed a fish-oil diet supplemented with cholesterol (Surette, 1992; Kubow, 2003; de Silva, 2004)

The hyperlipidaemic effect of fish oil was dependent on the level of dietary cholesterol and n-3 PUFA content (Surette, 1992). It is possible that female hamsters are susceptible to n-3 PUFA induced hyperlipidaemia, especially at high fat levels, and this increase is partially explained by the inhibition of hepatic LDL-receptor mRNA expression (de Silva, 2004).

Previous similar studies (Arjmandi, 1998) found flaxseed supplementation to have an anti-atherogenous effect in ovariectomized hamsters independently of lipid changes. In our study as well, the atherosclerotic lesions were in a less advanced stage in ovariectomized hamsters fed with ground flaxseed and the flaxseed effect was not proportional with the cholesterol-lowering result. Lucas (2004) demonstrated a flaxseed dose diminishing effect on atherosclerotic lesions without dose lowering effect on serum cholesterol. In rabbits, Prasad et al. (1997, 2000) showed that the formation of atherosclerotic lesions was decreased without noticeable cholesterol lowering effects. These results suggested that the cardioprotective property of flaxseed could be due to its hypocholesterolemic effect but also to other potential mechanisms such as being antioxidative, anti-inflammatory and/or antithrombotic.

Flaxseed reduced plasma triglycerides and this effect was consistent with previous studies using high dose of flaxseed during prolonged hypercholesterolemia in mice (Pellizzon, 2007) and rabbits (Dupasquier, 2006). In hamsters, Morise (2005) proved that the ALA substitution for oleic acid in the diet reaching 10% of total energy intake decreased triglyceridemia. The mechanisms involved in the decreased triglyceridemia could rely on gene regulation resulting in a decreased activity and expression of key lipogenic enzymes and sometimes on a rise in mitochondrial oxidation (Morise, 2005). Therefore, it is possible that the discrepancies between studies on TG concentration could be explained by the diversity of fatty acids which were substituted for by ALA. Also, there are differences in the dose and type of flaxseed as well as use of different animal models (Prasad, 1998, Babu, 2000).

The high dose of flaxseed used in our study slightly decreased ADP-induced platelet aggregation in sham operated animals suggesting that 15 g ground flaxseed/100 g of food provided the necessary levels of EPA and DHA to inhibit platelet aggregation. Similar to other studies (Jayachandran, 2003) the ovariectomy increased the platelet aggregation in our experiment as well, possibly by changes in the platelet phenotype related to the low estradiol status (Jayachandran, 2003, Aldrighi, 2005, Naimushin, 2003). Our aggregation studies were able to show only a non-significantly increased platelet aggregation in Ovx hamsters fed with flaxseed diet. Other studies have also reported minor changes in ADP aggregation after flaxseed supplementation (Allman, 1995).

In contrast to the aggregation studies, platelet adhesion was found to be significantly affected by the administration of flaxseed in our study. In estrogen deficiency states, the depression of platelet eNOS could contribute to platelet adhesion/activation and expression of the adhesive glycoprotein P-selectin. The EPA and DHA content of platelet phospholipids is increased by flaxseed diet (Mantzioris, 1995) and these results in increased e-NOS (Casos, 2010), decrease of P-selectin expression (Nomura, 2003) and a reduction in the number of pseudopodia (Li, X.L. and Steiner, 1990). This could explain the flaxseed's effect of reducing platelet adhesion in Ovx hamsters in our studies.

Our results are similar to those of previous studies that proved decrease platelet adhesion following fish oil supplementation in animals (Wines, 1989) and humans (Li and

Steiner, 1990). In our experiment, the effects of high dose of flaxseed on platelet activation could contribute to diminishing endothelial lesions in ovariectomized hamsters (the percentage of hamsters with atherosclerotic lesions was reduced by 40% compared to unsupplemented group). However, the aortic cholesterol content was unaffected by diet.

Although the addition of ground flaxseed has been demonstrated to have an antioxidative effect (Lee, 2008, Prasad, 2000) high doses of flaxseed required for protection against platelet adhesiveness and vascular diseases may lead to n-3 PUFA accumulation in membranes and increase lipid peroxidation (Reaven, 1992). Moreover, the 40% fat diet rich in polyunsaturated fatty acids was associated with a fall in plasma vitamin E in the female rat (Wiesenfeld, 2003).

In our study the supplementation of diet with high doses of ground flaxseed was beneficial in improving platelet functions in both Sham and Ovx animals without an increase of oxidative stress. We noticed an antioxidant effect of 15 g ground flaxseed/100 g of food revealed by a significant decrease of serum and liver TBARS and significant increase of liver GSH by the addition of flaxseed as compared to unsupplemented groups. This effect could be explained by the lignan content of flaxseed. SDG isolated from flaxseed has oxygen radical scavenging properties demonstrated in vitro by direct hydroxyl radical scavenging activity (Prasad, 2000) or by inhibition of lipid peroxidation (Kitts, 1999).

The effect of vitamin E against the pro-oxidative status associated with estrogen deficiency in Ovx hamsters was demonstrated in our study by the increased liver GSH and SOD together with decreased liver TBARS. The antioxidant effect of vitamin E was similar to that achieved by whole grain flaxseed supplementation. Moreover, the combined supplementation of vitamin E and flaxseed in ovariectomized animals non-significantly augmented the effect of flaxseed on platelet adhesion and the protection against oxidative stress in Ovx animals.

The additional effect on platelet activity brought by vitamin E supplementation could be explained by its powerful antioxidant activity which could reduce oxidative changes of plasma lipoproteins and therefore decrease platelet adhesion to oxidized LDLs (Szuwart, 2000). In platelets, vitamin E led to a decrease in lipid peroxidation and TxA2 synthesis (Chan, 1998), to an increase in NO release, eNOS activation, SOD protein content, and to a decrease in PKC activation, which may contribute to the effect on platelet activation (Liu, 2003). Non-antioxidant effects of vitamin E by modulation of gene expression of collagen α1 and glycoprotein IIbIIIa (Munteanu, 2004) could also contribute to decreased platelet activation.

Vitamin E-enriched platelets that adhered to adhesive surfaces failed to show the usual long thin pseudopodia which may explain the vitamin E-induced reduction of platelet adhesiveness (Steiner, 1991). Previous studies have shown the supplementation of vitamin E to be associated with a reduction (Kubow, 1996) or with no effect (Poirier, 2002) on plasma total cholesterol in the presence of fish oil feeding in hamsters.

In the present work, the vitamin E supplementation alone or combined to flaxseed had deleterious effects on lipid profile by increasing the total cholesterol, non-HDL cholesterol and triglycerides in ovariectomized hamsters. The hypercholesterolemic effect of alpha-tocopherol is dose-dependent and the high dose of vitamin E used in this study could exhibit a stimulatory effect on HMG CoA reductase and cholesterol 7α-hydroxylase (Khor, 2000). Similar effects of high doses of vitamin E on lipid profile have been observed by other authors, changes of non-HDL and HDL cholesterol fractions being explained by tocopherol-mediated enhancement oxidation.

The antioxidative and antithrombotic effect brought by additional supplementation of vitamin E was not significant in our experiment suggesting that the vitamin E content of high

dose of flaxseed (flaxseed contains 20 mg γ-tocopherol/100 g) could efficiently act on oxidative stress and platelet functions.

Taken together, our results suggested that a high dose of ground flaxseed (*Linum usitatissimum*) did not increase the oxidative stress and may have beneficial effects on platelet adherence and atherosclerosis progression in ovariectomized hamster fed with high fat diet. The vitamin E supplementation had similar effects to flaxseed in ovariectomized animals and the combined diet (Linum + vitamin E) did not seem to bring more benefits than flaxseed alone. Also, the high dose of ground flaxseed alone may have a cardioprotective effect by reducing hypercholesterolemia.

I.5.5. Conclusion

In conclusion health benefits related to flaxseed consumption may not be offset by increased oxidative stress. The use of whole grain flaxseed (high contents of n-3 polyunsaturated fatty acids and lignans) seems to considerably reduce platelet thrombotic functions and reactive oxygen species generation. High amount of ground flaxseed incorporated into diet is required to ensure significant increase in ALA into the body, to reduce hypercholesterolemia and to prevent the progression of atherosclerotic lesions in estrogen deficiency states. The flaxseed enriched diet (cereals) could regulate thrombosis and could consequently play a role in preventing the major cardiovascular complications in menopausal women.

I.6. Targeting the inflammatory chain of atherosclerosis using liposomes

I.6.1. Using liposomes to target the subendothelial space in rat aorta

I.6.1.1.Introduction

The goal of the actual study was represented by the construction of one type of liposomes able to target specifically the subendothelial space in rat aorta. This might represent the first step in targeting specifically the macrophages representing the turnover in the physiopathology of atherosclerosis. To emphasize the vascular tissues capture of liposomes we used rat aortic rings model, subjected to all normal stages encountered in experiments of organ bath contractility. MLV and SUV liposomes loaded with R-phycoerithrin were added to the organ bath in a ratio of 1/9 v/v for 15 minutes. R-phycoerithrin encapsulated in SUV liposomes was released only in the endothelium of isolated rat aorta. In contrast, R-phycoerithrin encapsulated in MLV liposomes was released in the aortic smooth muscle layer of isolated rat aorta. Thus, we might conclude that liposomes with a single layer of lipid or a small number of lipid layers, through merge, would release their contents only in the first row (or first rows) of impact cells. The presence of many vesicular lipid layers in the wall will allow liposomes to "cross" several rows of cells and to release their content in tissue depth. Thus, multi lamellar liposomes are very reliable and useful instruments for administration of various substances in the depth of tissues and to target the macrophages from sub-endothelial space.

Atherosclerosis is an inflammatory disease characterized by the progressive accumulation of lipids in the vessel wall. The first step is the internalization of lipids (LDL) in the intima with endothelial activation which enhances the permeability of the endothelial layer and the expression of cytokines/chemokine and adhesion molecules. These events increase LDL particles accumulation in the extracellular matrix where they aggregate/fuse, are retained by proteoglycans and become targets for oxidative and enzymatic modifications. In turn, LDLs enhance selective leukocyte recruitment and attachment to the endothelial layer inducing their transmigration across the endothelium into the intima. While smooth muscle cell numbers decline with the severity of plaque progression, monocytes differentiate into macrophages, a process associated with the upregulation of pattern recognition receptors

including scavenger receptors and Toll-like receptors leading to foam cell formation. Foam cells release growth factors, cytokines, metalloproteinases and reactive oxygen species all of which perpetuate and amplify the vascular remodeling process. In addition, macrophages release tissue factor that, upon plaque rupture, contributes to thrombus formation. Smooth muscle cells exposed in eroded lesions are also able to internalize LDL through LRP-1 receptors acquiring a pro-thrombotic phenotype and releasing tissue factor. Platelets recognise ligands in the ruptured or eroded atherosclerotic plaque, initiate platelet activation and aggregation leading to thrombosis and to the clinical manifestation of the atherothrombotic disease. Additionally, platelets contribute to the local inflammatory response and may also participate in progenitor cell recruitment (Badimon et al., 2011).

Experimental models of atherosclerosis suggest that recruitment of monocytes into plaques drives the progression of this chronic inflammatory condition. Cholesterol-lowering therapy leads to plaque stabilization or regression in human atherosclerosis, characterized by reduced macrophage content, but the mechanisms that underlie this reduction are incompletely understood. Mice lacking the gene Apoe (Apoe-/- mice) have high levels of cholesterol and spontaneously develop atherosclerotic lesions. Here, we treated Apoe-/- mice with apoE-encoding adenoviral vectors that induce plaque regression, and investigated whether macrophage removal from plaques during this regression resulted from quantitative alterations in the ability of monocytes to either enter or exit plaques. Within 2 days after apoE complementation, plasma cholesterol was normalized to wild-type levels, and HDL levels were increased 4-fold. Oil red O staining and quantitative mass spectroscopy revealed that esterified cholesterol content was markedly reduced. Plaque macrophage content decreased gradually and was 72% lower than baseline 4 weeks after apoE complementation. Importantly, this reduction in macrophages did not involve migratory egress from plaques or CCR7, a mediator of leukocyte emigration. Instead, marked suppression of monocyte recruitment coupled with a stable rate of apoptosis accounted for loss of plaque macrophages.

These data suggest that therapies to inhibit monocyte recruitment to plaques may constitute a more viable strategy to reduce plaque macrophage burden than attempts to promote migratory egress (Potteaux et al., 2011). As the role of monocytes and macrophages in a range of diseases is better understood, strategies to target these cell types are of growing importance both scientifically and therapeutically. As particulate carriers, liposomes naturally target cells of the mononuclear phagocytic system (MPS), particularly macrophages. Loading drugs into liposomes can therefore offer an efficient means of drug targeting to MPS cells. Physicochemical properties including size, charge and lipid composition can have a very significant effect on the efficiency with which liposomes target MPS cells. MPS cells express a range of receptors including scavenger receptors, integrins, mannose receptors and Fcreceptors that can be targeted by the addition of ligands to liposome surfaces. These ligands include peptides, antibodies and lectins and have the advantages of increasing target specificity and avoiding the need for cationic lipids to trigger intracellular delivery. The goal for targeting mono-cytes/macrophages using liposomes includes not only drug delivery but also potentially a role in cell ablation and cell activation for the treatment of conditions including cancer, atherosclerosis, HIV, and chronic inflammation (Kelly et al., 2011). The goal of the actual study was represented by the construction of one type of liposomes able to target specifically the subendothelial space in rat aorta. This might represent the first step in targeting specifically the macrophages representing the turnover in the physiopathology of atherosclerosis.

I.6.1.2. Material and methods

For the experiments we used 48 Wistar adult male rats (Băneasa source), weighing 150-200 g, kept under normal and the same laboratory conditions. They were decapitated and exsanguinated after anesthesia. Thoracic aorta was quickly removed and cut into rings 2 mm

long and mounted in a 2 ml organ bath. Mechanical activity was recorded using an isometric force transducer and recorders (type Radelkis, Budapest, or Carl Zeiss) or a data acquisition card PC-LPM-16 Multifunction I/O and associated software, NiDaqWin v.4.8. (National Instruments Inc.). 2 ml organ bath contained Krebs-Henseleit solution (pH=7.4) with the following composition (in mM): NaCl 118, KCl 4.8, CaCl2 2.5, MgSO4 1.6, KH2PO4 1.2, NaHCO3 25, glucose 5.5. Serum containing 40 mM K+ had the same composition, but Na+ was replaced with equimolar K^+. Krebs-Henseleit serum was kept at 37°C and continuously aerated with 95% O2 and 5% CO2 (Carbogen, Linde Romania). The initial tension was set to 2 g and preparations were left to equilibrate in the organ bath 2 hours before starting the experiments.

All experiments were performed on rings capable of reproduction of 40 mM K+ contractions without changes greater than 10% after stabilization. Liposomes used were prepared from egg phosphatidylcholine (type X-E, 12.5 mg lipid per ml of built, Sigma-Aldrich, St. Louis, USA), with slight modification of the method described by Kirby and Gregoriadis (1993). Thus, the chloroform in which was solved egg phosphatidylcholine was removed with a negative pressure rotavaporizator, obtaining a thin film on the walls of a flask of 50 ml. After removing the last visible traces of chloroform rotavaporization was continued for 15 minutes; this stage was followed by drying under nitrogen stream an additional 15 minutes. Then, there were added a few glass beads of 0.5 mm. After adding the desired volume of solution, the flask was sealed with a rubber stopper secured with Parafilm and then shaken vigorously with a Vortex. Blank liposomes included only 25 mM NaCl solution (pH=7.2 adjusted with NaOH if necessary).

Other series of liposomes have included 1 μM R-phyco-erythrin, solved in 25 mM NaCl (pH=7.2 adjusted with NaOH if necessary) (MLV liposomes). To obtain SUV liposomes loaded with Rphycoerythrin, MLV suspensions were subjected to short periods and consecutive sonications (30 sec), to clarify the solutions. To remove the unincorporated R-phycoerythrin, liposomes were undergoing dialysis in 25 mM NaCl solution (pH 7.2, for 120 minutes, volume 1/1000, the buffer being changed every 30 minutes). After stable contractions induced by K+ 40 mM, 200 μl liposomes (both MLV and SUV) containing R-phycoerythrin (1 μM in aqueous solution) were administered at a rate of 1/9 v/v in organ bath for 15 minutes. Then, the aorta was fixed in 4% paraformaldehyde (freshly prepared, solved in 0.1 M phosphate buffer, pH = 7.4 at 80°C) for 60 minutes and then cut using cryostat in sections of less than 50 μm thick. There followed three washes with PBS. Aortic slices were then transferred to glass plates with thickness of less than 0.17 mm, left 10 minutes to dry slightly and then mounted on glass slides using a solution based on glycerol and DABCO. Fixing the plates to slides was made with colorless nail polish.

Analysis of preparations was possible after a minimum of 24 hours of drying. Image acquisition was performed using a Nikon Eclipse TE-300 inverted microscope equipped with an oil immersion objective with a magnification of x60 and epifluorescence. Collection of fluorescence (emission) of R-phycoerythrin required the use of a filter 570LP that allows capturing its optimal peak emission at 578 nm. We aimed the fluorescence in the red spectrum to avoid interference with the green one of elastic fibers in rat aorta (autofluorescence). Final processing of images, i.e. their merge and deconvolution were performed using ImageJ software for Windows. Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996 and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iaşi.

I.6.1.3. Results and discussion

To emphasize the vascular tissues capture of liposomes we used rat aortic rings model, subjected to all normal stages encountered in experiments of organ bath contractility. MLV

and SUV liposomes loaded with R-phycoerithrin were added to the organ bath in a ratio of 1/9 v/v for 15 minutes. As can be seen from Figure I.6.1, R- phycoerithrin encapsulated in SUV liposomes is released only in the endothelium of isolated rat aorta.

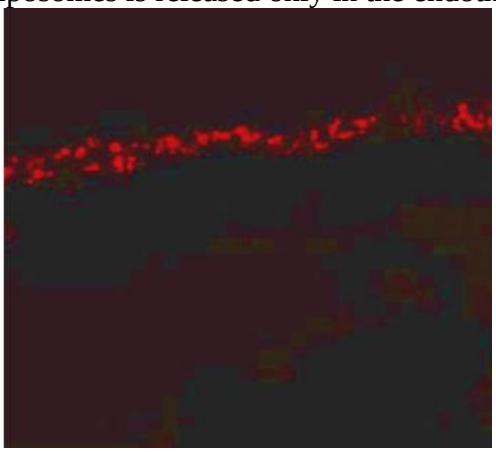


Figure I.6.1
R-phycoerithrin encapsulated in SUV liposomes is released only in the endothelium of isolated rat aorta (red fluorescence)

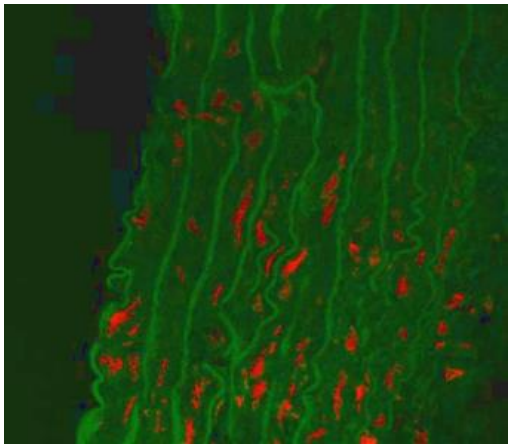


Figure I.6.2
R-phycoerithrin encapsulated in MLV liposomes is released in the rat aortic smooth muscle (red fluorescence).

In contrast, R-phycoerithrin encapsulated in MLV liposomes is released in the aortic smooth muscle layer of isolated rat aorta (Figure I.6.2). Thus, we might conclude that liposomes with a single layer of lipid or a small number of lipid layers, through merger, would release their contents only in the first row (or first rows) of impact cells. The presence of many vesicular lipid layers in the wall will allow liposomes to "cross" several rows of cells and to release their content in tissue depth. Thus, multilamellar liposomes are very reliable and useful instruments for administration of various substances in the depth of tissues and to target the macrophages from subendothelial space.

Macrophages play a key role in the initiation, progression and complications of atherosclerosis. In this article we describe the synthesis of biocompatible, paramagnetic, fluorescent phosphatidylserine vesicles containing cholesterol ester with a free carboxylic acid function and its use for targeted imaging of macrophages. There were synthesized anionic vesicles containing a combination of phosphatidylserine and a novel synthetic oxidized cholesterol ester derivative (cholesterol-9- carboxynonanoate (9-CCN)). In vitro studies to characterize particle size, MRI relaxation times and stability were performed. Vesicles containing 9-CCN demonstrated enhanced ability to bind human low-density lipoprotein and to be internalized by macrophages.

Experiments in cultured macrophages with 9-CCN vesicles, alone and in the presence of lowdensity lipoprotein, indicated uptake of vesicles through scavenger receptor and integrin-dependent pathways. In vivo MRI using 9-CCN vesicles containing gadolinium in a rabbit model of atherosclerosis revealed protracted enhancement of 9-CCN vesicles and colocalization with arterial macrophages not seen with control vesicles. Pharmacokinetic experiments demonstrated prolonged plasma residence time of 9-CCN vesicles, perhaps due to its capacity to bind to low-density lipoprotein. Vesicles containing 9-CCN demonstrate prolonged plasma and plaque retention in experimental atherosclerosis. Such a strategy may represent a simple yet clinically relevant approach for macrophage imaging (Maiseyeu et al., 2010). To confirm the efficacy of dexamethasone incorporated into liposomes in the treatment of atherosclerosis, the uptake of dexamethasone-liposomes by macrophages and foam cells and its inhibitory effect on cellular cholesterol ester accumulation in these cells were investigated in vitro. Dexamethasone-liposomes were prepared with egg yolk phosphatidylcholine, cholesterol and dicetylphosphate in a lipid molar ratio of 7/2/1 by the

hydration method. This was adjusted to three different particle sizes to clarify the influence of particle size on the uptake by the macrophages and foam cells, and the inhibitory effect on cellular cholesterol ester accumulation. The distribution of particle sizes of dexamethasone-liposomes was 518.7±49.5 nm (L500), 202.2±23.1 nm (L200), and 68.6±6.5 nm (L70), respectively. For each size, dexamethasone concentration and dexamethasone/lipid molar ratio in dexamethasone-liposome suspension were 1 mg dexamethasone mL-1 and 0.134 mol dexamethasone mol-1 total lipids, respectively. The zeta potential was approximately - 70 mV for all sizes. Dexamethasoneliposomes or free dexamethasone were added to the macrophages in the presence of oxidized low density lipoprotein (oxLDL) and foam cells, and then incubated at 37^0 C. The uptake amount of dexamethasone by the macrophages and foam cells after a 24-h incubation was L500 > L200 > free dexamethasone > L70. The macrophages in the presence of oxLDL and foam cells were incubated with dexamethasone-liposomes or free dexamethasone for 24 h at 37o C to evaluate the inhibitory effect on the cellular cholesterol ester accumulation. The cellular cholesterol ester level in the macrophages treated with oxLDL was significantly increased compared with that in macrophages without additives. L500, L200 and free dexamethasone significantly inhibited this cholesterol ester accumulation. L500, L200 and free dexamethasone also significantly reduced cellular cholesterol ester accumulation in foam cells. In addition, the relationship between the area under the uptake amount of dexamethasone-time curve (AUC) and the inhibition rate of cholesterol ester accumulation in macrophages and foam cells was evaluated.

The inhibition rate of cholesterol ester accumulation (%) was related to the AUC in both types of cell. These results suggested that dexamethasone-liposomes would be a useful approach to the development of a novel drug delivery system for atherosclerotic therapy (Chono and Morimoto, 2006). Phosphatidylserine (PS), which is normally located on the inner leaflet of the plasma membrane, translocates to the outer leaflet at the early stage of apoptosis. The PS externalization provides a signal for phagocytes to initiate uptake of apoptotic cells. After phagocytosis of apoptotic cells, phagocytes induce the secretion of anti-inflammatory mediators including prostaglandin E2 (PGE2). PS-containing liposomes (PSLs) can mimic the effects of apoptotic cells on phagocytes to induce the secretion of PGE2. PSLs induce the PGE2 secretion from microglia without induction of either cyclooxygenase (COX)-2 or microsomal prostaglandin E synthase (mPGES)-1. PSLs are found to rather utilize COX-1/mPGES-2 system to produce PGE2 secretion and then shift microglia and macrophages from pro- to anti-inflammatory phenotype by an autocrine action of PGE2. Moreover, PSLs inhibit the maturation of dendritic cells and osteoclast precursors. Therefore, PSLs will be potential pharmacological interventions for inflammatory and immune diseases through feedback mechanism utilizing PGE2 (Wu and Nakanishi, 2011).

I.6.1.4. Conclusions

MLV and SUV liposomes loaded with R-phycoerithrin were added to the organ bath in a ratio of 1/9 v/v for 15 minutes. R-phycoerithrin encapsulated in SUV liposomes is released only in the endothelium of isolated rat aorta. In contrast, R-phycoerithrin encapsulated in MLV liposomes is released in the aortic smooth muscle layer of isolated rat aorta. Thus, we might conclude that liposomes with a single layer of lipid or a small number of lipid layers, through merger, would release their contents only in the first row (or first rows) of impact cells. The presence of many vesicular lipid layers in the wall will allow liposomes to "cross" several rows of cells and to release their content in tissue depth. Thus, multilamellar liposomes are very reliable and useful instruments for administration of various substances in the depth of tissues and to target the macrophages from subendothelial space.

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I.6.2. Interactions between angiotensin II and polyamines incorporated in liposomes in experimental inflammation

I.6.2.1. Introduction

Oxidative stress and endothelial dysfunction are consistently observed in hypertensive subjects with atheromatous lesions, but emerging evidence suggests that they also have a causal role in the molecular processes leading to development of ATS and hypertensive disease. The aim of the actual study was represented by the interactions between angiotensin II and polyamine antioxidants incorporated in liposomes (agmatine, spermine, spermidine, cadaverine, and putrescine) in inflammation, as compared to indomethacin. Incorporation of antioxidants of polyamine type in liposomes induces at least interesting effects toward the experimental inflammation. Spermine, spermidine and agmatine incorporated into liposomes, associated anti-inflammatory effects extremely important, as compared even with substances unincorporated.

Renin-angiotensin system develops its effects predominantly through angiotensin II (Ang II) peptide. Although it was thought that Ang II is a typically hormone, which arises in the torrent blood, several authors consider that there is also a renin-angiotensin system (RAS) independent of the circulation one (Danser, 2009). Production of reactive oxygen species is regulated by several cytokines and growth factors, including Ang II, which increase O2 .- and H2O2 in cardiac cells, vascular smooth muscle, endothelial, adventicial and mesangial cells. Generation of oxygen free radicals has been implicated in the pathogenesis of ATS and hypertension induced by Ang II (Androulakis et al., 2009).

Systemic arterial hypertension is a highly prevalent cardiovascular risk factor that causes significant morbidity and mortality, and aggravates the risk of rupture of atheroma plaques. The pathophysiology of hypertension and ATS involves a complex interaction of multiple vascular effectors including the activation of the sympathetic nervous system, of the reninangiotensin-aldosterone system and of the inflammatory mediators. Subsequent vasoconstriction and inflammation ensue, leading to vessel wall remodeling and, finally, to the formation of atherosclerotic lesions as the hallmark of advanced disease.

Oxidative stress and endothelial dysfunction are consistently observed in hypertensive subjects, but emerging evidence suggests that they also have a causal role in the molecular processes leading to hypertension. Reactive oxygen species (ROS) may directly alter vascular function or cause changes in vascular tone by several mechanisms including altered nitric oxide (NO) bioavailability or signa-ing. ROS-producing enzymes involved in the increased vascular oxidative stress observed during hypertension include the NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain and an uncoupled endothelial NO synthase (Schulz et al., 2011).

Beside, polyamines are multifunctional molecules with anti-inflammatory potential, acting both by modulating respiratory combustion and by adjusting the lymphocyte multiplication. Polyamines inhibit NADPHoxidase complex formation by 2 different mechanisms: binding of spermine to phosphatidyl-inositol-4-phos-phate (PIP), making it inaccessible to phospholipase C (PLC), or inhibition of protein kinase C (PKC) coupling process to the membrane (Zhu et al., 2009). Our previous data showed that antioxidant agmatine (decarboxylated arginine) significantly reduced the experimentally carageenan inflammatory process enhanced by Ang II in rats. Noteworthy was the strong action of agmatine, very close to that of spermine, although surpassed by that of indomethacin.

In contrast, some other polyamines (spermidine, cadaverine and putrescine) had no significant reducing effects. Inhibition of polyamines synthesis by DL-αdifluoromethylornithine (DFMO) has further significantly enhanced the pro-inflammatory effects of Ang II at 6 hours. The above findings might demonstrate the involvement of agmatine synthesis (beyond other polyamines) as a reactive response to proinflammatory

action of angiotensins (Chelariu, 2010). The goal of the actual study was represented by the interactions between Ang II and antioxidants incorporated in liposomes (agmatine, spermine, spermi-dine, cadaverine, putrescine) in inflamma-tion, as compared to indomethacin.

I.6.2.2. Material and methods

For the experiments we used 48 Wistar adult male rats (Băneasa source), weighing 150-200 g, kept under normal and the same laboratory conditions. Experiments were carried out by the method of inflammation named air pouch. To achieve the "bag of air", rats were injected with 10 ml of sterile air into the back (the default and relatively the same for all rats) for 3 days consecutively. Rats were then divided into equal series and relatively homogeneous in weight, 6 each series. Inflammation was induced by injecting the air pockets with 2% carrageenan in saline. Series I witnessed and received only saline, 1 ml i.p. Series II received spermine, series III spermidine, series IV agamatine, series V putrescine, series VI cadaverine, all incorporated into liposomes, i.p., 1 ml, 10 μM each antioxidant in aqueous solution. For comparison, to series VII control liposomes were administered without the active substances, i.p., 1 ml. For comparison, series VIII was given i.p. indomethacin 10 mg/kg b.w. in 1 ml saline (Chelariu, 2010). Meanwhile, Ang II was administered to all above groups intravenously into a vein outside of the rear feet, 100 nM in 10 μl sterile saline solution for 2-3 minutes, 30 minutes after the specific treatments already mentioned. Moreover, all rats received Evans blue, 20 mg/kg b.w., i.p., in 200 μl saline. After 6 hours the rats were killed and the granulomatous tissue or the existing liquid in the air bag were harvested, and then introduced, after weighing, in formamide for 48 hours to extract the Evans blue. Evans blue was spectrophotometrically quantified at 620 nm using a UV-VIS spectrophotometer HP 8453. Evans blue values were expressed in μg/g tissue. Quantification was done using a standard curve made with 2 concentra-tions of Evans blue (0.1 μM and 10 μM), while knowing that Evans blue absorbance is linear with concentration in these areas. Concentration calibration equation was as follows: concentration = 12.79100μM/ml x absorbance (A). Liposomes used were prepared from egg phosphatidylcholine (type X-E, 12.5 mg lipid per ml of built), with slight modification of the method described by Gregoriadis et al. (1985).

Thus, the chloroform in which was solved egg phosphatidylcholine was removed with a negative pressure rotavaporizator, obtaining a thin film on the walls of a flask of 50 ml. After removing the last visible traces of chloroform rotavaporization was continued for 15 minutes, followed by drying under nitrogen stream an additional 15 minutes. Then, there were added a few glass beads of 0.5 mm. After adding the desired volume of solution, the flask was sealed with a rubber stopper secured with Parafilm and then shaken vigorously with a Vortex. Blank liposomes included only 25 mM NaCl solution (pH 7.2 adjusted with NaOH if necessary). Other series of liposomes have included spermine, spermidine, agmatine, cadaverine and putrescine 10 μM each, solved in 25 mM NaCl (pH 7.2 adjusted with NaOH if necessary). To remove the unincorporated substances, liposomes were undergoing dialysis in 25 mM NaCl solution (pH 7.2, for 120 minutes, volume 1/1000, the buffer being changed every 30 minutes). The statistical significance of test results was highlighted using the Variance One-Way ANOVA (possibly complemen-ted by Bonferroni test) and Student t-test and the results were expressed as mean ± S.E.M (n = 6). Value of $p < 0.05$ was considered statistically significant always. Present studies were carried out in accordance with the "Guide for Care and Use of Animal Experiments" of U.S. National Institutes of Health (NIH), published by the U.S. National Academy in 1996 and approved by the Ethics Committee of the University of Medicine and Pharmacy "Gr T. Popa" Iaşi. Chemicals, compounds and reagents used: spermine, spermidine, agmatine sulfate (argamine), cadaverine (1,5-diaminopentan), putrescine (1,4-diaminobutan), angiotensin II (Ang II), L-α-egg phosphatidylcholine type X-E, Evans blue, formamide, indomethacin and carrageenan were purchased from

SigmaAldrich Company, St. Louis, U.S.A. The remaining reagents used were of analytical grade.

Results and discussion

Administrations of spermine and agmatine significantly reduced the extru-sion of Evans blue into the granulomatous tissue, enhanced by Ang II, in rats (in fact, the amplifying of the experimentally inflammatory process). Noteworthy is the strong action of agmatine. On the other hand, spermidine, cadaverine and putrescine had no significant reducing effects on the amplification of the inflammatory process by Ang II. Inhibition of polyamines synthesis by difluorome-thylornithine (DFMO) has further ampli-fied the pro-inflammatory effects of Ang II at 6 hours. This demonstrates the involvement of polyamine synthesis as a reaction response to anti-inflammatory action of angiotensins, as we showed in other studies (Chelariu et al., 2010).

However, none of these answers was as strong as that of indomethacin, an extremely potent anti-inflammatory. Incorporation of antioxidants polyamines in liposomes induces at least interesting effects toward the experimental inflammation. Spermine, spermidine and agmatine incorporated into liposomes, associated anti-inflammatory effects extremely important, compared even with substances unincorporated.

The effects of agmatine incorporated into liposomes were almost identical to those induced by indomethacin (Figure I.6.3). If spermidine incorporated into liposomes has an anti-inflammatory effect, unlike unincorpo-rated spermidine, cadaverine and putrescine are without effects, even incorporated into liposomes. The mentioned effects, to reduce the amplification of the inflammatory process induced by Ang II, is not due to components of the liposomal membranes since control liposomes (empty) do not have anti-inflammatory effects.

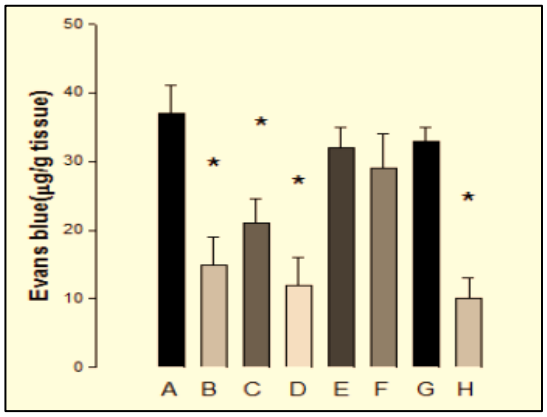

Figure I.6.3

Effects of liposomes loaded with spermine (B), spermidine (C), agmatine (D), putrescine (E) cadaverine (F), the control liposomes (G) and indomethacin (H) on the amplification of the inflammatory process induced by Ang II 100 nM in rats, *p<0.05, as compared with Ang II in saline

Polyamines are small cationic molecules required for cellular proliferation and are detected at higher concentrations in most tumors tissues, compared to normal tissues. Agmatine (AGM), a biogenic amine, is able to arrest proliferation in cell lines by depleting intracellular polyamine levels. It enters mammalian cells via the polyamine transport system. Agmatine is able to induce oxidative stress in mitochondria at low concentrations (10 or 100 microM), while at higher concentrations (e.g. 1-2 mM) it does not affect mitochondrial respiration and is ineffective in inducing any oxidative stress. As this effect is strictly correlated with the mitochondrial permeability transition induction and the triggering of the proapoptotic pathway, AGM may be considered as a regulator of this type of cell death

Furthermore, polyamine transport is positively correlated with the rate of cellular proliferation. By increasing the expression of antizyme, a protein that inhibits polyamine biosynthesis and transport, AGM also exhibits a regulatory effect on cell proliferation (Agostinelli et al., 2010). Agmatine, an endogenous arginine metabolite, has been proposed as a novel neuromodulator that plays protective roles in the CNS in several models of cellular damage. However, the mechanisms involved in these protective effects in neurodegenerative diseases are poorly understood. Some studies were undertaken to investigate the effects of agmatine on cell injury induced by rotenone, commonly used in establishing in vivo and in vitro models of Parkinson's disease, in human-derived dopaminergic neuroblastoma cell line (SHSY5Y).

We report that agmatine dose dependently suppressed rotenone-induced cellular injury through a reduction of oxidative stress. Similar effects were obtained by spermine, suggesting a scavenging effect for these compounds. However, unlike spermine, agmatine also prevented rotenoneinduced nuclear factor-κB nuclear translocation and mitochondrial membrane potential dissipation. Further-more, rotenone induced increase in apoptotic markers, such as caspase 3 activities, Bax expression and cytochrome c release was significantly attenuated with agmatine treatment. These findings demonstrate mitochondrial preservation with agmatine in a rotenone model of apoptotic cell death, and that the neuroprotective action of agmatine appears because of suppressing apoptotic signalling mechanisms. It is established that agmatine, an endogenously formed decarboxylated arginine, is a weak competitive inhibitor of neuronal nitric-oxide synthase (nNOS) with an apparent Ki value of 660 microM.

Although agmatine is known to bind to alphaadrenergic and imidazoline recep-tors, it has been suggested that some of the pharmacological actions of agmatine, such as the prevention of morphine tolerance, may be due to the inhibition of nNOS. In the current study, we have discovered that agmatine, at concentrations much lower than the reported Ki value, leads to a time-, concentration-, NADPH-, and calmodulin-dependent irreversible inactivation of nNOS. The kinetics of inactivation could be described by an apparent dissociation constant for the initial reversible complex (Ki) and a pseudo first-order inactivation constant (kinact) of 29 microM and 0.01 min-1, respectively. As determined by high-performance liquid chromatography analysis, the mechanism of inactivation involves alteration of the prosthetic heme moiety of nNOS, in part to protein-bound products. Moreover, we discovered that agmatine causes a 3-fold increase in the NADPH oxidase activity of nNOS leading to the production of H2O2 and is a likely cause for the inactivation of the enzyme. Both the inactivation of nNOS and the oxidative stress produced should now be considered in the pharmacological actions of agmatine as well as provide insight into the potential biological effects of endogenously formed agmatine (Demady et al., 2001).

Conclusions

Incorporation of antioxidants polyamines in liposomes induces at least interesting effects toward the experimental inflammation. Spermine, spermidine and agmatine incorporated into liposomes, associated anti-inflammatory effects extremely important, as compared even with substances unincorporated. The effects of agmatine incorporated into liposomes were almost identical to those induced by indomethacin. If spermidine incorporated into liposomes has anti-inflammatory effects, unlike unincorporated spermidine, cadaverine and putrescine are without effects, even incorporated into liposomes. The mentioned effects, to reduce the amplification of the inflammatory process induced by Ang II, is not due to components of the liposomal membranes since control liposomes (empty) do not have anti-inflammatory effects. Thus, the reduction of the antioxidative stress by polyamines, and especially by agmatine incorporated into liposomes might represent a mean to counteract the hypertensive condition development.

Chapter II

ORAL CANCER FROM PATHOPHYSIOLOGY TO NOVEL THERAPEUTICALLY APPROACHES

Head and neck cancers are a therapeutic challenge due to the high recurrence rate after multimodal treatment, especially in the advanced stages. The oral cavity consists of the lips, oral tongue, and floor of the mouth, retro molar trigon, alveolar ridge, oral mucosa, and hard palate (Figure 40). Classification of tumors by subside is useful because patterns of spread and clinical outcomes vary by specific subside, partly reflecting the variable risk of nodal spread by anatomic site of presentation.

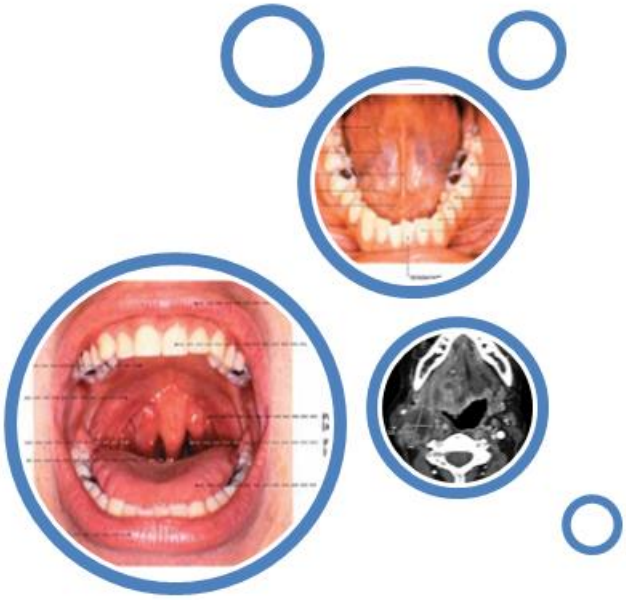

Figure II.1
Oral cavity surface anatomy.Floor of mouth surface anatomy adapted from Sobotta ed.©2008

The anterior boundary of the oral cavity is the skin–vermilion junction. The superior portion of the oral cavity extends posteriorly to the junction between the hard and soft palate, while the inferior portion extends to the circumvallate papillae. The specific anatomic subsides of this region are listed in the figure 40.

Cancer of the oral cavity makes up approximately 30% of head and neck region tumors and 3% of all cancers. The incidence rate of oral cancer is more than twice as high in men as in women (Greenlee, 2000). George Crile's seminal article in 1906 heralded the beginning of the modern era in treating oral cancer (Figure II.2)

The belief of physicians and surgeons that identifying and treating a tumor using anatomically directed therapies (radiation and surgery) were primordial for oral cancer treatment and rest the main stain in this type of cancer.

Bose et al., (2013) define this period like “anatomic era” the steps being followed: define a target, treat the target and cure the patient. However, the overall survival rate of has not changed since the 1960's (~50%). The “biologic era” of cancer treatment debuted in 1950's after the discovery of DNA structure by Watson and Crick.

The subsequent studies have led to a profound understanding of molecular mechanisms that drive neoplastic transformation, progression and response to therapy. Cancer treatments

focused on specific molecular targets and pathways are becoming increasingly common (Nowak, 2012).

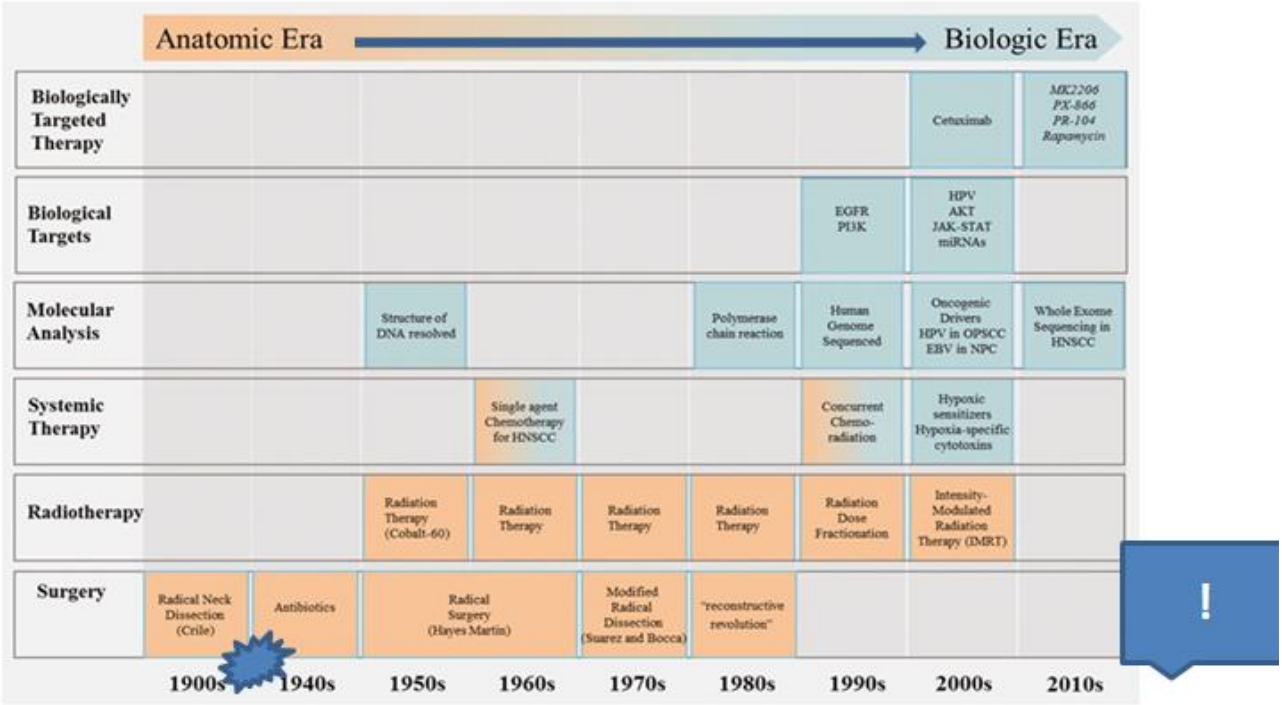

Figure II.2
Head and neck cancer: from anatomy to biology, adapted from Bose, 2013

The epidemiology of oral cancer strongly reflects exposure to certain environmental agents, particularly tobacco and alcohol. Worldwide, the incidence of oral cancer varies considerably. There is a strong causal relationship between smoking and cancer of the oral cavity. Smoking is identified as an independent risk factor in 80% to 90% of patients (Garavello, 2010).

Tobacco users have a fivefold to 25-fold higher risk of oral cavity cancer. Cessation of smoking is associated with a decline in the risk of cancer of the oral cavity. The oral cavity is the most common site for head and neck cancer in the US. Carcinoma of the oral cavity commonly afflicts patients in the sixth to seventh decades of life (Lambert, 2011).

Carcinogenesis and Progression

Aberrant cellular proliferation is a hallmark in cancer (Hanahan, 2011)**.** The critical drivers of proliferation in oral include signaling cascades such as the epidermal growth factor receptor (EGFR), phosphatidylinositol-3-kinase-AKT-mammalian target of rapamycin (PI3K-AKT-mTOR) and Janus kinase-signal transducer and activator of transcription JAK-STAT pathway (Molinolo, 2011, Bose, 2013)**.**

Considerable cross-talk exists between these pathways and therapeutic targeting of one pathway can modulate signaling through others (Bose, 2013). In addition, HPV produces oncoproteins that can overthrow the cellular proliferation and lead to malignant transformation.

PERSONAL CONTRIBUTION RELATED TO THE SYNTHESIZED IN THE FOLLOWING PAPERS:

BDI Articles	
1.	**Zonda GI, Goriuc A, Indrei A, Iancu RI, Chelaru L, Carasevici E, Costuleanu M.** **Ionomycin-Induced Ca 2 Cytosolic Increase Is Not Inducing Massive Apoptosis Of Ba/F3 Cells ,** **Jurnalul de Chirurgie, Iaşi, 2010, Vol. 6, Nr. 3 (ISSN 1584 – 9341)** **http://www.jurnaluldechirurgie.ro/jurnal/docs/jurnal310/art%2005_vol%206_2010_nr%203.pdf**
2.	**Albu M, Hatescu I, Buzea CG, Mireștean CC, Borcia C, Iancu RI, Iancu DT** **Comparative Evaluation Of The Risk Of Xerostomia Using Radiobiological Models For Patients With Locally Advanced Nasopharynx And Oropharynx Neoplasm, Irradiated With IMRT and VMAT Techniques** **Polytechnic Institute Bulletin – Mathematics. Theoretical Mechanics. Physics Section.Volumul 63 (67), Numărul 4, 2017**
3.	**Chelariu R, Gurzu B, Dumitriu I, Chelaru L, Iancu RI, Slătineanu SM, Petrescu G, Costuleanu M** **Interactions between angiotensin II and agmatine in experimental inflammation** **Anal. Şt. UAIC Iaşi. Sect. a IIa. Biol. Gen. Molec., XI, 2-3**
4.	**Goriuc A, Petreus T, Peptenariu T, Iancu R, Costuleanu M** **The Influence Of Ca 2 Ionophor Ionomycin On The Mitochondrial Transition Permeability Pore In Normal And Drug Treated Gingival Fibroblasts** **Annals of the Romanian Society for Cell Biology 2010, 15 (2)**
5.	**Goriuc A, Jipu R, Iancu RI, Costuleanu M** **Endoplasmic Reticulum Involvement In Apoptosis Of Normal And Treated Gingival Fibroblasts.** **Annals of the Romanian Society for Cell Biology 2011, 16 (1)**
6.	**Jipu R, Amititeloaie C, Zonda GI, Iancu RI, Carasevici E, Costuleanu M** **Thapsigargin-induced endoplasmic reticulum stress is not accompanied by mitochondrial membrane potential dissipation in murine pro-B cells** **The Medical-Surgical Journal 2012, 116 (2), 557-562**
7.	**D Iancu, RI Iancu** **The Influence Of New Irradiation Techniques Of Parotid Tumors For Oral Rehabilitation** **Romanian Journal of Oral Rehabilitation 2015, 7 (3)**
8.	**DT Iancu, RI Iancu** **Oral Cavity Cancers-General Review.** **Romanian Journal of Functional & Clinical, Macro-& Microscopical Anatomy 2015, 14(4), p610-618.**
9.	**Mirestean CC, Pagute ON, Cardei S, Apostu R, Buzea C, Teaca C, Roman R, Iancu RI, Iancu DT** **Evaluation of the Diabetes Contribution to the Occurrence of Treatment Related Toxicities in Multimodal Treated Locally Advanced Head and Neck Cancers** **Modern Medicine 2019, 26 (2), 70**
10.	**Iancu RI, Mirestean CC, Buzea CG, Iancu DT** **Radiotherapy Induced Toxicity For Oropharyngeal Cancer Patients: Implications For Oral Health** **Romanian Journal of Oral Rehabilitation 2019, 11 (3)**
11.	**Mirestean CC, Pagute O, Buzea C, Iancu RI, Iancu DT**

	Radiomic Machine Learning and Texture Analysis–New Horizons for Head and Neck Oncology **Maedica 2019, 14 (2), 126**
12.	**Iancu RI, Zara AD, Mirestean CC, Iancu DPT** **Radiomics in Head and Neck Cancers Radiotherapy. Promises and Challenges** **Maedica 2021 16 (3), 482**
13.	**Mirestean CC, Iancu RI, Iancu DT** **Targeting EGFR Exon 20 Insertion-mutated Cancers–New Perspectives in Head and Neck Cancers–Lessons to Learn from Non-small Cell Carcinoma (NSCLC)** **Modern Medicine 2022, 29 (3)**
14.	**Mirestean CC, Iancu RI, Iancu DT** **Delta-radiomics Entropy Based on Tumor Heterogeneity Concept –Response Predictor to Irradiation for Unresectable/recurrent Glioblastoma** **Modern Medicine | 2022, 29(4), 273-278**
15.	**Mirestean CC, Buzea CG, Iancu RI, Iancu DT** **Modern Radiotherapy for Head and Neck Cancers Benefits and Pitfalls – a Literature Review** **Modern Medicine | 2019, 26(3), 97-100**
ISI Articles	
1.	**CC Mireștean, RI Iancu, DPT Iancu** **Micro-RNAs, the Cornerstones of the Future of Radiobiology in Head and Neck Cancers?** **Current Oncology 2022, 29 (2), 816-833** **IF (2021)=3,109**
2.	**Mireștean CC, Iancu RI and Iancu DPT** **New horizons in modulating the radio-sensitivity of head and neck cancer - 100 years after Warburg' effect discovery.** **Front. Oncol. 2022, 12:908695. doi: 10.3389/fonc.2022.908695** **IF (2021) = 5,738**
3.	**Mireștean CC, Iancu RI and Iancu DPT** **Immunotherapy & radiotherapy – a future partnership Focus on radiation induced lymphopenia (RIL) implications** **Bratislava Medical Journal, 2023, Vol.124, No.1, p.70–73** **Bratislava Medical Journal Vol.124, No.1, p.70–73, 2023 (elis.sk)** **FI (2021)= 1,564**
4.	**Mireștean CC, Iancu RI, Iancu DPT** **Simultaneous integrated boost (SIB) vs. sequential boost in head and neck cancer (HNC) radiotherapy a radiomics-based decision proof of concept.** **J. Clin. Med. 2023, *12,* 2413. https://doi.org/10.3390/jcm12062413** **IF (2021)=4,964**
5.	**CC Mirestean, RI Iancu, DPT Iancu,.** **p53 Modulates Radiosensitivity in Head and Neck Cancers—From Classic to Future Horizons.** **Diagnostics 2022, 12, 3052** **IF (2021)=3.992**
6.	**CC Mireștean, RI Iancu, DPT Iancu** **Capecitabine—A "Permanent Mission" in Head and Neck Cancers "War Council"?** **Journal of Clinical Medicine 2022, 11 (19), 558** **IF (2021)=4,964**
7.	**CC Mirestean, AD Zara, RI Iancu, DPT Iancu** **Radiobiological approach to treatment gaps in locally advanced head and neck cancers**

	radical radiotherapy arising from the COVID-19 pandemic **Bratisl Med J 2022; 123 (5) 362 – 365** **IF (2021)= 1,564**
8.	**R Iancu, SA Irimiciuc, M Agop, M Frasila, MA Paun, VA Paun, VP Paun, Sorin Stratulat** **5-fluorouracil release from chitosan-based matrix. Experimental and theoretical aspects** **Materiale Plastice 57, 180-188** **IF (2021)=0,782**
9.	**D Vasincu, RI Iancu, L Chelaru, G Stoleriu, M Costuleanu** **Biochemical effects of some endoplasmic reticulum stress inducers on mesenchymal stem cells in vitro** **Revista de Chimie 69 (6), 2018** **IF (2018)=1,605**
10.	**TV Barnea, A Sava, C Gentimir, A Goriuc, O Boișteanu, L Chelaru, RI Iancu, CA Avram, DD Acatrinei, EG Bogza, OC Răducanu, DP Cioloca, D Vasincu, M Costuleanu** **Genetic polymorphisms of TNFA and IL-1A and generalized aggressive periodontitis** **Rom J Morphol Embryol 56 (2), 459-464** **IF (2015)=0,811**
11.	**Mireștean CC, Iancu RI, Iancu DPT.** **Simultaneous Integrated Boost (SIB) vs. Sequential Boost in Head and Neck Cancer (HNC) Radiotherapy: A Radiomics-Based Decision Proof of Concept.** **Journal of Clinical Medicine. 2023; 12(6):2413. https://doi.org/10.3390/jcm12062413** **IF (2021)=4,964**
Projects	
I.	**Contract grant CNCSIS 61GR/16.05.2006** **Title - ASSESSMENT OF THE ANTI-TUMOR EFFECT OF VITAMIN D IN PATIENTS WITH HEAD AND NECK CARCINOMA** **Director - Prof. Dr. Veronica Mocanu**
II.	**Grant contract CNCSIS GR215/15.09.2006-2008, project type A, code 1478** **Title - INTEGRATION OF THE MOLECULAR MECHANISMS OF B PROLYMPHOCYTE APOPTOSIS** **Director - Prof. Dr. Costuleanu Marcel**
III.	**Grant contract, A Type code 1128, 2003-2005** **Title - RELATIONS OF CYTOSOLIC AND MITOCHONDRIAL CALCULATION WITH APOTOSIS** **Director - Prof. Dr. Costuleanu Marcel**
Chapter book	
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II.1. HPV-Mediated Oral Carcinogenesis

In recent years, there has been recorded a reduction of head and neck cancers associated with smoking and alcohol consumption, but an alarming increase in a subtype associated with Human Papilloma Virus (HPV) infection. The 6th "R" of radiobiology - reactivation of the immune system can be identified in this subtype of cancers, the substrate being multiple mechanisms and pathways which differentiates HPV driven head and neck cancers from the HPV negative subtype. Even if programmed death ligand (PD-1) is already a target in clinical practice, modulation of radio-sensitivity by the other pathways involved is the subject of translational research and will be the basis for adapting treatment and fractionation regimens for HPV+ head and neck cancers radiotherapy in the near future. Approximately 80% of cancers associated with HPV infection arise from the oropharynx (the base of the tongue and tonsils) and HPV-positive head and neck cancers (HPV+) account for 25% of all head and neck cancer cases. HPV+ head and neck higher radio-sensitivity may have as a substrate the triggering effect of radiation induced DNA on the repair response of DNA damage (Özcan-Wahlbrink, 2019, Steel, 1989, Boustani, 2019, Arenz, 2014).

The molecular basis of the mechanisms involving the increased radio-sensitivity of cancers involves the cGasSTING pathway, having effects on both DNA damage repair and modulation of apoptosis, but also to cell distribution in the phases of the cell cycle and to hypoxia modulation. A score consisting of 13 genes called RadR has been shown to be useful in predicting both radiation sensitivity and prognosis. The treatment response is also correlated with important pathways including epithelial to mesenchymal transition (EMT), angiogenesis, DNA' damage repair. Lower score values were found for HPV + head and neck cancers, the data being in line with the conceptual higher radio-sensitivity level of this tumor subtype (Özcan-Wahlbrink, 2019, Rödel, 2019, Zhou, 2020).

Among the mechanisms involved in modulating the radio-sensitivity of head and neck cancers, DNA repair systems is one of the essential HPV+ cases, being associated with TGFβ mediated DNA repair deficiencies. As a result HPV is associated with an increased sensitivity to irradiation by blocking the repair of DNA lesions in the tumor cell. HPV has overexpressed proteins that modulate both single-strand break and dual-strand break (DSB), either by excision of the bases or by overexpressed onco-protein E6 which regulates factors involved in repairing of DNA lesions. HPV+ and HPV- also manipulate in opposite ways non-homologous recombination of DNA. The E7 protein associated with subtype 16 of HPV modifies the repair ability of double stranded DNA damage by suppressing non-homologous end joing, mechanisms involving RAD53 and TRIP12 (Özcan-Wahlbrink, 2019; Rödel, 2019; Zhou, 2020; Constanzo 2021; Nickson, 2017).

Other directions of radio-sensitivity modulation by HPV infection involve the regulation of the cell cycle and the control of tumor metabolism. P53, a p53 gene-regulated suppressor protein is usually mutant in smoking-induced head and neck cancers, but not in those associated with HPV. However, the effect of protease inhibition induced by the onco-protein E6 associated with HPV+ cancers leads to loss of function for this tumor suppressor. P53 protease degradation and p53 mutation in smoking-associated head and neck cancers are "loss of function" events associated with radio-resistance. P53 is one of the important players in modulating radio-sensitivity, but it is certainly not the only factor involved in this phenomenon (Özcan-Wahlbrink, 2019; Rödel, 2019; Zhou, 2020; Nickson, 2017).

Regulation of tumor metabolism can modulate tumor radio-sensitivity and HPV- head and neck cancers have much higher glycolysis rates than HPV+. HPV induced oxidative stress has a synergistic effect with radiation, being a radio sensitizing factor. Reactive oxygen species (ROS) found in 100% higher amounts in HPV + cancers are additional factors that increase the intrinsic radio-sensitivity of HPV+ head and neck cancer cells. Both the impairment of the function of some enzymes associated with HPV infection and the excess of

oxygen and nitrogen radicals contribute to the increased radio sensitivity of HPV+ tumors. Elevated levels of HIF1α in HPV head and neck cancers - justify high levels of hypoxia and higher resistance to these cancers compared to HPV + cancers. Modulation of the glycolytic phenotype of HPV- cancers could be a strategy to restore the radio-sensitivity of this category of radiation-resistant cancers (Özcan-Wahlbrink, 2019, Fleming, 2019, Chandel, 2020, Jayaraman, 2018).

HPV+ head and neck cancers have an important component of involving the immune system in modulating radio-sensitivity, being a good example to justify the introduction of the 6th R of radiobiology. HPV viral infection is a trigger for the immune response and in this context the effect of radiotherapy to stimulate immunogenicity and immune-mediated cell death causes a synergistic effect. Antigen presentation, inflammation potentiation, T cell activation, and dendritic cell maturation are processes associated with this synergistic effect of HPV infection and radiation therapy. Levels of tumor infiltrating lymphocytes (TILs) are considered significantly higher in HPV+ cancers, and CD8+ cytotoxic lymphocytes, a positive prognostic factor identified in higher amounts in HPV-driven cancers is associated with the inflammatory profile of this head and neck cancer subtype. TILs and CD8+ cytotoxic lymphocytes are considered also modulators of the tumor intrinsic radio sensitivity. Tumor associated macrophages (TAMs), programmed cell death protein 1 (PD-1), regulatory T cells (Tregs) are factors that orchestrates radio-sensitivity, having different expression in HPV-driven head and neck cancers. TAMs are associated with chronic inflammation, but Tregs and PD-1 are considered correlated with a favorable prognosis. The inflammatory response but also the immunosuppression promoted by myeloid-derived suppressor cells (MDSCs) can be modulated by combining irradiation with TGFβ-MDSCs, considered capable to convert an immunosuppressive tumor microenvironment profile into an immune-stimulatory one. PD-1 is already being targeted with approved Phase III clinical trials drugs that have already demonstrated synergistic potential with irradiation. Weiss and collaborators demonstrate the feasibility of combining PD-1 (Pembrolizumab) inhibitors with radiation therapy in squamous cell carcinoma of the head and neck, a combination considered effective but also advantageous in terms of toxicity profile (Özcan-Wahlbrink, 2019, Cao, 2019, Botticelli, 2021, Qiao, 2020, Marullo, 2013, Weiss, 2020).

In the case of the HPV-16 subtype, the E1–E5 genes encode proteins involved in the replication and transcription of the viral genome and the E6 and E7 genes are associated with carcinogenesis. The E6 gene acts through the synthetic protein product on the tumor suppressor p53, degrading it. E7, via retinoblastoma-associated protein (RB1), acts at the nickel control points of the cell cycle. Alteration of RB1 function also has the effect of up-regulating the p16 protein, well known as a surrogate biomarker in the identification of HPV− or HPV+ oro-pharyngeal cancer discrimination. All these totally different mechanisms in the case of HNSCC HPV− and HPV + highlight that we can talk about two different diseases not only as an etiology, but also as a pathophysiological mechanism of carcinogenesis (Scheffner, 1993, Johnson, 2020)

In the case of HPV-negative cancers, the most incriminated carcinogens are polycyclic aromatic hydrocarbons and especially nitrosamines in the case of tobacco exposure, but for situations where the main carcinogen is betel quid or areca nut, the mechanism is not so much studied. A destabilization of the balance between metabolic activation and detoxification of carcinogen-induced DNA damage underlies carcinogenesis in HPV negative HNSCC. The metabolism of alcohol to acetic aldehyde increases the number of lesions accumulated, so alcohol is a potentiating factor of carcinogenesis. In the case of HPV+ cancers, carcinogenesis begins in the crypts of the palatal and lingual tonsils. Along with the HPV-16 subtype, which is undoubtedly dominant as a risk factor, in a minority of cases HPV-52, HPV-18, HPV-31, and HPV-33 subtypes could also be identified (Michaud, 2014).

Conclusions

HPV driven tumors are a radiosensitive cancers, multiple factors and pathways being involved at the molecular level in increasing radio-sensitivity but also radio-resistance. If PD-1 is already a target included in clinical protocols, modulation of radio-sensitivity by the other pathways involved is the subject of translational research and in the near future will be the basis for adapting treatment and fractionation schemes for HPV-driven oral cancers.

II.2.EGFR pathway in oral carcinogenesis

EGFR belongs to the ErbB family of trans-membrane receptor tyrosine kinases and is activated by ligands including EGF, transforming growth factor-alpha (TGF) amphiregulin and β–cellulin. Ligand binding is followed by the dimerization of EGFR monomers leading to tyrosine kinase activation and auto-phosphorylation. This results in downstream signal transduction cascades regulating cellular proliferation and survival. EGFR can also translocate to the nucleus and regulate diverse cellular processes such as cell-cycle progression, metastasis and resistance to radiotherapy. In the nucleus, EGFR functions as a transcription factor or co-activator of other transcription factors, such as signal transducer and activator of transcription (STAT) (Bose, 2013, Lin, 2001, Lo, 2005).

EGFR is a recognized oncogene in oral cancer, mutations and gene amplification signaled without that elevated EGFR protein levels to be accompanied by poor prognosis in oral cancer. Approximately 60% of the studies reported an association between EGFR overexpression and poor clinical outcome, whereas 40% of the studies report a lack of association between EGFR and prognosis (Leemans, 2011) The signaling pathways activated by EGFR include PI3K-AKT-mTOR, Ras-MAPK, PLCγ-PKC and the JAK-STAT pathway (Choong, 2006, Vivanco 2002, Hynes, 2005).

EGFR immunotherapy was the first targeted "biologic" therapy approved for oral cancer. Cetuximab is a monoclonal antibody targeting the extracellular ligand-binding domain of EGFR blocking EGFR signaling. The resistance to EGFR inhibition by Cetuximab is a common occurrence one of the proposed mechanisms of Cetuximab resistance is the expression of the tumor-specific EGFR deletion mutant EGFRvIII. (Wheeler, 2010, Sok, 2006). Other mechanisms of resistance to Cetuximab include epithelial mesenchymal transition and activation of redundant RTK signaling pathways (Bose, 2013).

Mutations in the tyrosine kinase (TKD) domain of the epidermal growth factor receptor (EGFR) are involved also in the unfavorable therapeutic response through resistance to targeted molecular therapy. Data from the clinical experience of non-small cell lung carcinoma treatment demonstrate the benefit of tyrosine kinase inhibitors (TKIs) in cases of EGFR mutation. The next generation sequencing technique (NGS) allows the identification of hot spots involved in mutations, exon 20 insertion being associated with the unfavorable response. Exon 20 insertions are more common in head and neck squamous cell carcinoma (HNSCC) compared to NSCLC, which could explain a resistance to targeted therapy in head and neck cancers. Taking into account the data reported Amivantamab, a bi-specific EGFR-MET antibody with potential immune cell modulation of activity, but also other innovative therapies validated in exon20 EGFR mutation could be part of the therapy of sino-nasal cancer, but also of other oral cancers sites exon 20 mutant EFGR.

Mutations in the tyrosine kinase (TKD) domain of the epidermal growth factor receptor (EGFR) are involved in the unfavorable response through resistance to molecular targeted therapy. Given the data obtained from lung cancer patients proving that cases with mutations in the TKD domain of EGFR respond to small-molecule inhibitors such as, Erlotinib, Gefitinib, Osimertinib and Afatinib, the approach to identify potential therapeutic targets involving this. The receptor in oral cancers is a topic of interest. The identification of a particular subtype of HNSCC (head and neck squamous cell carcinoma) with better response

to multimodal treatment and better prognosis, a disease variant linked to Human Papilloma Virus (HPV) infection make it necessary the personalization of the therapy, including identification of new valid biomarkers.

Evaluating EGFR mutations in patients with oral cancer, Perisanidis (2017) analyzed in a systematic review of the literature the data regarding prevalence of EGFR in HNSCC. A 2.8% percent of the 4,122 patients included in 53 clinical trials expressed a mutation in EGFR. Approximately 90% of HNSCC express EGFR of the trans-membrane cell surface receptor in the tyrosine kinase receptor family. The over-expression of EGFR in HNSCC is associated with unfavorable prognosis. EGFR is involved in pathways that modulate carcinogenesis, being a therapeutic target of current interest. Testing in fundamental and clinical research of monoclonal antibodies and tyrosine kinase inhibitors (TKIs) are strategies currently being evaluated, but de novo or acquired resistance to these classes of agents are the causes of therapeutic failures (Perisanidis, 2017).

II.2.1. EGFR exon 20 insertions targeting – lesson to learn from oral cancers

Next-generation sequencing tests (NGS) are used to detect EGFR exon 20 insertions, mutations that may confer special therapeutic features compared to exon 19 deletions, mutations encountered in most cases. The National Comprehensive Cancer Network (NCCN) also supports NGS testing for all cases, taking into account data on differences in disease progression for NSCLC subtypes classified based on EGFR mutation types and the fact that conventional PCR detection methods have a rate of over 50% to omit the correct identification of EGFR mutations. In head and neck cancers, mutations in the TKD tyrosine kinase domain of exon were identified in exon 18, exon 19, exon 20 and exon 21 in percentages of 9.4%, 41.5%, 32.1% and 17%, respectively. A percentage of 5% of EGFR mutations represent insertions in exon 20, a type of mutation that benefits from a new therapy in non-small cell lung carcinoma. The missense T790M mutations in exon 20 are associated with resistance to TKI. It is worth mentioning the higher percentage of insertions in exon 20 in the case of HNSCC compared to NSCLC (5% *vs.* 3%) which justifies the interest for a possible implementation of the innovative target therapy used in lung cancer. Three clinical trials analyzed the results of platinum-based chemotherapy in HSCLC with EGFR mutation in exon 20. With a progression free survival (PFS) between 6.4 and 7.6 months, platinum-based chemotherapy was associated with an overall response. Lower rates of ORR (approximately 19%) compared to Pemetrexed-based treatment (41.6%). In terms of PFS, Pemetrexed based chemotherapy was inferior (5.5 months) to platinum based chemotherapy. Although overall survival (OS) was analyzed only in one clinical trial that included platinum-based chemotherapy, the results were inferior to Pemetrexed (19.9 vs. 25 months). Three generations of TKI were tested in combination with EGFR-mutated NSCLC chemotherapy in exon 20: Gefitinib, Erlotinib and Icotinib, first-generation TKI, Afatinib and Dacomitins, second-generation TKI, and Osimetrinib, a third-generation targeted therapy (Wang, 2020, Shi, 2017, Xu, 2020).

Platinum-based chemotherapy was the treatment of choice for lung cancer before the identification of EGFR mutations and before the implementation of TKI in clinical practice. For this category of patients carrying the EGFR mutation, the use of TKI brings benefits in tumor control being preferred compared to platinum-based chemotherapy. However, for the subgroup of patients with common mutations (EGFR ex19del and EGFR L858R) they have a better prognosis and respond better to TKI. NSLC patients carrying exon 20 insertion have an unfavorable prognosis and, even if TKIs have been approved for this category of patients, they will have minimal benefit to the target therapy, being also patients with an unfavorable prognosis.

A French multicenter study that included rare mutations identified a higher rate of exon 20 mutations in patients who never shared smokers with exon 18 mutations, and metastatic survival was almost double for non-smokers vs. smokers (21 months *vs.* 14 months). Patients treated with TKI had a median OS of 14 months, but clinical results were more favorable in cases with exon 18 mutations or complex mutation compared with patients which expresses EGFR exon 20 mutations alone. Clinical data validated the hypothesis from preclinical studies regarding the benefit of high doses of Osimertinib in cases of EGFR T790M resistance time insertion in exon 20. POSITION20 of single arm phase II showed a modest benefit of high doses of Osimertinib, median ORR being 28 % with acceptable toxicity. Piotrowska et al. performed a single-arm phase II study with Osimertinib 160 mg in NSCLC patients with EGFR exon 20 insertion mutations. Of the 20 patients with EGFR mutant insertion in exon 20 tested in a phase II study for high doses of Osimertinb (160 mg), the ORR rate was 25% of which one was fully responsive in term of disease control (Hou, 2022; Beau-Faller, 2014; Piotrowska, 2018).

II.2.2. Amivantamab – a dual EGFR and MET inhibitor with promising potential

Amivantamab, a double-acting antibody on both EGFR mutation and mesenchymal epithelial transition factor receptor (MET) activity and on immune cells was evaluated in the clinic in the phase I study of CHRYSALIS including EGFR NSCLC cases with exon20 insertion. The study established the recommended dose for phase II trials in order to keep toxicities within normal limits. Administration of Amivantamab at a dose of 1050 mg (1400 mg ≥80 kg) given once a week for the first 4 weeks and then every 2 weeks from week 5, after platinum-based chemotherapy. The 40% response rate including 3 complete responses from 40 patients and a median response time of 11.1 months recommends treatment as effective after progression of NSCLC cases with exon 20 EGFR insertions. Rash, infusion reactions, paronychia, hypokalemia, pulmonary embolism, neutropenia and diarrhea have been reported as toxicities, with dose reductions and treatment discontinuations reported in 13% and 4% of cases, respectively. Vyse et al. hypothesizes that Amivantamab, being a large molecule, will not cross the brain blood barrier and will have reduced activity on brain metastases, but through its extracellular action will help delay the onset of resistance to other therapies. Amivantamab also demonstrates benefit in patients with NSCLC MET exon 14 skipping mutation (METex14) previously treated with MET inhibitors. Thus ORR was 33% 46 and 21% for naïve treatment cases, with no previous MET inhibitors, respectively for the patient previously treated with MET inhibitors (Park, 2021, Vyse, 2022, Zwierenga, 2022, Krebs, 2022).

Pozitinib, a next-generation TKI for targeting exon20 aberration in EGFR Pozitinib, a next-generation TKI demonstrated efficacy in the treatment of NSCLC with aberrations in EGFR exon 20. A case of a 62-year-old patient who progressed after surgery and adjuvant chemotherapy showed a favorable, long-term response after Pozitinib therapy, the patient being in the metastatic stage of disease at the moment of TKI treatment initiation. Data from the ZENITH20 trial mention alopecia, skin rash, conjunctivitis as well as diarrhea and xerostomia associated with Pozitinib as toxicities (Prelaj, 2022).

Amivantamab and chemotherapy – a successful partnership? By now, the safety profile of Amivantamab has been evaluated in 250 cases and the PAPILLON phase III trial aims to identify a possible benefit of the combination of Amivantamab and Carboplatin-Pemetrexed vs. Carboplatin-Pemetrexed. The trial includes patients with EGFR mutants with exon 20 insertion in advanced or metastatic NSCLC. The results of this trial will determine the value of an association between EGFR-MET therapy and chemotherapy in this subgroup of patients (Minchom, 2022).

II.2.3. EGFR exon 20 insertion – a surprising predominance in sino-nasal cancers

Recurrent mutations involved in EGFR activation have been reported in squamous cell carcinoma, a rarer form of head and neck cancer but with a mortality of over 40% at 5 years. Although in most oral cancers mutations other associated with exon 19 deletion and L858R mutation predominate, in the case of sino-nasal carcinoma it is noted by the predominance of exon 20 insertions. By analogy with data obtained from lung cancers we can assume that this mutation is associated with pathogenesis as well as with treatment resistance and testing the strategies validated in NSCLC would open new therapeutic perspectives for this subtype of cancers. Pacini (2022) also mention the possible involvement of mutated EGFR by inserting exon 20 into the conversion of inverted sino-nasal papilloma, a locally aggressive benign tumor, into sino-nasal carcinoma. Perisanidis also mentions the major difference between EGFR mutations in NSCLC and HNSCC, noting an association of lung cancer with hot spots in exons 18 and 21. Head and neck cancers also involve hot spot regions in exons 19 and 21. Given that some studies have reported only mutations in exons 19 and 21 it is possible that there is an under-reported prevalence of EGFR mutations in HNSCC. In the systematic review, the author identifies a 2.8% prevalence of EGFR mutations in HNSCC with a minor variation between geographic regions (Perisanidis, 2017).

Conclusions

The NGS technique will play an essential role in accurately detecting the type of mutation and the exon involved in the detailed characterization of the EGFR mutation in HNSCC. Taking into account the data reported in HSCLC, Amivantamab, a bispecific EGFR-MET antibody with potential immune cell modulation of activity but also other innovative therapies validated in exon20 and other types of HNSCC.

II.3. Micro-RNAs, the cornerstones of the future of radiobiology in head and neck cancers

II.3.1. Introduction

MicroRNAs (miRNAs) are evolutionarily conserved noncoding RNAs that are between 18 and 25 nucleotides in length. miRNAs regulate the expression of genes involved in fundamental cellular processes like development, proliferation, differentiation and apoptosis. miRNAs are initially transcribed by RNA polymerase II and processed in the nucleus by ribonucleases to form pre-miRNAs. After being transported to the cytoplasm by exportin 5, further processing of the pre-miRNA by the ribonuclease Dicer produces a mature miRNA. One of the strands of the double-stranded miRNA gets incorporated into the RNA-induced silencing complex (RISC) and the other is generally degraded. The miRNA strand imparts specificity to the RISC and facilitates the incorporation of mRNAs into the complex to affect gene silencing. Overexpression of oncogenic miRNAs and loss of tumor suppressor miRNAs have been associated with tumor genesis, progression and metastasis in cancer (Garzon, 2010, Bose, 2013).

Micro-RNAs (miRNAs, miRs), small single-stranded non-coding RNA molecules are currently being extensively evaluated as potential biomarkers in numerous diseases, including cancer. The evaluation of the potential of miRNAs to modulate or predict radio sensitivity or radio resistance, to anticipate the risk of recurrence and metastasis, and to differentiate different tumor subtypes is based on multiple mechanisms by which mRNAs control proliferation and apoptosis and interact with cell cycle phases or act as oncogenes with the potential to influence invasion promotion or tumor suppression. A refinement of radio sensitivity based on miRNAs with clinical and radiobiological application in head and neck cancers can lead to a personalization of radiotherapy. Thus, a miRNA signature can anticipate the risk of toxicity associated with chemo radiation, the possibility of obtaining loco-regional control after treatment, and the recurrence and distant metastasis risk. The potential of

miRNAs as an intrinsic predictor of sensitivity to chemotherapy may also guide the therapeutic decision toward choosing an escalation or de-escalation of concurrent or sequential systemic treatment. The choice of the irradiated dose, the fractional dose, the fractionation scheme, and the refining of the dose-volume constraints depending on the radio sensitivity of each tissue type estimated on a case-by-case basis by miRNAs profile are possible concepts for the future radiotherapy and radiobiology of head and neck cancers.

With an annual incidence of over 500,000 new cases worldwide, the severity of oral cancer is given by the unfavorable prognosis, the 5-year survival being about 40% even if maximum treatment is administered (Vigneswaran, 2014). The pattern of therapeutic failure is mainly loco regional recurrence which occurs in 15–50% of cases, but also distant metastasis can be a cause of disease progression (Chang, 2017). Resistance to oncological therapies (chemotherapy, radiotherapy, molecular therapy, and immunotherapy) is the main pathophysiological phenomenon that underlies the therapeutic failure of this type of cancer. Radiotherapy is part of the adjuvant or definitive treatment of HNSCC as a single treatment or in combination with therapies with synergistic and radio sensitizing potential, such as chemotherapy or targeted molecular therapy. Although the combination of radiation therapy with chemotherapy demonstrates a potential benefit on tumor control, the toxic effects associated with concurrent treatment are severe and may alter the quality of life (QoL) or may even limit the survival of these patients (Langendijk, 2008). In this context, the identification of agents that increase tumor radio-sensitivity could lead to increased local control rates without increasing the rate of toxicity. Even if radio-sensitizing agents are not used, there are significant variations in the tumor response to irradiation, modulated by intrinsic factors that modulate radio-sensitivity. Protein-encoding genes can modulate the response of tumor and normal tissues to irradiation, but direct gene manipulation is difficult (Pardo-Reoyo, 2016).

MiRNAs, a class of small endogenous non-coding RNA, generally composed of 22 nucleotides, can provide new horizons in the control of intrinsic radio-sensitivity by post-transcriptional regulation of gene expression. It has been shown that miRNA overexpression or knock-down may both alter radio-sensitivity of a tumor or normal tissue (Bourhis, 2007; Pardo-Reoyo, 2016; Zhao, 2012). Thus, evaluation of this miRNAs' expression can predict DNA damage or lead to cell-cycle checkpoint manipulation. Knowledge of each miRNA role may result in the design of radiobiological models based on miRNAs with direct clinical applications (Bourhis, 2007).

II.3.2. Aim of Study

Without intending to study the depth of the mechanisms of involvement of each miRNA in modulating radio-sensitivity, we want to provide a starting point for radiation oncologist clinicians with an interest in translational research in understanding and applying in clinical trials data obtained from fundamental miRNA research in head and neck cancers. By presenting epidemiological and etiopathogenic data, we wish to provide a miRNA-based bridge to understanding the different response to irradiation with a special focus on differentiating HPV+ and HPV− subtypes from oral cancers. Last but not least, the current context created by the COVID-19 pandemic with consequences in the rapid implementation of hypo fractionation schemes simultaneously with the large-scale implementation of immunotherapy having potentially synergistic with irradiation requires an in-depth understanding and refinement of the head and neck cancers radiobiology, beyond the basics of the linear quadratic model.

II.3.3. MicroRNA Involvement in Oral Cancer Development

Human papillomaviruses (HPV) are involved in the etiopathogenesis of at least three type of cancers (cervical, anal, and HNSCC). Although the involvement of HPV infection in the case of cervical cancer is incriminated in almost all cases, there is only a part of head and neck cancers associated with this viral infection. It is obvious that the HPV infection causes the hijacking of the host cell pathways, but the identification of the targets within this cell as well as their contribution to the malignancy process is also of major importance.

The Hippo pathway, involved in epithelial homeostasis, is thought to be involved in the carcinogenesis and progression of HPV-induced cancer. Morgan and collaborators propose the activation of the Hippo pathway as a therapeutic target in cancers associated with HPV infection. Serine/threonine-protein kinase 4 (STK4), the master Hippo kinase is identified as low in HPV-associated cancers and HPV-associated proteins E6 and E7 up-regulate the miR-18, thus promoting tumor genesis by inhibiting STK4. The suppressive effect of miR-18 on STK4 and indirectly tumor promotion is identified not only in HPV-induced cancers but also in prostate cancer (Morgan, 2020, Klein, 2010).

The development of the tumor phenotype is based on alterations in tumor suppressor oncogenes in tumor cells and stromal cells. These phenomena have the effect of transforming the normal epithelium into carcinoma in situ and subsequently into invasive squamous cell carcinoma. Loss of p53 control by viral mutations has the final effect of carcinogenesis by inhibiting apoptosis. In HPV-positive cancers, inhibition of p53 is mediated by the E6 protein characteristic of the HPV16 subtype. Tumor proliferation is also supported by p16 inactivation and overexpression of cyclin D in HNSCC. Epidermal growth factor (EGFR) is involved in the control of multiple pathways that mediate both proliferation and invasion and migration. EGFR also modulates tumor survival and angiogenesis, and EGF-induced STAT3 signaling initiates the transcription of genes that modulate cell growth, survival, and angiogenesis (Cyclyn D1, Bcl-XL, and VEGF, respectively) (Morgan, 2020, Klein, 2010, Shi, 2021, Acunzo, 2016, Jérôme, 2007, Hui, 2009, Spadaccino, 2021, Cai, 2017).

Progression and metastasis involve the interaction of the tumor with the tumor microenvironment and the remodeling of it and the stromal cells, a cytokine-mediated interaction. Angiogenesis, basement membrane modification, and tumor proliferation are also processes in which cytokines are involved. Infiltrating immune and endothelial cells but also cancer fibroblasts have been associated with the process of distant metastasis. Cancer-associated fibroblasts already shown to be associated with tumor progression have been correlated by Shi et al. with the occurrence of lung metastasis. Evaluated in a preclinical model, TGFβ-enriched fibroblasts and TGFβ activation were correlated with the risk of micro metastases (Alural, 2017, Cai, 2017, Kishore, 2014).

The role of miRNA in the carcinogenesis process is both to regulate genes that suppress tumors, their blocking being associated with carcinogenesis, but also to block oncogenes. Deletion of miRNA genes leads to increased oncogenic production, favoring tumor progression (Cai, 2017; Hui, 2009). Moreover, miR-15a and miR-16-1 are the first miRNAs identified as involved in carcinogenesis, being detected in more than 50% of cases of chronic leukemia. The oncogenic effect of these two miRNAs is mediated by the modulation of the BCL-2 anti-apoptotic gene (Acunzo, 2016, Jérôme, 2007). MiR-21 has been shown to be frequently identified in HNSCC and has the role of reducing the expression of PTEN, a modulator of the phosphoinositide 3-kinasePI3K pathway, the most commonly mutated pathway in HNSCC. Another miRNA involved in the carcinogenesis of head and neck cancers is miR-31. It promotes tumor progression and angioneogenesis by activating the hypoxia-inducible factor (HIF) pathway. MiR-375, identified in over 90% of HNSCC, is considered a tumor suppressor (Cai, 2017, Hui, 2009, Spadaccino, 2021). miRNA-34a is involved in tumor suppression, its reduced expression being identified in pancreatic, breast,

and lung cancer (Chang, 2007, Bommer, 2007, Amit, 2020). MiR-34a demonstrated a significant role in mediating apoptosis, senescence, and related to p53-mediated cell cycle arrest, directly targeting cyclin E2, BCL-2cyclin-dependent kinases (CDK4 and CDK6). MiR-34 family loss in tumors was also associated with tumorigenesis (Chang, 2007, Bommer, 2007).

Recent studies demonstrate the potential of miR-34a to modulate tumor growth by modification of the p53-mediated tumor microenvironment (Amit, 2020). P53 modulates cell survival through indirect control of hypoxia and angiogenesis. Knockdown of endogenous miR-107 may act to amplify inducible factor-1beta hypoxia (HIF-1beta) and overexpression of miR-107 reduces angiogenesis. MiR-192 and miR-215 are effectors and regulators of p53 and may suppress tumor genesis by cell cycle arrest (Yamakuchi, 2010). No miRNA has been shown to directly modulate capillary tumor extravasation to favor metastasis, but miR-520/373, miR-204, and miR-200 modulate tumor angiogenesis via TGFβ by tumor-associated fibroblasts (TAFs) (Braun, 2008). MiR-30a-5p interacts with the MET and EGFR pathways thus being an indirect suppressor of tumor growth (Kanchan, 2020).

Mammalian target of rapamycin (mTOR) of serine-threonine kinase protein with PI3K/Akt pathway signaling role also has a role in tumor proliferation independent of EGFR and p53. The mTOR pathway can also modulate epithelial-mesenchymal transition (EMT), thus being a promoter of tumor migration. MiR-7, miR-99a, miR-100, and miR-101 are just some of the miRNAs involved in modulating this pathway in cancer (Saleh, 2019). MiRNA-7 inhibits tumor growth and metastasis by targeting the phosphatidylinositol 3-kinase/Akt pathway in hepatocellular carcinoma (Dong, 2014). Silencing of miR-17-5p can block HNSCC tumor cells in the G2/M phase, thus demonstrating the potential of this miRNA to promote tumor growth and progression (Fang, 2014).

II.3.4. miRNAs and cancer - implications in clinical practice, focus on Radiobiology

The interest in the value of miRNA in the radiobiology of the future is justified by the vast number of reports that mention the involvement of these small non-coding RNAs both in the development and normal function of organs (brain development and functioning) and in the pathogenesis of diseases such as neuropsychiatric disorders, schizophrenia or bipolar disorder, atherosclerosis, cardiac hypertrophy, and systemic lupus erythematous. Their huge potential for regulatory molecules opens new horizons in the medicine of the future and in the treatment and diagnosis of many diseases (Alural, 2017, Kishore, 2014, Ardekani, 2010). The modulation potential of radio-sensitivity is given by the involvement of miRNAs in the differentiation and proliferation and cell death. By specifically altering these cellular functions of both cancer and other diseases, miRNA demonstrates its potential to be used as a circulating noninvasive biomarker. Even if the many prognostic and predictive risk factors for the cancer evolution are currently used in daily clinical practice (such as tumor staging according to TNM classification, histological type, degree of cell differentiation, genetic mutations, and expressions of some proteins with biomarker value), a refining of these biomarkers is necessary, especially regarding both the tumor and the normal tissue response to irradiation. Thus, it will be easier to anticipate as accurately as possible the risk of toxicity but also the possibility of tumor control for each individual case (Alural, 2017, Ardekani, 2010, Aravin, 2005).

As early as 25 years ago, the different response to irradiation was considered a consequence of the variation in intrinsic radio-sensitivity. Pekkola-Heino and collaborators, 2005, evaluated the average value of radio-sensitivity at 1.9 Gy with variations between 1.0 Gy and 2.8 Gy, finding sensitive differences even between cell populations (Pekkola-Heino, 2005). Cancers are also among the diseases in which miRNAs are involved both in down-

regulation and up-regulation of different genes involved in tumor radio-sensitivity by activating mechanisms of radio-resistance or radio-sensitization.

MiRNAs' impact can be augmented via a carcinogenesis effect exercised by down-regulation of the tumor suppressor gene or by modulating cell proliferation and apoptosis. The large amount of evidence that makes the connection between down-regulation, up-regulation, knockdown, overexpression, or other dys-regulation of miRNAs in various cancers justifies the hypothesis that these small single-stranded non-coding RNA molecules will play an essential role in the oncology of the near future. MiRNAs are modulators of radio-sensitivity through effects on phenomena such as cell damage repair, apoptosis, and free radical generation (Lhakhang, 2012, Petrovi´c, 2021, Metheetrairut, 2013). Mi-R-139-5p is associated with the accumulation of DNA damage caused by irradiation via the methionine adenosyltransferase 2A (MAT2A) gene.

Another pathway for modulating DNA damage involved in radio-sensitivity is the mutant ataxia-telangiectasia (ATM) and ataxia-telangiectasia (ATR) genes that modulate DNA damage repair via cyclin-dependent kinase (CDK) (Saleh, 2019, Dong, 2014). MiR-16 and miiR-15/ab are involved in the modulation of this pathway. Blocking the formation of CDK/Cyclin complexes limits the transition of the cell from phase G1 to phase S and from phase G2 to phase M. MiR-15 family and miR-449 are involved in G2 and G2/M phase arrest. ATM/P53/P21 is another pathway with cell cycle implications and miR-200c, miR-375, and miR-106b controlled this pathway (Chen, 2021, Pajic, 2018, Yoo, 2007). Also worth mentioning is miR-208a, a radiation-induced mi-RNA that can produce radio-resistance by activating the AKT/mTOR pathway (Chen, 2021, Pajic, 2018, Yoo, 2007, Zheng, 2015, Tang, 2016). A direct or inverse correlation between miRNA and specific proteins, determinants of tumor radio-sensitivity in breast cancer, has been identified.

Among these proteins, we mention proteins that are therapeutic targets in several types of cancers: vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR), human epidermal growth factor receptor-2 (Her-2/neu) whose status is involved in the molecular classification of breast cancer, and p53, a well-known predictor both of radio-sensitivity and of sensitivity to platinum-based chemotherapy. The mechanism of direct and inverse correlation is given by the role of these proteins in regulating the expression of some miRNAs, but miRNAs also have a role in regulating proteins at the post-transcriptional level (Shi, 2013, Dittmann, 2017). MiR-18a has the potential to be a radio-sensitivity biomarker and an actor that modulates radio-resistance, having a suppressive effect on ATM genes, with direct influence on the ability to repair double-strand DNA breaks after irradiation (Alural, 2017, Kishore, 2014). Without considering that we cover the entire vast field of radio-sensitivity modulation, the purpose of the example was to highlight the role of miRNAs in the radio-sensitivity of one of the most heterogeneous cancers, HNSCC. MiR-218 identified in tumor tissue is associated with unfavorable evolution and cancer progression. In fact, it has been shown that up-regulation of miR-218 is also involved in potentiating irradiation-induced apoptosis and due to this mechanism; miR-218 becomes a potential biomarker of radio-sensitivity in cervical cancer. EMT and angiogenesis are regulated in colorectal cancer by miR-218 (Lun, 2018). MiR-145 expression is associated with radio-sensitivity in high-risk human papilloma virus (HPV) associated with cervical cancer, the mechanism being one of synergistic interaction with long non-coding RNA mucosa-associated lymphoid tissue via the lymphoma translocation protein 1 (MALAT1) (Liu, 2015, Yuan, 2014, Lu, 2016).

MiR-139 can modulate radio-sensitivity by accumulating DNA damage, the mechanism being mediated by the MAT2A gene (Jérôme, 2007). The accumulation of radiation-induced DNA damage has as its substrate the inhibition of two mechanisms: non-homologous end joining (NHEJ) and homologous recombination (HR) pathways, methods by which under normal conditions DNA damage is repaired. If the total repair of the lesions has occurred, the

cell may die by entering in the apoptosis, suffering a mitotic catastrophe, or it may continue the normal cell cycle (Maier, 2016). MiR-208a-modulated radio-sensitivity has been demonstrated in both preclinical cell and animal models and miR-208a has been associated with radio-resistance and proliferation in lung cancer. In patients with non-small cell lung carcinoma (NSCLC), overexpression of hsa-miR-96-5p and hsa-miR-874-3p associated with irradiation may potentiate the tumoricid effect, the results being similar to the situation where systemic agents targeting HR and NHEJ pathways are added to radiotherapy (Piotto,2018, Chen , 2021). PTEN down-regulation has as a consequence the activation of the PI3K/AKT pathway, associated with radio-resistance and tumor proliferation. MiR-10b reduces the anti-proliferative effect of irradiation by activating caspase 3/7 and inhibiting Bcl-2 expression (Zhang, 2015, Li, 2009, Zhen, 2016, Kabzinski, 2021). Thus, activating p-AKT, miR-10b expression reduces the sensitivity of glioblastoma to irradiation promoting proliferation and tumoral invasion (Li, 2009, Zhen, 2016).

II.3.5. miRNA in Head and Neck Cancers—from the biomarker of the future to the orchestrator of radio-sensitivity

The huge potential of miRNAs to become valuable and accurate biomarkers for diagnostic and prognosis in the future is supported by several basic characteristics: they are rapidly synthesized in a certain clinical situation, have a high specificity, and remain in the environment from which they are identified a long period of time. It is generally considered that there are three types of diseases in which miRNAs have and will have a key role as a biomarker (cardiovascular diseases, infectious diseases, and neoplasms) (Lun, 2018, Liu, 2015, Yuan, 2014, Lu, 2016). Nearly a decade after the launch of the first miRNA panel that opened the horizons for the widespread use of miRNAs in medical practice, there are still problems standardizing and establishing relationships between a miRNA or set of miRNAs and a particular disease. In clinical practice, the first use of miRNA as a biomarker is attributed to Lawrie and collaborators who, in 2008, identified higher levels of tumor-specific miRNAs in patients with large B-cell lymphoma. MiR-138 was associated with the control of transcriptional activity of E-cadherin and elevated levels were negatively correlated with the risk of metastasis. Being commonly reported in HNSCC miR-138, associated with EMT this miRNA is a potential prognostic biomarker for HNSCC. Different results are reported for miR-205-5p; identified in peritumoral tissue, this miRNA is associated with early detection of minimal residual disease involved in tumor development.

MiR-205-5p is also considered a tumor suppressor and a limiter of tumor migration and invasion in squamous cell carcinoma of the oral cavity. An association with let-7d of this miRNA may constitute a combined biomarker of survival and prognosis in HNSCC (Liu, 2011, Sha, 2017, Bao, 2017, Childs, 2009). Reduced miR-29c-5p expression is associated not only with the malignant phenotype in HNSCC but also has prognostic and therapeutic implications. Up-regulation of miR-29c-5p is correlated with the arrest of tumor cells in the G2/M phase, being associated with reduced tumor proliferation both in vivo and in vitro. This miRNA proves to be not only a biomarker but also a potential therapeutic target (Li, 2009).

Intensity Modulated Radiation Therapy (IMRT) in a dose of at least 70Gy in 35 daily fraction, 5 fractions per week over 7 weeks, and concurrent chemotherapy with Cisplatin is currently the therapeutic standard for the treatment of locally advanced non-metastatic head and neck cancers. Cisplatin, a platinum-based alkylating agent, has the potential for radio-sensitization, being, so far, the backbone of the concurrent association between radiation therapy and systemic therapy. Even if given weekly at a dose of 40mg/m2 or 100 mg/m2 every 3 weeks, new evidence indicates that a cumulative dose of at least 200 mg/m2 is associated with therapeutic benefit. In addition, the stratification of patients with squamous cell carcinomas of the head and neck (HNSCCs) according to human papilloma virus (HPV)

status in HPV+ and HPV− tumors showed differences in response to chemo-radiation with a higher response rate in non-smokers, HPV+ (Van Gestel, 2011, Rühle, 2021, Tao, 2020, Wittekindt, 2017, Strojan, 2016, Al-Mamgani, 2017).

Although the role of induction chemotherapy is still controversial, the triple combination of TPF (Docetaxel, Carboplatin, and Fluorouracil) followed by platinum-based chemo-radiation (CCRT) may be considered with benefits in loco-regional control for certain categories of patients. Contrary to expectations, trials that proposed de-escalating treatment for oro-pharyngeal HNSCC HPV + cases were negative. However, some controversial topics exist regarding the concept of de-escalation, requiring a refinement of results. Analyzing the data from a small Italian phase II trial, the significantly lower (50% *vs*. 83%) OS at 2 years for patients who received Cetuximab and not Cisplatin suggests that it can be estimated that it is not the reduction of irradiation dose that would be the cause of treatment failure but de-escalation of chemotherapy by substituting Cisplatin with Cetuximab (Anderson, 2019, Magrini, 2016, Haddad, 2018). If currently these biomarkers of radio-sensitivity have no implications in the clinical decision, the de-escalation of chemo-radiotherapy treatment in certain groups of patients being only the subject of clinical trials, miRNAs open new horizons in customizing treatments based on different radio-chemo-sensitivity (Korpela, 2015, Ahmad, 2017, Nowicka, 2019).

Analyzing data obtained from 515 HNSCC samples and 44 normal tissues, Luo et al. highlights deregulated miRNAs for both positive and negative HPV HNSCC cases (96). In the patient lot including 97 HPV + patients, 282 miRNAs were identified and after statistical analysis, a 7 miRNA signature was considered proper for its prognostic value. In the lot including 418 HPV− HNSCC patients, among 289 miRNAs, 6 were included in the prognostic signature. Importantly, the signature of miRNAs for the two types of HNSCC is completely different. In the HPV+ group, miRNAs associated with the unfavorable prognosis are negatively correlated with CD8+ T lymphocytes. MiRNAs associated with a better OS have been associated with NK cells and T regulatory cells (Tregs). In the HPV− tumors group, miR-605-5p was associated with CD8 + T cells, activated CD4 T cells (considered tumor suppressors), and M1 macrophages. MiR-135-3p was associated with a better survival and it was negatively correlated with M2 macrophages. The authors also propose a risk score based on miRNA, mentioning the involvement of miRNAs correlated with CD8 + T lymphocytes and NK cells, Tregs, and T follicular helper cells (TFH) in the tumor microenvironment in pre-existing antitumor immune response. Cases of HPV− with a high risk of miRNAs could benefit more from target therapies and HPV+ patients with a low risk of miRNAs could benefit more from immunotherapy. The authors mention metabolic disorder as a possible cause of therapeutic failure in HPV− patients with an increased miRNA risk score (Kabzinski, 2021, Bartels, 2009, Luo, 2021).

Resistance to Cisplatin is modulated by cancer-associated fibroblasts (CAF) which in turn regulate cell survival and proliferation in head and neck cancers. The mechanism of restoration of miR-196a sensitivity to Cisplatin is related to depletion of miR-196a in CAF, and the authors demonstrate the exosomal capacity of miR-196a to be a biomarker of Cisplatin sensitivity (Salazar-Ruales, 2018). Factors involved in HNSCC radio-resistance or radio-sensitivity are numerous and are generally common to all cancers including traditional variable like cells lesions repair capacity, hypoxia, cell cycle position, and cell growth fraction, but also new "actors" such as hepatocyte growth factor receptor (HGFR) or programmed cell death protein 1 and programmed cell death protein 1 (PD-1) and its ligand, programmed death-ligand 1 (PD-L1) (Menegakis, 2021, Zölzer, 2002, Fiedler, 2018, Chang, 2014).

HNSCC is a malignancy of a special interest for the improvement of therapeutic ratio due to the importance of radiotherapy in both adjuvant treatment and definitive treatment in

association with chemotherapy or in particular cases with target therapy. The proximity of radiosensitive organs which are exposed to potentially severe or even life-threatening toxicities by irradiation with tumoricid doses is a serious argument both for improving the technique and conformity of delivered radiation dose as accurately as possible to target volumes in prescribed isodose but also for improving prediction by radiobiological modeling based on new evidence of radio-sensitivity (Van Gestel, 2011, Rühle, 2021, Tao, 2020, Wittekindt, 2017, Strojan, 2016). The RAS, EGFR, and PI3K/AKT pathways are involved in this process of modulating tumor radio-sensitivity. EGFR is currently a clinically validated therapeutic target by using anti-EGFR monoclonal antibodies (cetuximab) in both metastatic and locally advanced settings but also has prognostic value, increased EGFR expression being associated with radio-resistance and therapeutic failure. The TP53 suppressor gene, a regulator of genome stability and consequently a manipulator of DNA degradation, is mutant in 40–70% of HNSCC and thus inactivates the product or protein with effect on the cell cycle. Tumor hypoxia induces neovascularization and, by modulating the response to DNA damage, indirectly influences intrinsic radio-sensitivity (Mehanna, 2017; Huang, 2020). The mutation in the TP53 gene leads to the inactivation of its protein (p53) product and the change in p53 leads to an impaired ability to stop the cell cycle and inhibit apoptosis. Hypoxia-induced neovascularization and epithelial-mesenchymal transition (EMT), a phenomenon that modulates invasion and resistance to apoptosis, are also involved in tumor radio-resistance in HNSCC. Cancer stem cell (SCC) populations, by their ability to renew and proliferate indefinitely and the potential for differentiation, are also associated with radio-resistance. A signature consisting of 4 and 12 miRNAs associated with TP53 were identified in HNSCC as being correlated with non-recurrent survival and cancer-specific survival, respectively. MiR-96-5p is overexpressed in tumors that show a p53 mutant, being involved in chemo-resistance and radio-resistance by activating the PI3K-AKT pathway (Horn, 2015, Mohammadi, 2021, Steinbichler, 2019).

The role of miRNAs in HNSCC radio-resistance phenomena has been demonstrated for a group of miR-16, miR-29b, miR-1254, and miR-150 up-regulated miRNAs and the down-regulated let-7e miRNA for the situation where radio-sensitivity is not mediated by ATM gene (Van Gestel, 2011, Tao, 2020, Ahmad, 2017). MiR-196a and miR-1323 are considered to have oncogenic potential and down-regulation of miR-205 and up-regulation of miR-96 have been associated with radio-resistance but through different signaling pathways. Of note is the different effect that the same miRNA has in two different anatomical localizations of cancer for the same pathological tumoral type. The low level of miR-203 predicts an unfavorable prognosis and early recurrence after radiotherapy in laryngeal cancer and the same miRNA is associated with radio-sensitivity in nasopharyngeal cancer cell populations (Kabzinski, 2021, Vahabi, 2021, Ahmad, 2017, Nowicka, 2019). Given the need for a validated biomarker to be accessible and evaluable in dynamics, the conceptual use of miRNAs as biomarkers obtained from body fluids is a goal of translational research in personalized medicine and particularly in oncology. One of the most studied miRNAs that can be obtained from serum, plasma, or saliva is miR-21. In head and neck cancers, the high expression of this miRNA is associated with an unfavorable prognosis, the risk of distant metastasis. As demonstrated by a study that included 50 HNSCC patients, elevated postoperative miR-21 levels were associated with unfavorable 1-year survival and this observation offers the opportunity for miRNAs to be used as biomarkers in head and neck cancer surgery (Ahmad, 2017, Luo, 2013). Down-regulated serum miR-9 has been associated with recurrence and metastasis in nasopharyngeal cancer and miR-31 is involved in the regulation of the hypoxia pathway, a well-known radio-resistance factor. A low level of miR-31 in the blood was also observed in nasopharyngeal cancer compared to the level of this miRNA in normal nasopharyngeal tissues. HPV status with an essential role in radio-

sensitivity of head and neck cancers can be differentiated based on miRNA (miR-9, miR-122, miR-124, miR-134) in p16 positive and negative, as was demonstrated by Salazar-Ruales et al. (2018) and Wan 2017, Vahabi, 2019, John, 2013, Hsu, 2012 and Arantes, 2017). In oral cancer, miR-802 was identified as down-regulated in approximately 60% of cases using this level of miRNA in normal tissue as a reference.

For a relatively "orphan" tumor subtype in terms of systemic treatments with curative potential (cystic adenoid carcinoma of the head and neck), it has been shown that high levels of miR-374c are associated with reduced recurrence rate and miR-21 inhibitors in association with Sinvastatin have shown anti-proliferative potential in lung metastases of cystic adenoid carcinoma of the salivary glands (Ahmad, 2017, Salazar-Ruales, 2018, Wang, 2018, Maia, 2015).

Without proposing to include the vast number of research papers that analyze the involvement of miRNAs in the radio-sensitivity of head and neck cancers, we present in a table the main studies in the field, also mentioning the mechanism of action, binding site, and the radio-sensitizing or the radio-resistance augmentation effect for each mentioned miRNA. (Table II.3.1).

II.3.6. Conclusions

MiRNAs are currently intensively evaluated as potential biomarkers in numerous diseases and cancers. Determining the ability of miRNAs to modulate or predict radio-sensitivity and resistance, to anticipate the risk of recurrence and metastasis, and to differentiate different tumor subtypes is based on multiple mechanisms by which mRNAs control proliferation and apoptosis, interact with cell cycle phases, and mechanisms by which miRNAs act as oncogenes with the potential to influence invasion promotion or tumor suppression.

A refining of biomarkers of radio-sensitivity miRNAs with clinical application in head and neck cancers can lead to a personalization of radiotherapy by anticipating the risk of toxicity, loco-regional tumor control, recurrence, and metastasis. The potential to be an intrinsic predictor of sensitivity to chemotherapy may also guide the decision to choose an escalation or de-escalation of concurrent or sequential systemic treatment. Choosing the total irradiation dose, the dose per fraction, the fractionation scheme, and refining the dose-volume constraints according to the radio-sensitivity of each tissue type estimated on a case-by-case basis by miRNAs profile are possible concepts and may be the basis for radiotherapy customization and radiobiology of head and neck cancers in the near future.

miRNAs will be able to answer questions about the groups that will benefit from de-escalation of treatment and miRNA signatures could stratify patients who will benefit from hypo-fractionated radiotherapy and we can anticipate that they will answer the controversies regarding synergistic irradiation with immunotherapy in oral cancer.

Table II.3.1
MiRNAs modulate the radio-sensitivity of head and neck cancer

mi-RNA	Tissue of Origin	Cancer Type	Mechanism of Action	Binding Site	Radiosensitivity	References
miR-23a	cells	nasopharynx	targeting IL-8/Stat3 pathway	GJA1; p53 (Gindin et al. 2015) [154]	radiosensitivity	Qu et al. 2015 [126]
miR-24	tissue and cells	nasopharynx	inhibits Jab1/CSN5 translation	dihydrofolate reductase gene (Mihsra et al. 2007) [150]	radiosensitivity	Wang et al.2016 [127]
miR-494-3p	cells	oral cavity SCC	downregulation of Bmi1 pathway	SIRT3 (Zeng et al. 2021) [156]	radiosensitivity	Weng et al. 2016 [166]
miR-375	tissue	oral cavity SCC	targeting insulin-like growth factor 1 receptor	KIT; JAK2 (Gyvyte et al. 2020) [157] (Ding et al. 2010) [158]	radiosensitivity	Zhang et al. 2017 [120]
miR-182-5p	cells	HNSCC	radiation-induced antioxidant effect through SESN2	STARD13 (Wu et al. 2021) [159]	radiosensitivity	Lin et al. 2021 [128]
miR-503	cells	HNSCC	inhibition of WEE1	CUGBP1; CCND1; VEGF; E2 F3 (Cui et al. 2021) (Xu et al. 2013) (Ikari et. al 2017) [160–162]	radiosensitivity	Ma et al. 2017 [129]
miR-150	cells	nasopharyngeal	targeting glycogen synthase kinase-3β	MALAT1 (Liu et al. 2021) [163]	radioresistance	Huang et al. 2018 [125]
miR-138-1-3p	cells	nasopharynx	EMT and the JAK2/STAT3 signaling pathway	CRIPTO (Du et al. 2021) [130]	radiosensitivity	Du et al. 2021 [130]
miR-195-3p	tissue and cells	nasopharynx	inhibits cyclin dependent kinase 1	CDK1 (Xie at al. 2021) [131]	radiosensitivity	Xie at al. 2021 [131]
miR-19b-3p	cells	nasopharynx	activating the TNFAIP3/NF-κB axis	TNFAIP3 (Huang et al.) 2016 [132]	radioresistance	Huang et al. 2016 [132]
miR-BART4	tissue and cells	nasopharynx	targeting PTEN	PTEN (Wu et al. 2018) [133]	radioresistance	Wu et al. 2018 [133]
miR-96-5p	cells	HNSCC	PI3K-Akt signaling pathway	PTEN (Vahabi et al. 2019) [105]	radioresistance	Vahabi et al. 2019 [105]

Table II.3.1
MiRNAs that modulate the radio-sensitivity of head and neck cancer

mi-RNA	Tissue of Origin	Cancer Type	Mechanism of Action	Binding Site	Radiosensitivity	References
miR-296-5p	tissue	larynx	not specified PIN1	PIN1(Lee et al. 2014) [135]	radioresistance	Maia et al. 2015 [113]
miR-324-3p	cells and tissue	nasopharynx	targeting WNT2B	RelA promoter (Dharap et al. 2013) [136]	radiosensitivity	Liu et al. 2017 [114]
miR-203	Cells	HNSCC nasopharyngeal	EMT modulation targeting IL8/AKT	PKCα (Wang et al. 2013) [137]	radiosesitivity	de Jong et al. 2015 [115] Qu et al. 2015 [119]
miR-324-3p, miR-93-3p miR-4501	tissue	nasopharynx	down-regulation/*CDH1, PTENP1* and *HSP90AA1*	PEDF (Wang et al. 2017) [138]	radioresistance	Li et al. 2013 [116]
miR-371a-5p, miR-34c-5p and miR-1323	tissue	nasopharynx	up-regulation/*ICAM1, WNT2B, MYC, HLA-F,* and *TGF-β1 pathways*	Xiap (Du et al. 2016) [139] XBP1(Bartoszewska et al. 2019) [140] BMP4 and SMAD4; PRKDC (Xie et al. 2020) [141]	radioresistance	Li et al. 2013 [116]
miR-27a-3p	cells	HPV+ HNSCC	up-regulation/DGCR8/miR-27a-3p/SMG1 axis	FBXW7 (Lu et al. 2021) [143]	radiosensitivity	Long et al. 2021 [117]
miR-106a	cells	HPV+ HNSCC	up-regulation/DGCR8/miR-106a/RUNX3 axis	L10; ASK1 (Sharma et al. 2020) [144] (Hong et al. 2018) [145]	radiosensitivity	Zhang et al. 2020 [118]
miR-375	tissue	oropharyngeal	targeting IGF-1R/cycle arrest in G0/G1 phase, increases apoptosis	YBX1 (Liu et al. 2016) [146]	radiosensitivity	Zhang et al. 2017 [120]
miR-9	cells tissue, cells, saliva	HPV + HNSCC	inducing M1 macrophage polarization via down-regulation of PPARδ by targeting KLF5, positively regulates the expression of Sp1	cyclin D1 and Ets1 (Zheng et al. 2013) [147]	radiosensitivity radioreistance	Tong et al. 2020 [164] Citron et al. 2021 [134]
miR-210	cells	HNSCC	modulation of hypoxia	MNT (Zhang et al. 2009) [148]	radioresistance	Gee et al. 2010 [121]
miR-196a	tissue and cells	HNSCC	suppressing annexin A1 /induce EMT	SNP (Wang et al. 2012) [149]	radioresistance	Suh et al. 2015 [122]
miR-24	tissue and cells	Larynx SCC	targeting X-linked inhibitor of apoptosis protein	dihydrofolate reductase gene (Mihsra et al. 2007) [150]	radiosensitivity	Xu et al. 2015 [165]
miR-495	tissue and cells	nasphparynx	targeting GRP78 to regulate EMT	Sox9 (Lee et al. 2014) [151]	radiosensitivity	Feng et al. 2018 [124]
miR-150	cells	nasopharynx	targeting glycogen synthase kinase-3β	Rab1a and Rab31 (Liu et al. 2015) [152]	radiresistance	Huang et al. 2018 [125]
miR-205	cells	nasopharynx	directly targeting PTEN	ErbB3; VEGF-A (Wu et al. 2009) [153]	radioresistance	Qu et al. 2012 [119]

II.4. p53 in oral cancers radio-sensitivity- from classic to future horizons

p53, initially considered a tumor suppressor, has been the subject of research relate with cancer treatment resistance in the last 30 years. The unfavorable response to multimodal therapy and the higher recurrence rate despite an aggressive approach of head and neck cancers, even if it is only the 6'th most common malignancy worldwide, is a research topic of interest for improving therapeutic outcomes of HNSCC. New advances in molecular biology and genetics include the involvement of mi RNA's in the control of the p53 pathway, the understanding of GOF mechanisms and loss of function, the development of different methods to restore p53 function both for HVP - and HPV + HNSCC cases. The different ratio between mutant p53 status in the primary tumor and distant metastasis originating HNSCC may serve to select the best therapeutic target for activating an abscopal effect by irradiation, as an activator of the immune system. P53 may also be a key player in choosing the radiotherapy fractionation regimen. Targeting any pathway involving p53, including tumor metabolism and in particular the Warburg effect could modulate radio-sensitivity and chemo-sensitivity of oral cancers.

Discovered in 1979 as a protein of about 53kDa expressed highly in cancer cells and then called p53 is considered today not only involved in DNA damage, but also a mediator of responses to cellular stress, being associated with sensitivity to irradiation and chemotherapy of malignant tumors. Scientific knowledge of current molecular biology has made a decisive contribution to understanding the mechanisms involved in p53 pathway by identifying an increasing number of post-transcriptional targets, but also by understanding p53-mediated apoptotic mechanisms. Testing and validation of agents and mechanisms targeting mutant and wild-type variants of p53 opens new perspectives for improving the therapeutic ratio of oncological therapies by enhancing tumor destruction and simultaneously protecting healthy tissues. Recently, immune checkpoint inhibitors (ICIs) entered the therapeutic spectrum of HNSCC and the potential biomarker value of the TP53 gene mutation, the most common genetic mutation in these cancers, associated with the accumulation of p53 in the malignant cell is investigated in correlation with tumor mutation burden (TMB) and tumor neo-antigens (TNA), both for HNSCC primary tumor and for distant metastases. Three therapeutic strategies are proposed for the restoration of p53 function and consequently for the improvement of therapeutic results in HNSCC: targeting the degradation or direct inhibition of wild-type p53 (WT), reactivation of transcriptional activity by binding mutant p53 and restoration of WT p53 status. The 3rdstrategy is associated with HPV + head and neck cancers and includes targeting E6/E7 enzymes (de Bakker, 2022, Lu, 2009, Kong, 2021).

II.4.1. p53 in immunotherapy ERA

The role of p53 is once again at the forefront with the introduction of immunotherapy in the multimodal treatment of cancers. Intracellular accumulation of hotspot mutations may be immunogenic resulting in the triggering of p53 neo-antigens associated with T lymphocyte-mediated intra-tumoral immune responses. The potential to use p53 antigens as therapeutic targets is proposed by Chasov (2021), monoclonal antibodies (mAbs) being part of the new strategy (Cortez, 2015).

P53 also seems to play an essential role in the era of immune-oncology. The accumulation effect of mutant p53 in the cancer cell induced by multiple mechanisms of viral infections such as degradation of p53 WT, inhibition the Rb protein has already been demonstrated, being related to tumor apoptosis. Not associated with the characteristic regulatory mechanisms of p53 WT including the inability to bind to DNA to promote MDM2 transcription, mutant p53 accumulated in the cell will become active antigen and generate a

more intense response to immunotherapy, resulting in cancer cell death. The expression of programmed cell death ligand 1 (PD-L1), one of the preferred targets of immunotherapy is down-regulated by mutant p53 via miR-34, thus demonstrating the involvement of p53 expression in the amplitude of the response to ICI therapy (Low, 2019, Sobhani, 2020, Sun, 2020).

Although KRAS/ATM/ EGFR/ TK11 co-mutations are considered independently predictive factors of the ICI response, not all types of p53 mutations appear to have the same predictive power. Although the missense and nonsense p53 alterations have not been mentioned before, the study by Sun et al. also evaluates these two types of mutations in relation to PD-L1 to anticipate the response to immunotherapy in lung adenocarcinoma (Marcu, 2019).

II.4.2. p53, hew horizons for head neck cancer treatment

Cancer stem cells (CSC) and hypoxia are key factors in radiation resistance being evaluated in personalization strategies for head and neck cancers radiation therapy in order to increase the rate of tumor control. Doses escalation, but also an increasingly popular method (hypo-fractionation) is a possible strategy to overcome the radio-resistance as even historical radiobiological studies mention. Hyper-fractionation, although considered a potential method, requires additional resources and can be difficult to implement. Marcu et al. use an experimental model *in silico* in order to evaluate the cell division probability, the average time of a cell cycle and the doubling time of the tumor volume for HNSCC. The values obtained (1.9%, 33 hours and 52 days respectively) for the variables mentioned above justify the authors' hypothesis that incipient toxic and hypoxic tumors may benefit from hypo-fractionation, but tumors with oxygen levels below 6 mmHg and a percentage of 5.9% CSC pre-treatment require either systemic adjuvant treatment or dose escalation to 81.6Gy. In the case of advanced tumors, hyper-fractionation is the authors' choice in the concept of overcoming radio-resistance.

Cisplatin and anti-epidermal growth factor receptor (EGFR) antibody Cetuximab are agents with demonstrated radio-sensitizing potential, already included in the HNSCC therapeutic protocol. Radiotherapy with weekly Cisplatin ($40mg/m^2$) administered until a total dose 70Gy in 35 daily fractions over 7 weeks or bio-radiotherapy with Cetuximab for platinum non-eligible cases is currently the definitive, non-surgical, standard treatment. De-escalation of treatment in cases of HNSCC HPV+ and the use of induction chemotherapy followed by chemo-radiotherapy for cases with potentially unfavorable outcome are options evaluated with possible benefits for carefully selected groups of patients with radio-sensitive and chemo-sensitive tumors (Homma. 2011, Gebre-Medhin, 2021 Yamamoto, 2016, Haddad, 2018, Anderson, 2019, Brachman, 1993).

Interest in p53 as a modulator of radio-sensitivity in head and neck cancers is not a recent research concern. A historical hypothesis associating the p53 mutation with the possible increase in radio-sensitivity in HNSCC has been contradicted more than 2 decades after the study conduce by Brachman et al. Ras, myc, and raf expression mutations have been associated with radio-resistance, thus demonstrating that the p53 mutation is not directly involved in tumor sensibility to irradiation (Jung, 1992, Tojyo, 2019,).

PD-L1 bound to the p53 protein is thought to influence both prognosis and response to treatment, particularly ICI. The study by Tojyo and collaborators evaluates the correlation between cytokeratin 17 (CK17), PD-L1, p53 and its value of diagnostic and prognostic biomarker of HNSCC. Analyzing data obtained from 48 patients with squamous cell carcinoma of the oral cavity PD-L1, p53 and CK17 were evaluated regarding possible clinical and pathological correlation. p53 status was associated with tumor stage T, TNM stage and PD-L1 expression, but CK17 was not correlated with p53 or prognosis (Seltzsam, 2018).

HNSCC associated with HPV infection has different biological, clinical, and therapeutic characteristics compared to classic HNSCC, which is generally associated with a long history of smoking. At the molecular level, there are differences that may explain different prognosis, evolution and response to therapeutic agents, whether it is chemotherapy, biological therapy, radiotherapy or ICI therapy. The p53 gene is not usually mutagen in HPV+ HNSCC, but the E6 viral onco-protein has the effect of inhibiting and proteasome degradation of HPV induced p53. A striking difference between HPV related HNSCC subtypes are that the tumor suppressor gene TP53 is not usually mutated into HPV+ cancer cells. However, p53 is inhibited by the viral onco-protein E6, leading to premature proteasomal degradation for this cancer subtype. Implications with therapeutic potential result in the design of methods to restore p53 function. Bortezomib, a proteasome inhibitor active in the treatment of multiple myeloma and mantle cell lymphoma, has been evaluated in cell lines in HNSCC HPV +, demonstrating its ability to restore p53 function and hypothetical restore radio-sensitivity via p21/p53 transactivation. However, in combination with radiotherapy or chemotherapy with Cisplatin, Bortezomide has no radio-sensitivity and chemo-sensitivity modulator effect on HNSCC HPV+ and HPV- cell lines (de Bakker, 2022).

For HPV- HNSCC multiple strategies are proposed for the restoration of p53 function in order to modulate radio-sensitivity. Targeting factors that degrade, inhibit or prevent p53 WT breakdown such as PM2, RITA nutlin-3, Ch1iB, MDMX/4, but also direct modulators of p53 binding and reactivation such as COTI-2, PRIMA-1, CP-31398, APR-246 are options proposed and evaluated for HNSCC HPV- (Waitzberg, 2004).

C-MYC, whose positive expression was identified in 35.7% HNSCC, is considered a mediator of p53 GOF, being associated with radio-resistance of head and neck cancers, but the association of C-MYC with p53 is also a negative prognostic factor. BYL719 (alpelisib), a PI3Kα-selective inhibitor, is proposed as a therapy for restoring radio-sensitivity by breaking the C-MYC p53 bond. A chemical chaperone (glycerol) has the potential to restore p53 function on HNSCC cell lines, being considered an agent with a possible role in p53-mediated radio-sensitization (Ganci, 2020, Ohnishi, 2000).

CIP2A is considered another possible therapeutic target of rapamycin for inducing senescence in HNSCC radio-resistant cells. The presence of large amounts of CIP2A in HNSCC radio-resistant cells with mutant p53 justifies strategies for modulating radio-resistance by controlling this pathway (Kim, 2019).

Oct4 and CIP2A in combination are considered to be potentiating factors for radiation resistance in head and neck cancers, and Ventelä and collaborators propose the use of the Oct4/CIP2A combination as a biomarker for the selection of radiation-resistant tumors. Analyzing p53, NDFIP1, EGFR and nuclear positivity of stem cell Oct4 marker and CIP2A, Routila et al did not identify a correlation between p53, EGFR, CIP2A and intrinsic radio-sensitivity, but stem cell Oct4 and NDFIP1 were correlated with radio-resistance in HNSCC (Ventelä. 2015).

The study by Klinakis et al. hypothesizes that mutant TP53 is more common in primary tumor than in distant metastases and the impact of TP53 mutation in metastatic disease regarding ICI treatment is was also assessed. The study group included 512 primary tumor biopsies and 134 distant metastases biopsies, all from HNSCC. The results indicate a lower frequency of TP53 mutations in metastatic disease, but the predominance of missense mutations. A higher TMB in metastases than primary tumors also justified a unfavorable response to immunotherapy for primary tumors. Ginkel's study highlights a 95% and a 91% concordance of p53 mutation in distant metastases respectively in loco-regional recurrences, by analyzing a lot of 239 HNSCC. Authors propose the use of p53 as a biomarker of response to treatment. A stratification of the prognosis and prediction of the response to chemotherapy and radiotherapy is also proposed by Zhou et al. based on p53 status, with a focus on the

possibilities of GOF for both WT and mutant p53 HNSCC cases (Routila, 2021, van Ginkel, 2016, Klinakis, 2020).

Variations in cell death rates and radio-sensitivity of tongue SCC classified according p53 status after X-rays (low-linear energy transfer (LET)) or carbon-ion beams (high-LET heavy ion) irradiation were evaluated by Asakawa and collaborators two decades ago. The study highlights a significant dependence of p53-mediated radio-sensitivity depending of the ionizing radiation type used. A limited ability to modulate radio-sensitivity and a lower rate of apoptosis associated with X-rays were used explains the authors' conclusion. In the case of carbon ions therapy the biological effect of irradiation does not involve the p53 pathway. The results become significant in current clinical practice with increasing interest for heavy ions radiotherapy in head and neck cancers, especially in HPV- subtype (Chen, 2019, Asakawa, 2002).

Loss of p53 function creates an Achilles heel in HNSCC as observed by Wilkie et al. by potentiating the Warburg effect. Loss of p53 function or mutation or down-regulation of p53 causes a lack of metabolic flexibility, malignant cells being more dependent on glycolysis by losing the ability to oxidative phosphorylation. The authors propose a strategy to increase the radio-sensitivity of HNSCC to HNSCC cells with impaired p53 function if a glycolysis inhibitor is used (Wilkie, 2020).

Without intending to cover the vast number of studies evaluating the involvement of p53 in HNSCC, we have synthesized data on all anatomical structures of head and neck. We also tried to synthesize in Table II.4.1 and Table II.4.2 different implications of the p53 pathway and some suggestive studies evaluating p53 as a modulator of radio-sensitivity in HNSCC

P53 acquires new valence due to testing of agents with the potential torestore/control p53 function for potential clinical benefit. Regardless of whether geno-toxic agents or inhibitors of the p53 pathways including the Warburg effect are used, steps forward have been made for testing of strategies that target p53 in clinical practice for both HNSCC HPV- and the viral etiology HPV+ subtype. Table II.4.1 include clinical trial initiatives including p53 pathway modulation therapies.

Table II.4.1
Various roles of p53 in different anatomical sites of oral cancer

Cancer type	**Mechanism of action**	**Results/Clinical implication**	**References**
All type of HNSCC	36 to 39 TP53 mutations detection	not specified	Peltonen et al. 2010
All type of HNSCC	identification of p53 as the most common somatic mutation	biomarker for prognosis and a predictor of clinical response to radiotherapy and chemotherapy	Zhou et al. 2016
All type of HNSCC	identification of p53 TP53 mutations in DNA-binding regions (L2, L3 and LSH motif)	marker for predicting prognosis and response to radiation	Peltonen et al. 2011
All type of HNSCC	TP53 mutation detection in 53.3% of patients	TP53 mutation is associated with reduced survival	Poeta et al. 2007
HNSCC treated surgically with curative intent	HPV16-positive and p53 mutation coexistence	possible implication in patient outcome	Westra et al. 2008
Oral cavity, oropharynx or larynx surgically treated	TP53 mutation detection	tobacco and alcohol consumption tumor histological grading correlation	Golusinski et al. 2016
HNSCC with	p53 mutations detection in	identification of p53 as in surgical	van Houten et al.

radical tumor resection	surgical margins	magins as prognostic factor for high recurrence risk	2002
Oral cavity SCC (OCSCC)	P53 mutation detection	not specified	Ragos et al. 2018
OCSCC	P53 identification in relation with carcinogens	high incidence of P53 mutation in Tabaco users.	Lazarus et al. 1996
OCSCC	betel quid chewing, alcohol use and smoking in relation to the p53 mutation	not specified	Hsieh et al. 2001
All type of HNSCC	correlation of the 36 TP53 mutations confirmed with carcinogens	smoking, alcohol and work history and no clinical correlation specified	Peltonen et al. 2010
Nasopharyngeal carcinoma (NPC)	identification of p53 protein in NPC primary tumor and metastatic nodes	no statistically significant correlation with p53 immuno-reactivity and overall and disease-free survival identified.	van Houten et al. 2002
All type of HNSCC	loss of p53 function	adrenergic trans-differentiation of tumor-associated sensory nerves with inhibition of tumor growth as a consequence	Amit et al. 2020
All type of HNSCC	overexpression of p53 protein was detection	not specified, only the association of the p53 mutation with carcinogens such as tobacco are mentioned	Somers et al. 1992
		p53 mutations is uncommon in virus-related HNSCC but common in oro-pharyngeal and hypo-pharyngeal carcinoma	Maruyama et al. 2014
All type of HNSCC	p53 protein degradation by the viral onco-protein E6 and p53 mutations inHPV16-positive tumors	inverse relationship between human papillomavirus-16 infection and disruptive p53 gene mutations, clinical implications not specified	Westra et al. 2008
All type of HNSCC	restoring the tumor suppressor activity of p53	Ad-E6 / E7-As and bortezomib may restore p53 function to improve therapeutic outcomes	de Bakker et al. 2022
Larynx HNSCC	evaluation of p53 as a predictor for larynx preservation	p53 and Bcl-xL are strong predictors of larynx preservation after induction chemotherapy and radiotherapy	Kumar et al. 2008
All type of HNSCC	evaluation of differences in the mutation profile of TP53 in primary and metastatic disease	TP53 mutations are associated with higher TMB score in only metastatic NHSCC associating an unfavorable response to ICI	Klinakis et al. 2022
All type of HNSCC	evidence of concordance between p53 mutations in primary disease and metastasis	TP53 is associated with metastases, recurrence and as post-treatment biomarker of disease evolution	van Ginkel et al. 2016
All type of HNSCC	p63 and p73 may act synergic with p53	p63 and p73 profile modulate sensitivity to chemotherapy	Gwosdz et al. 2005
OSCC and oropharynx SCC	evaluation of Ki-67, PCNA and p53 as prognostic factors	no relationship found between p53 or PCNA status and tumor prognosis	Sittel et al. 1999

Table II.4.2
Clinical trials based on p53 restoration therapy for HNSCC

Cancer type	p53 function restoration therapy	Clinical Trial/Endpoint	References
recurrent HNSCC	INGN 201	NCT00041626/Phase III/ Cisplatin and 5-Fluoruracil sensitivity	ClinicalTrials.gov NCT00041626
HNSCC	COTI-2	Phase I/ Tolerability	ClinicalTrials.gov NCT02433626
HNSCC	adenovirus-p53 gene (Gendicine) + radiotherapy	randomized controlled clinical trial/safety and efficacy	Zhang et al. 2005
liver Metastases of Solid Tumors and Recurrent HNSCC	Ad-p53 With Capecitabine (Xeloda) or Anti-PD-1	phase 1-20/ Safety and Efficacy	ClinicalTrials.gov NCT02842125
recurrent HNSCC	Ad5CMV-p53	phase II/ objective response rate	ClinicalTrials.gov NCT00003257
newly Diagnosed stage III/IV resecable Oral Cavity,Oropharynx, Hypo-pharynx, or Larynx SCC	Ad5CMV-p53 gene followed by Cisplatin and radiotherapy	phase II/ effectiveness	ClinicalTrials.gov NCT00017173
reccurent or metasatic HNSCC	Ad-p53 + immune checkpoint inhibitors	safety and efficacy	ClinicalTrials.gov NCT03544723
HNSCC	ONYX-015+ cisplatin / fluorouracil	Phase I / feasibility and maximum tolerated dose (MTD) - Withdrawn	ClinicalTrials.gov NCT0000610

II.4.3. Conclusions

p53 is implicated in HNSCC radio-sensitivity as evidenced by studies over the past 30 years. However, new advances in molecular biology and genetics include the involvement of miRNAs in the control of the p53 pathway, the understanding of GOF mechanisms and loss of function, the development of different methods to restore p53 function both for HVP - and HPV + HNSCC cases. The different ratio between mutant p53 in the primary tumor and distant metastasis originating HNSCC may serve to select the best therapeutic target for activating an abs copal effect by irradiation, as an activator of the immune system. p53 may be a key player in choosing the radiotherapy fractionation regimen. Evaluation in clinical trials of targeted therapies that can restore/modulate p53 function opens new horizons for synergistic associations with chemo-radiotherapy and radiotherapy for sensitization. The possibility of using the status of HPV, p53, and miRNAs as biomarkers for the selection of therapy, as well as updating the interest in tumor metabolism and in particular the Warburg effect as a possible target involving the restoration of p53 function and implicitly the net benefit in the therapeutic response. With increasing interest in high-LET in clinical practice, the involvement of the p53 pathway in the different radiobiological response to HNSCC depending on the type of ionizing radiation chosen could argue for the use of heavy ion therapy especially for HPV+ cases. CSC, hypoxia and modulation of the p53 pathway tested in silico models may underpin the future concept of precision radiotherapy in terms of dose, fractionation, radiation type and association with systemic therapies for HNSCC.

II.5. **Endoplasmic reticulum (ER) stress response - an adaptive program occurs during cancer progression**

The endoplasmic reticulum (ER) stress of cancer cells not only determined cancer cell fate but also indirectly triggered pro-inflammatory or immunosuppressive responses of macrophages. In addition, ER stressed neutrophils were known to acquire immunosuppressive activity with surface expression of lectin-like oxidized low-density lipoprotein receptor-1.

Endoplasmic reticulum (ER) stress response is an adaptive program to cope with cellular stress that disturbs the function and homeostasis of ER, which commonly occurs during cancer progression to late stage. Late-stage cancers, mostly requiring chemotherapy, often develop treatment resistance. Chemo-resistance has been linked to ER stress response; however, most of the evidence has come from studies that correlate the expression of stress markers with poor prognosis or demonstrate pro-apoptosis by the knockdown of stress-responsive genes. Since ER stress in cancers usually persists and is essentially not induced by genetic manipulations, we used low doses of ER stress inducers at levels that allowed cell adaptation to occur in order to investigate the effect of stress response on chemo-resistance (Chen, 2021).

The endoplasmic reticulum (ER) is a subcellular organelle involved in the synthesis of secretory/membrane proteins and lipids. The synthesis, folding and processing of the secretory/membrane proteins by the endoplasmic reticulum (ER) requires the functioning of ER chaperones, maintenance of ER calcium pools, and an oxidative environment. To maintain homeostasis against any ER dysfunction, the ER responds through a complex and coordinated adaptive signaling mechanism called the unfolded protein response (UPR). The UPR relays ER stress to the cytosol and nucleus to counter the imbalance in protein synthesis, folding, modification, translocation and degradation. Several physiological and pathological conditions such as nutrient or glucose deprivation, elevated protein synthesis, virus infection, disturbances in Ca^{2+} fluxes and redox regulation have been shown to promote ER dysfunction and elicit UPR (Zhao and Ackerman, 2006).

Analysis of the UPR signaling pathways has become possible using various pharmacological agents such as tunicamycin (an inhibitor of protein glycosylation), cycloheximide (an inhibitor of translational elongation), thapsigargin (that affects ER Ca2+ levels), redox agents such as DTT, GSH or GSSG (that influence disulphide bond formation), brefeldin A (an inhibitor of ER to Golgi protein transport) and inhibitors of protein degradation such as bortezomib. The UPR plays an important role in embryonic development, maturation of secretory cell types such as antibody producing plasma cells, osteoblasts that secrete collagen, and insulin-secreting pancreatic β-cells. It is also implicated in normal biological processes like aging, liver development, sleep deprivation, and in nutrient sensing in yeast (Wu and Kaufman, 2006).

Incessant ER stress beyond the limits of adaptation can trigger the pro-apoptotic potential of the UPR. The suicide of unhealthy cells via apoptosis represents the last resort of multicellular organisms to clear the non-functional cells. Cell death results in loss of cell/tissue function and may be the primary reason for the manifestation of disease in several ER stress-related disorders. Although the exact mechanism is not known, the ER stress-induced apoptosis is mediated by the mitochondria (intrinsic pathway) and/or through the activation of pro-apoptotic downstream kinases that are triggered typically in the death-induced receptor mediated extrinsic apoptotic pathway (Yoshida, 2007).

ER stress and cell death pathways in fibroblasts from patients with gingival hypertrophy.

Accumulation of mis-folded proteins and alterations in Ca2+ homeostasis in the endoplasmic reticulum (ER) causes ER stress and leads to cell death. However, the signal transduction events that connect ER stress to cell death pathways are incompletely understood, especially in fibroblasts from patients with gingival hypertrophy. Gingival fibroblasts were achieved from 6 weeks old-male rats, 150-170 g body weight, from gingival explants, and grown up in specific culture medium, with and without cyclosporine A (CsA) treatment (1μg/ml), nifedipine (3mM) and phenytoin (2.5mM). The control group received no treatment. We aimed the involvement of ER in the apoptosis of normal fibroblasts and as well as of those treated with CsA, nifedipine and phenytoin. As technique we used flow cytometry (FACS) and calcein-AM (C-AM) as the marker for the mitochondrial permeability transition pore (mPTP) opening. As inductor of apoptosis we used thapsigargin (THP). THP is considered one inducer of apoptosis through the RE stress. Several studies indicate that THP might produce apoptosis mainly in malignant or genetically modified cells, but not in normal cells. Facs images and statistical analysis showed differences between normal fibroblasts under thapsigargin action and those treated with CsA, nifedipine and phenytoin in culture medium under the same substance. Induction of ER stress with THP in normal fibroblasts had no statistically significant effects on MPTP opening. In fibroblasts treated with CsA, nifedipine and phenytoin, thapsigargin reduced mitochondrial calcein, suggesting the opening of mitochondrial permeability transition pore as a result of endoplasmic reticulum stress.

The purpose of this study was to evaluate the involvement of endoplasmic reticulum in the apoptosis of normal gingival fibroblasts and treated gingival fibroblasts with CsA, nifedipine and phenytoin.

Material and methods

Gingival fibroblasts were achieved from 6 weeks old-male rats, 150-170 g body weight, from gingival explants, and grown up in specific culture medium, consisting of DMEM, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 mg/ml streptomycin in an atmosphere containing 5% CO2 at 37°C. Medium was supplemented with CsA treatment (1μg/ml), nifedipine (3mM) and phenytoin (2.5mM). The control group received no treatment.

We aimed the involvement of ER in the apoptosis of normal fibroblasts and as well as of those treated with CsA, nifedipine and phenytoin. As technique we used flow cytometry (FACS) and calcein-AM (C-AM) as the marker for the mitochondrial permeability transition pore (MPTP) opening.

As inductor of apoptosis we used thapsigargin (THP). THP is considered one inducer of apoptosis through the RE stress. Several studies indicate that THP might produce apoptosis mainly in malignant or genetically modified cells, but not in normal cells.

The grown cells were used after the third passage at least. The normal and treated fibroblasts were separated with trypsin-EDTA standard solution, flushed by several centrifugations at 300xg for 5 minutes, after which were re-suspended in 1 ml culture medium.

Cells were counted (about 1.000.000/ml) and were equally divided in tubes. A tube represented the control group for each treatment, in all other tubes we added calcein-AM 5μl/ml (2 μM) and 5μl/ml of CoCl2 (80 μM) for 20 minutes at 37°C and 5% CO2. As apoptosis inducer we added thapsigargin 10 μl/ml (10 μM) for 24 hours at 37°C and 5% CO2. After that we centrifuged the tubes at 300xg for 5 minutes, and then the cells were re-suspended in PBS and centrifuged again, thus applying a double wash. The flow cytometry settings were as follows: 623 V for FL1, 505 V for FL2, 10,000 events and 488 nm laser. Data were processed using FlowJo 7.6.1 software. Meanwhile, the same protocol was applied

for untreated fibroblasts. Statistical data were analyzed using One Way ANOVA method (completed with Student-Newman-Keuls method). Results were considered statistically significant for a p value < 0.05 and were expressed as mean ± S.E.M. (5 experiments).

Results and discussion

Added together with CoCl2, calcein-AM appears to be a good technique for MPT channel opening, following Ca2+ concentration variations in the cell cytoplasm or mitochondria. We aimed the involvement of ER in the apoptosis of normal fibroblasts and as well as of those treated with CsA, nifedipine and phenytoin. Thapsigargin is considered one inducer of apoptosis through the RE stress. Several studies indicate that THP might produce apoptosis mainly in malignant or genetically modified cells, but not in normal cells. Despite the importance and possible involvement of the ER stress in various diseases, the pathway of ER stress-induced cell death has not been fully elucidated. Thus, in the present experiments we examined the role of thapsigargin in normal and treated gingival fibroblasts apoptosis.

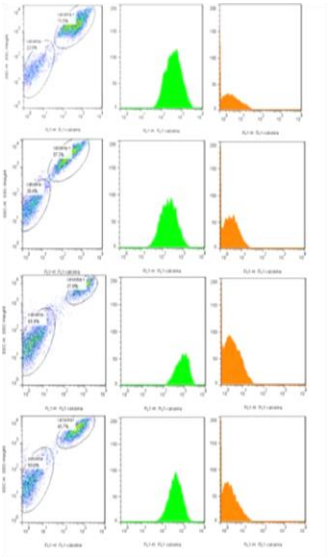

Figure II.5.1
FACS fluorescence images of calcein-AM loading, with reference to normal gingival fibroblasts (the upper image), of CsA-, nifedipine- and phenytoin-treated fibroblasts (second to fourth top to down images) under the action of THP for 24 hours.

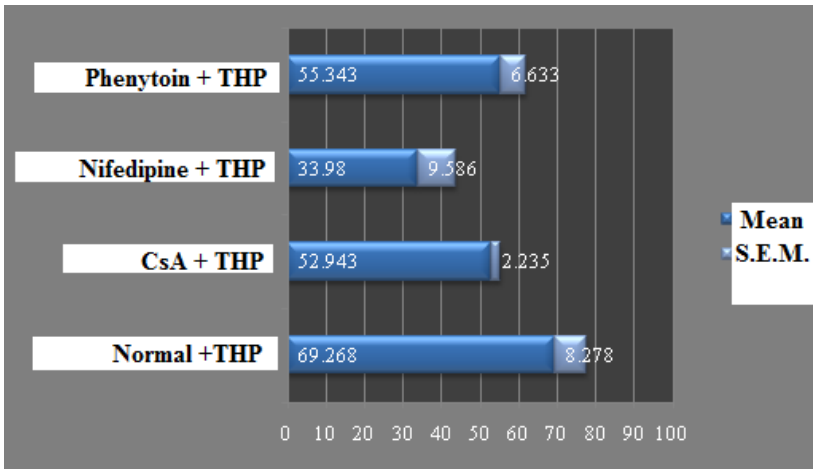

Figure II.5.2
Statistical analysis of calcein-AM loaded and treated fibroblasts with CsA, nifedipine and phenytoin under thapsigargin action, as compared to normal fibroblasts.

Since mitochondria are juxtaposed to the ER, in many cases ER stress is communicated to the mitochondria, and ER stress-induced apoptosis is mediated through a dysfunction in the mitochondria. Recent work suggests that BCL2 protein family regulates the ER-Ca2+ release and communication of ER stress signal to the mitochondria. Persistent ER stress can also induce a switch in the UPR signalling from pro-survival to pro-apoptotic pathways, like the induction of CHOP, a pro-apoptotic transcriptional factor and GADD34 (a cofactor of eIF2α phosphatase), through the PERK-eIF2α pathway, and activation of pro-apoptotic kinases such as ASK1 (apoptosis signal regulating kinase) and JNK (c-Jun-N-terminal kinase) through the IRE-1 pathway. The major players involved in ER stress-induced apoptosis and their roles are described below (Hussain and Ramaiah, 2007). Thapsigargin induces acute responses in a large variety of cell types, but thapsigargin-induced cellular activation appears, in all cases, to be initiated by a single common event: a rapid and pronounced increase in the concentration of cytosolic free Ca2+ that occurs via a direct discharge of intracellular stored Ca2+ without hydrolysis of inositol-phospholipids. These results imply that thapsigargin might act directly on a recognition site associated with the intracellular Ca2+ store (Thastrupt, 1990).

Our experiments showed that THP has no effect on normal gingival fibroblasts. But showed significant effects of thapsigargin on fibroblasts treated with CsA, nifedipine and less phenytoin as mitochondrial permeability transition pore opening, evidenced by calcein_AM release from mitochondria. Any alteration in the environment surrounding the endoplasmic reticulum, such as disturbance in Ca2+ homeostasis changes in secretory protein synthesis, deprivation of glucose or other sugar, altered glycosylation and accumulation of unfolded proteins in the endoplasmic reticulum stress can cause cell death (Park *et al*., 1999, Bullon *et al*., 2007).

All the elements involved in apoptosis are interconnected both physically and functionally, making possible the existence of a variety ways to initiate the response to very different stimuli and also the means of adjusting them. Mitochondrial permeability transition pore opening, as evidenced by reduction of calcein-AM fluorescence in the presence of cobalt chloride represents, according to current data, an initial step in the development of apoptosis. Induction of endoplasmic reticulum stress in normal fibroblasts with thapsigargin had no statistically significant effects on the opening of mitochondria permeability transition pore. In contrast, in fibroblasts treated with CsA, thapsigargin reduced mitochondrial calcein, suggesting the opening of permeability transition pore as a result of endoplasmic reticulum stress. Thapsigargin caused significant reduction of mitochondrial calcein loading even in fibroblasts treated with nifedipine and phenytoin.

II.6. The influence of Ca2+ Ionophor Ionomycin on the Mitochondrial transition permeability pore in normal, drug treated and cancer cells

Introduction

The mitochondrion is at the core of cellular energy metabolism, being the site of most ATP generation. Calcium is a key regulator of mitochondrial function and acts at several levels within the organelle to stimulate ATP synthesis. However, the dys-regulation of mitochondrial Ca^{2+} homeostasis is now recognized to play a key role in several pathologies. For example, mitochondrial matrix Ca^{2+} overload can lead to enhanced generation of reactive oxygen species, triggering of the permeability transition pore (MTP), and cytochrome C-release, leading to apoptosis. Despite progress regarding the independent roles of both Ca^{2+} and mitochondrial dysfunction in disease, the molecular mechanisms by which Ca^{2+} can elicit mitochondrial dysfunction remain elusive (Brookes, 200141).

Apoptosis represent a form of programmed cell death, characterized by well-defined morphological and biochemical attributes (Park et al. 1999). Apoptosis is process involved in

various diseases as cancer, IDS, Alzheimer, rheumatoid arthritis, periodontitis, and diabetes (Angosto, 2003) and is considered a main step during tissue and organ development. Actually, we acknowledge that the early important events in apoptosis take place at mitochondrial and endoplasmic reticulum level by c-cytocrome and calcium release in the cytoplasm (Wang, 2003). Mitochondria play an important role in apoptosis. It amplifies the signal initiated by caspases, insures the apoptosis development even if low pro-enzyme initiator levels are present. During Ca2+ accumulation (or as a result of ageing processes) mitochondria undergo a spontaneous process of internal membrane permeability increase for agents with a molecular weight of less than 1500 Da. This phenomenon is called transient membrane permeability (TPM).

Despite the recent popularity of this research topic, it is worth noting that Knyazeva et al. (1975) discovered mitochondrial cytochrome c release in ischemic liver nearly 30 years ago! Several studies suggest a role for the MPT in this process, including findings like MPT inhibitors (e.g., cyclosporin); the Bcl family proteins have been shown to functionally interact with MPT components and the loss membrane potential - a hallmark of apoptotic cell death and is thought to signal the recruitment of Bcl family proteins to the mitochondrion (De Giorgi, 2002, Brookes, 2004).

TPM, discovered in the 1970s, was consideration, even the beginning to be a strategic regulator of cell death in different pathological states from neurodegenerative and cardiac diseases to cancer. In the case of cancer, several studies have revealed alterations in the activity of the mitochondrial permeability transition pore (mPTP) and have determined its regulatory mechanism; these studies have also suggested that suppression of the activity of the mPTP, rather than its inactivation, commonly occurs in solid neoplasms (Bonora and Pinton, 2014).

Overall, it appears that mithocondrial Ca^{2+} overload and subsequent mitochondrial dysfunction play a key roles in cell death. Mithocondrial Ca^{2+} and oxidative stress are currently known as the two stereotypical activators of MPT. A role for the MPT in apoptotic cell death has been supported by several studies. During apoptosis, alteration of the mitochondrial membrane potential represent an early event and is produced due to the opening of a large size channel that is called pore for transient mitochondrial permeability (PPTM). The physiologic function for this pore is to allow Ca2+ delivery into cytoplasm and to allow the penetration for specific proteins, responsible for potential maintenance, toward mitochondrial matrix. The amplification of the apoptotic process or cell life extension depends on the presence of intracellular Ca2+. As previously described (Silvestri et al. 2003), cyclosporin A (CsA) inhibits this permeability pore thus inhibiting cation transport through MTP. Thus, Ca2+ cannot leave mitochondria using this pathway or the outward flow rate is very low. A recent study (Kantarci et al. 2007)) shows that apoptosis is significantly reduced in phenytoin gingival overgrowth or in gingival fibrous tissues compared to control tissues or treated by CsA. Inflammation induces apoptosis rate increase in non-fibrous tissues and also in tissues treated with CsA and nifedipine. The mentioned study shows that fibroblast apoptosis is decreased in gingival overgrowth.

Because defects in the cell death pathway are considered a hallmark of tumor initiation and progression, the MPT is a potential target for rescuing oncogenic defects; Functional mPTPs (mitochondria permeability transition) can be found in cancer cell lines, indicating that mPTPs are not completely inhibited in cancer. Furthermore, several structural elements of the pore are overexpressed and not down-regulated in these cell lines. More recent studies have proposed the existence of an “evolved aberrant conformation” of extremely efficient components that are indispensable for mitochondrial physiology (such as ion and solute transporters as well as chaperones), (Bonora and Pinton, 2014).

Figure II.6.1 present the mitochondrial permeability transition pore – a key effector in the mitochondrial pathways to cell death – and on the adaptive responses of tumor cells that desensitize the PTP to Ca2+ and reactive oxygen species (ROS),

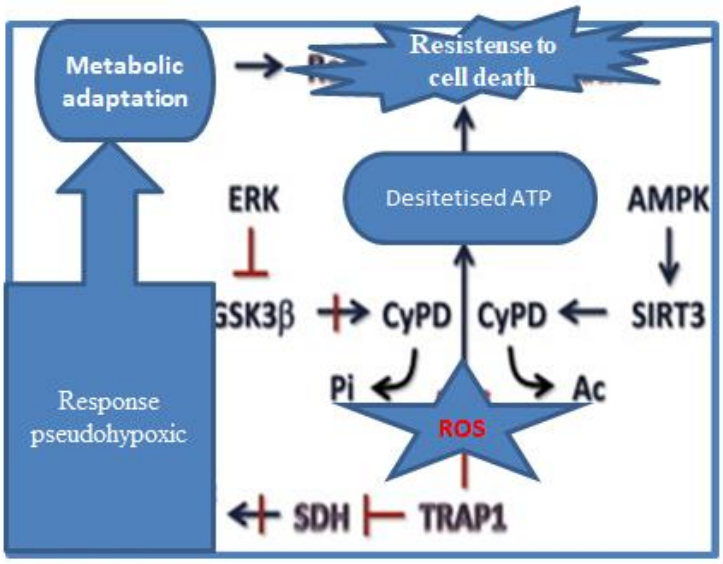

Figure II.6.1
Adaptive responses in tumor cells for mitochondrial permeability transition, adapted from Rasola (2014)

In normal cells, the transfer of substances between the endoplasmic reticulum and mitochondria is finely regulated by the phosphorylation status of the IP3Rs. Phosphorylation can be mediated by the AKT kinase, and proteins are dephosphorylated by the PP2A phosphatase. Normally this process is regulated by the onco-suppressors the loss of these in cancer cells, due to an increase in the phosphorylation of IP3R that inhibits Ca^{2+} transfer between the endoplasmic reticulum and the mitochondria.

Further accumulation of the oncogenic proteins Bcl-2 and RAS in the endoplasmic reticulum allows depletion of the Ca^{2+} store. The reduced amount of transferable Ca^{2+} prevents the occurrence of the MPT, despite the increased ROS levels often observed in cancer cells. Further, the increase in the levels of the ROS detoxifying enzymes superoxide dismutase-2 (SOD-2) and mitochondrial thioredoxin reductase (TRX-2) reduces the toxicity of oxidative stress. The region of contact between the mitochondria and ER, corresponding to the MAMs structure, is marked with a dashed circle. The yellow circle represents phosphorylated residues. Green circles represent Ca^{2+} ions (Bonora and Pinton, 2014).

So, apoptosis represent a form of programmed cell death, characterized by well-defined morphological and biochemical attributes. Until present time, few data regarding Ca2+ involvement in normal gingival fibroblasts apoptosis are available.

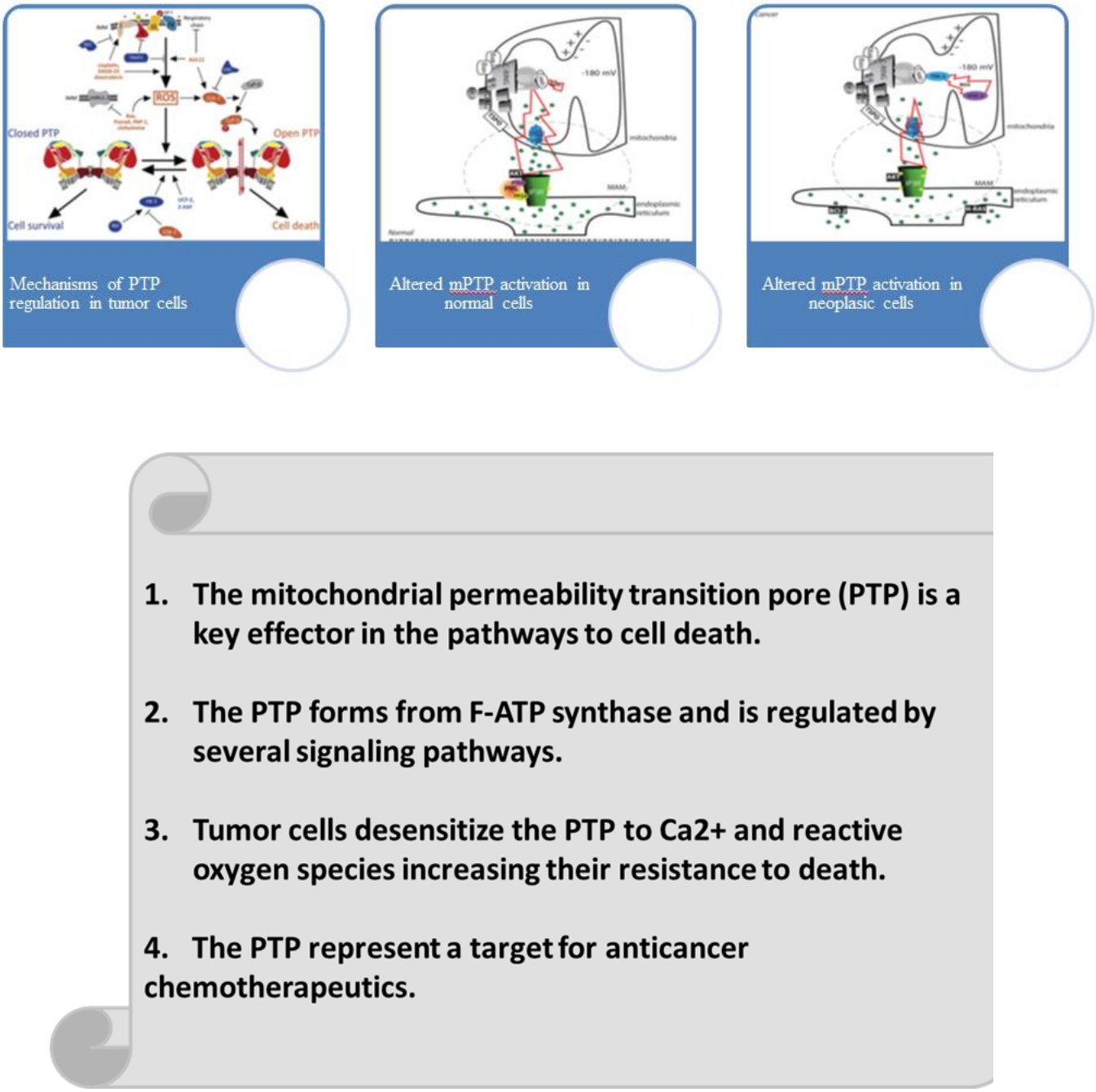

Figure II.6.2
The mitochondrial permeability transition pore and its adaptive responses in tumor cells, adapted from Rasola (2014)

II.6.1 The effect of ionomycin on normal and treated gingival fibroblasts

This studies purpose is to emphasize the effect of ionomycin on normal gingival fibroblasts and also on fibroblasts treated by Cyclosporine A (CsA), nifedipine, phenytoin, by using flow cytometry methods. Gingival fibroblasts were achieved from 6 week old male rats, 150 – 170 g weight, by gingival explants and grown up in specific culture medium consisting of DMEM, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in an atmosphere containing 5% CO2 at 37°C. Medium was supplemented with CsA treatment (1μg/ml), nifedipine (3mM) and phenytoin (2,5mM). We have used a control group that received no treatment. Following these steps, flow cytometry using FL1 settings 623 V, 505 V, FL2, 10,000 events, 488 nm lasers was performed. Data were processed using FlowJo 7.6.1 software. Statistical data were analyzed by One Way ANOVA method (completed by Student-Newman-Keuls method). Results were considered statistically significant for a p value <0,05 and were expressed as mean value (S.E.M). CsA, nifedipine and phenytoin treated fibroblasts were periodically examined and recorded by an image acquisition system attached to the inverted microscope. We have observed and recorded morphological changes and major count changes between cells treated by CsA, nifedipine and phenytoin and untreated cells. Our results show major morphological differences between treated and untreated fibroblasts. Calcein together with CoCl2 appears to be a viable mode; to evaluate mitochondrial transient permeability pore opens following intracellular and intra-mitochondrial Ca2+ concentrations variation. Mitochondrial calcein load for normal and treated fibroblasts does not show significant differences.

Nifedipine prevents apoptosis and inhibits its effects, as BAX proteins discharge, caspase activation, superoxide production and accumulation of condensed nuclei (Kantarci et al. 2007). One of the best described mechanisms in apoptosis induction is represented by the ionomycin intake that increases Ca2+ concentration in the cytoplasm for investigated cells. Until present time, few data regarding Ca2+ involvement in normal gingival fibroblasts apoptosis are available. This paper purpose is to emphasize the effect of ionomycin on normal gingival fibroblasts and also on fibroblasts treated by CsA, nifedipine, phenytoin, by using flow cytometry methods. The mitochondrial pore could constitute an important downstream effector in conferring crucial pathogenic traits, such as alterations in cell death responses, and an attractive pharmacological target.

Material and methods

Our purpose was to evaluate if Ca2+ overload in gingival fibroblasts obtained from gingival overgrowth induced by CsA, nifedipine and fenitoin, amplifies apoptosis in these cells. Gingival fibroblasts were achieved from 6 week old male rats, 150 – 170 g weight, by gingival explants and grown up in specific culture medium consisting of DMEM, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin in an atmosphere containing 5% CO2 at 37°C. Medium was supplemented with CsA treatment (1μg/ml), nifedipine (3mM) and phenytoin (2,5mM). We have used a control group that received no treatment. The protocol consisted in normal and treated fibroblasts trypsinisation with trypsin-EDTA, flushing them by centrifugation at 300 x g for 5 minutes, after which their resuspension in 1 ml culture medium. Cells were counted (about 1,000,000/ml) and were equally divided in tubes. A tube was depicted as control group, in other tubes we have added calcein 5μl/ml (2μM concentration) and 5μl/ml of CoCl2 (concentration of 80mM) and allowed for 20 minutes incubation at 37°C and 5%CO2. In the other four tubes we added tapsigargin 10μl/ml (10μM) 10μl/ml cyclo-piezonic acid (10μM), brefeldin A 10μl/ml (10μM) and 50μl/ml capsaicin (50μM concentration). All tubes are allowed for 24 hours incubation at 37°C and 5% CO2. Then, the tubes were centrifuged at 300 x g for 5 minutes after which the cells are re-suspended in PBS and centrifuged again, creating a double wash.

Following these steps, flow cytometry using FL1 settings 623 V, 505 V, FL2, 10,000 events, 488 nm lasers was performed. Data were processed using FlowJo 7.6.1 software. Statistical data were analyzed by One Way ANOVA method (completed by Student-Newman-Keuls method). Results were considered statistically significant for a p value <0,05 and were expressed as mean value (S.E.M).

Results

CsA, nifedipine and phenytoin treated fibroblasts were periodically examined and recorded by a image acquisition system attached to the inverted microscope. We have observed and recorded morphological changes and major count changes between cells treated by CsA, nifedipine and phenytoin and untreated cells (figure II.6.1.1-4).

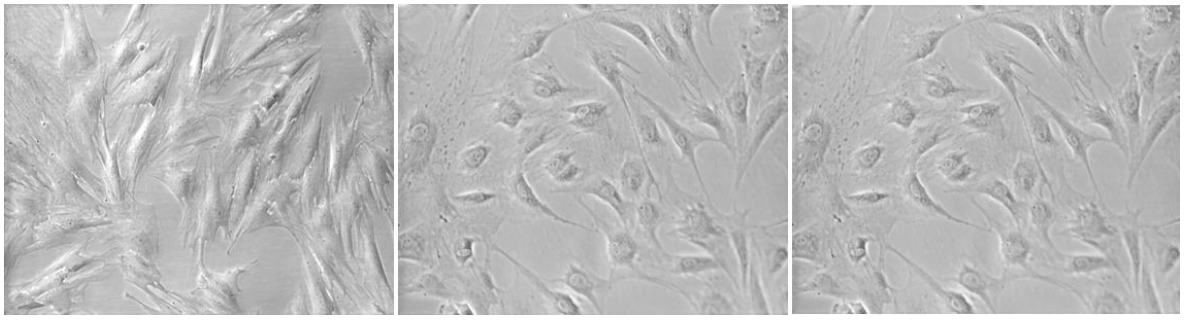

Figure II.6.1.1
Normal fibroblasts in culture at 7, 14 and 30 days (from left to right) (phase contrast x10)

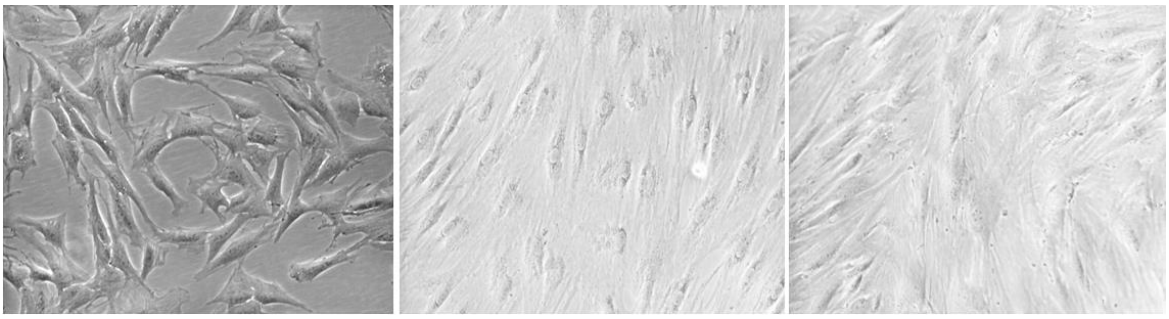

Figure II.6.1.2
CsA (1μm) treated fibroblasts in culture at 7, 14 and 30 days (from left to right) (phase contrast x10)

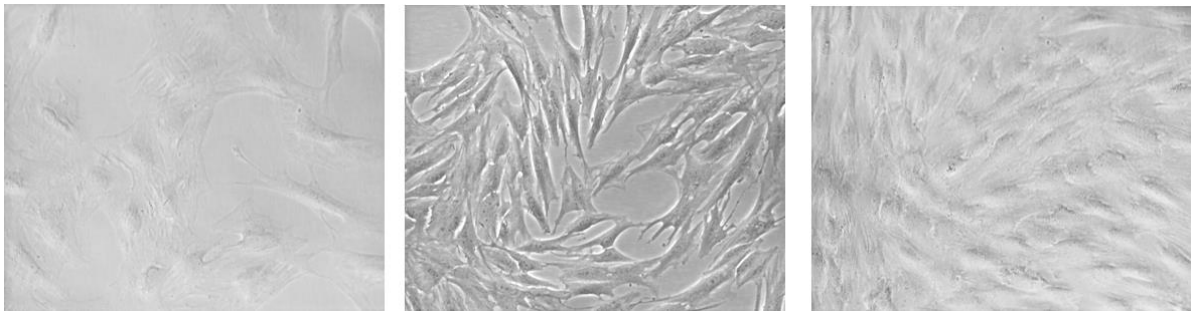

Figure II.6.1.3
Phenytoin treated fibroblasts in culture at 7, 14 and 30 days (from left to right) (phase contrast x10)

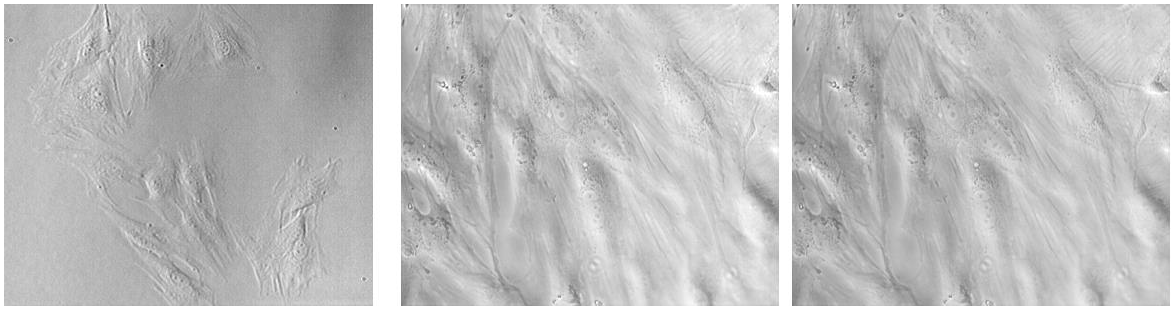

Figure II.6.1.4
Nifedipine (3mM) treated fibroblasts in culture at 7, 14 and 30 days (from left to right) (phase contrast x10)

Added to CoCl2, calcein appears to be a good model for MTP channel opening following Ca2+ concentration variations in the cell cytoplasm or mitochondria. By increasing Ca2+ concentration, we have induced the MTP opening by ionomycin administration (calcium ionophore). FACS (fluorescence activated cell sorting) results are shown in figures II.6.1.5-12).

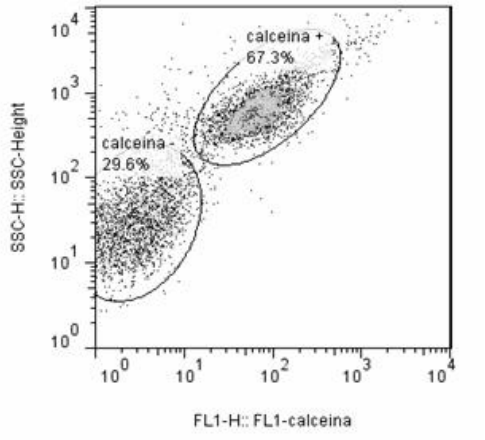

Figure II.6.1.5
FACS distribution for normal gingival fibroblasts loaded with calcein (2μM - 20 min)

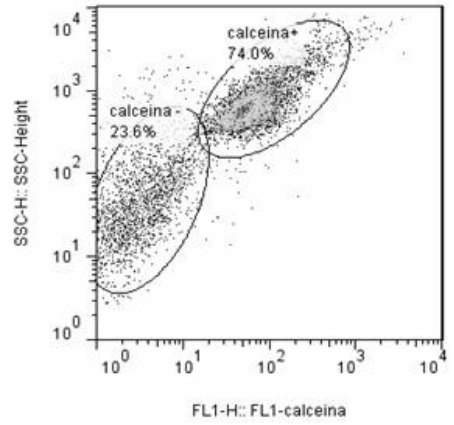

Figure II.6.1.6
FACS distribution for CsA treated gingival fibroblasts in standard culture

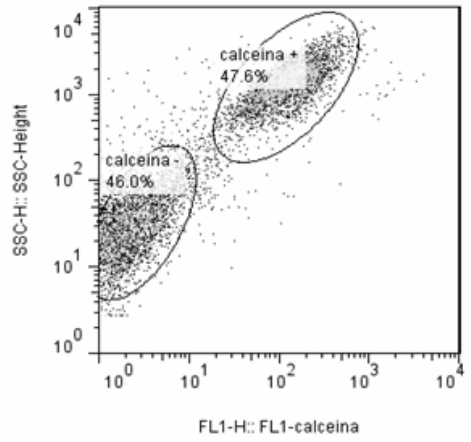

Figure II.6.1.7
FACS distribution for nifedipine treated gingival fibroblasts in standard culture

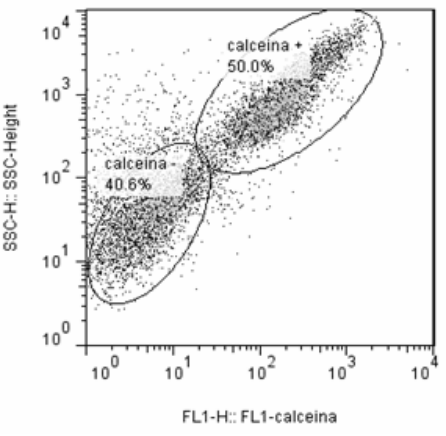

Figure II.6.1.8
FACS distribution for phenytointreated gingival fibroblasts in standard culture

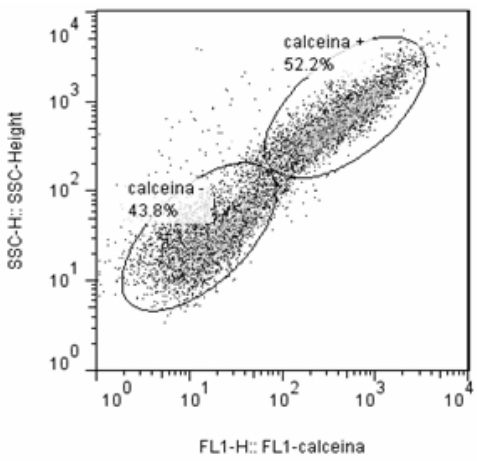

Figure II.6.1.9
FACS distribution for normal gingival fibroblasts under ionomycin action for 24h

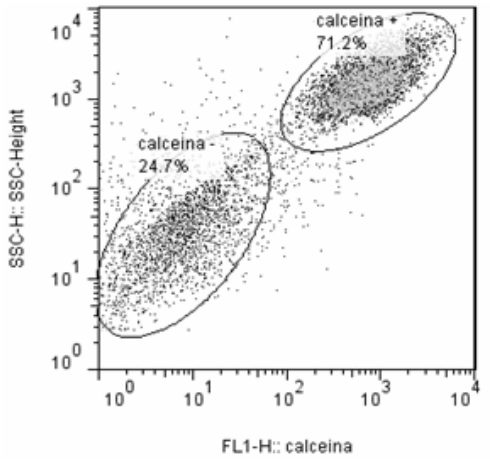

Figure II.6.1.10
FACS distribution for CsA treated gingival fibroblasts under ionomycin action for 24h

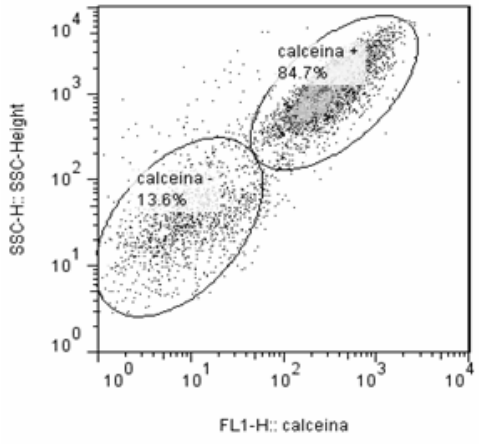

Figure II.6.1.11
FACS distribution for nifedipine treated gingival fibroblasts under ionomycin action for 24h

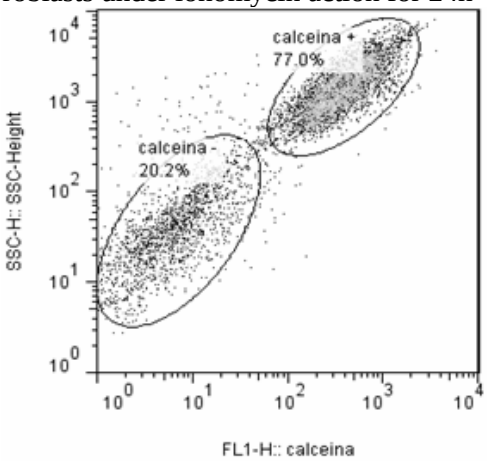

Figure II.6.1.12
FACS distribution for phenytoin treated gingival fibroblasts under ionomycin action for 24h

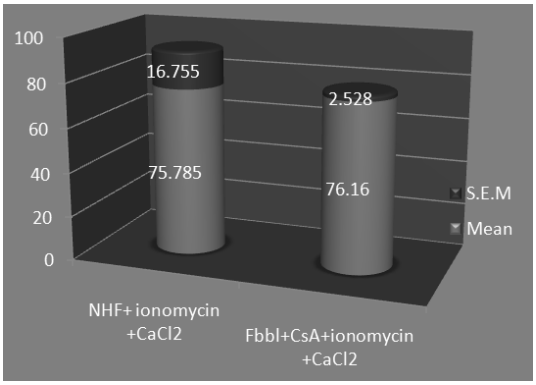

Figure II.6.1.13
Calcein loaded fibroblasts and treated by CsA under ionomycin action compared to normal fibroblasts treated by the same ionophore

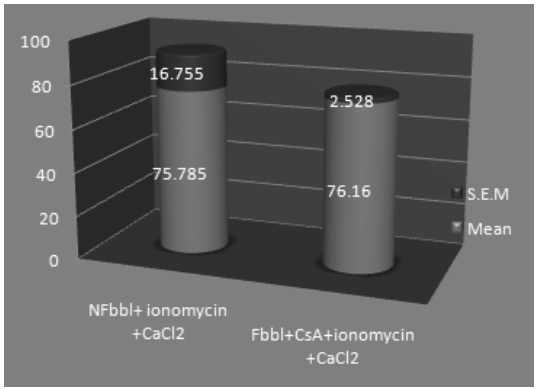

Figure II.6.1.14
Calcein loaded fibroblasts and treated by CsA under ionomycin action compared to normal fibroblasts treated by the same ionophore (NFbbl - normal

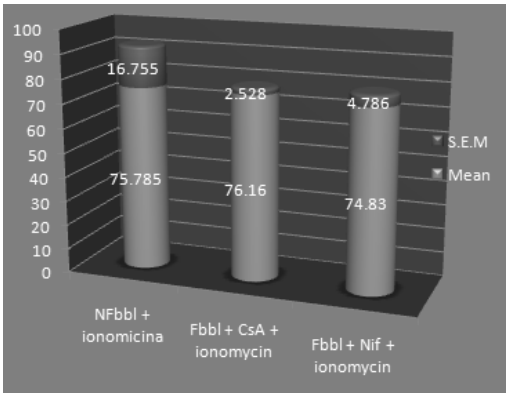

Figure II.6.1.15
Calcein loaded fibroblasts and treated by nifedipin (Nif) under ionomycin action compared to normal fibroblasts treated by the same ionophore (NFbbl – normal fibroblasts fbbl - fibroblasts)

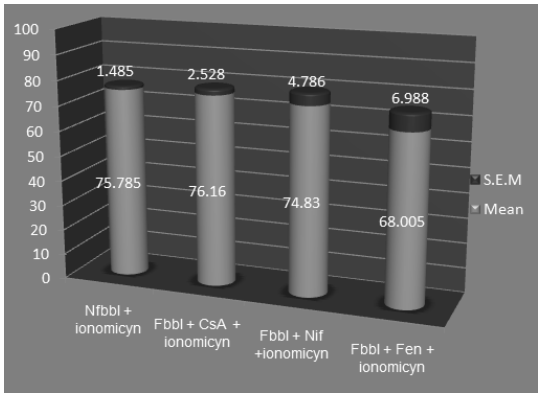

Figure II.6.1.16
Calcein loaded fibroblasts and treated by phenitoin (Fen) under ionomycin action compared to normal fibroblasts treated by the same ionophore (NFbbl – normal fibroblasts fbbl - fibroblasts)

Discussions

Mitochondrial transient permeability pore (mPTP) represent a protein complex of approximately 1-1,3 nm, permeable to less than 1500 Da solutes and including an anionic voltage gated channel (VGC), cyclophillin D and proteins, members of the BAX-BCL family (Salisbury, 2004). mPTP is activated as answer to various conditions as ATP release, oxygen reactive compounds and mitochondrial Ca2+ overload. Pore activation was associated to apoptosis inducers release from mitochondria (C cytochrome), apoptosome formation, caspase activation and apoptosis initiation. Pore activation can be observed by flow cytometry by following calcein fluorescence in cytoplasm by using cobalt chloride (Salisbury, 2004).

We have studied the effects of various apoptosis inducers on normal ginvival fibroblasts and also on CsA, nifedipine and phenytoin treated fibroblasts, by comparing flow cytometry results for treated cells and a control cell culture. Our purpose was to evaluate normal fibroblast sensitivity to Ca2+ induced apoptosis compared to CsA, nifedipine and phenytoin treated fibroblasts.

Conclusions

Our results show major morphological differences between treated and untreated fibroblasts. Calcein together with CoCl2 appears to be a viable mode; to evaluate PPTM opening following intracellular and intra-mitochondrial Ca2+ concentrations variation. Inside the cell, acetoxymethyl esters are hydrolysed by endogenous esterases, releasing negative polarized calcein that is intensely fluorescent (emission in green spectra); calcein is captured in cytoplasmic compartments of the living cells. To mask cytoplasmic fluorescent signal, cells are incubated with CoCl2 that does not affect signal intensity from mitochondria. Mitochondrial calcein load for normal and treated fibroblasts does not show significant differences.

ACKNOWLEDGMENTS Supported by Doctoral Contract/ “Gr.T.Popa” University of Medicine and Pharmacy Iasi

II.6.2. Ionomycin-Induced Ca2+ overload and mitochondrial membrane potential in immortalized pro-B lymphocytes type Ba/F3.

Other studies were performed on an immortal cell line and evaluated the influence on the mitochondrial membrane potential as an effect of Ionomycin-Induced Ca2+ overload. One of the mechanisms well known to induce apoptosis is the administration of ionomycin, a ionophore for Ca2+, which results in strong and sustained growth of citosolic calcium. Cellular clone Ba/F3 was derived from murine bone marrow. Ba/F3 cells are immortalized pro-B lymphocytes presenting anti-apoptotic phenotype. There are extremely few data concerning the involvement of Ca2+ fluxes in the apoptosis of the pro-B cell type Ba/F3. Thus, we aimed the characterization of ionomycin-induced effects on Ba/F3 cells in vitro, using laser confocal microscopy.

Materials and methods

The IL-3-dependent mouse pro-B cell line Ba/F3 was maintained in RPMI 1640 (Sigma-Aldrich) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% heat-inactivated fetal bovine serum and 10% WEHI-3-conditioned media as a source of murine IL-3 (Royer et al., 2005), in an atmosphere with 5% CO2 and at 37o C. Cells were grown at a density of around 5 x 105 per ml before treatment. For some experiments (in triplicate) Ba/F3 cells were treated with 1 μM and 10 μM ionomycin and 1 mM Ca2+ for 24 hours. To compare, we used as control the effects of staurosporine 10 μM, a well-known inducer of dissipation of mitochondrial membrane potential, also in triplicate. The control Ba/F3 cells received no treatment for 24 hours.

After that, all batches of Ba/F3 cells were incubated in the presence of 1 μM JC-1 (Sigma-Aldrich) at 37^{o} C for 30 minutes. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'- tetra-ethylbenzimidazolyl-carbocyanine iodide or CBIC2(3)) is a very sensitive marker for mitochondrial membrane potential. Ba/F3 cells were washed three times with phosphate-buffered saline (PBS, Sigma-Aldrich) at 300 x g for 5 minutes each and were plated in Nunc Lab-Tek II chamber slide systems (one well on glass, Sigma-Aldrich), being incubated for 20 minutes at 37^{o} C. Non-adhered cells were carefully washed out with PBS and the adhered ones were covered with normal culture medium at room temperature. The Nunc chamber slide systems have been prepared by pretreatment for 24 hours with poly-L-lysine (0.1 mg/ml) at room temperature, washed three times with PBS, dried and exposed to UV for 30 minutes. The final concentration for dimethyl sulfoxide (DMSO) used as a drug solvent in the medium did not exceed 0.1%, having no cellular effects at this concentration.

For the laser confocal microscopy we used a Microradiance (Bio-Rad/Zeiss) setup, with an argon ion laser (488 and 514 nm), mounted on an inverted Nikon Eclipse TE-300 microscope. The x100 magnification images were generated using an x100 oil-immersion objective, CFI Plan Fluor (1.30 N.A.), and LaserSharp software. We used the HQ515/530 emission filter for 488 nm excitations and HQ530/560 emission filter for 514 nm excitation in a sequential mode. The laser power was of 3%. For the analysis of the collected images (resolution 1280 x 1024) we used ImageJ, a public domain, Java-based image processing program developed at the National Institutes of Health (U.S.A.). The red and green emissions of JC-1 were merged to obtain the final images.

Results and discussions

As figure no. 63 shows, 1 μM ionomycin and 1 mM Ca2+ treatment of Ba/F3 cells for 24 hours did not induced significant effects on the mitochondrial membrane potential as compared with control cells (figure II.6.2.1-2).

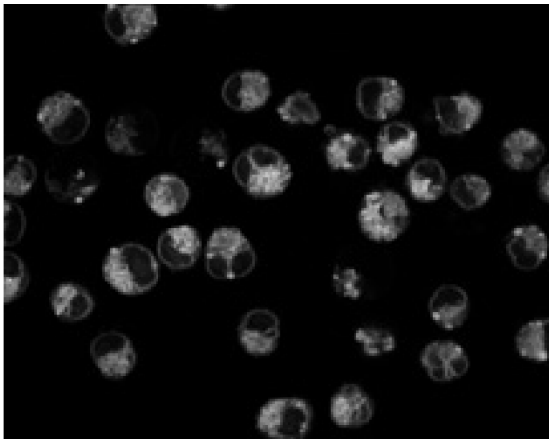

Figure II.6.2.1
Laser confocal microscopy of Ba/F3 cells treated with ionomycin 1 μM for 24 hours in the presence of 1 mM Ca2+. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide or CBIC2(3)), is showing that more than 90% of the cells are having high mitochondrial membrane potential (Ψmt), normal for live cells. This is reflected in the high intensity of red emission of JC-1, the energized mitochondria being clearly distinct in the citosol. Image shown is representative of many acquired from three independent experiments (100x).

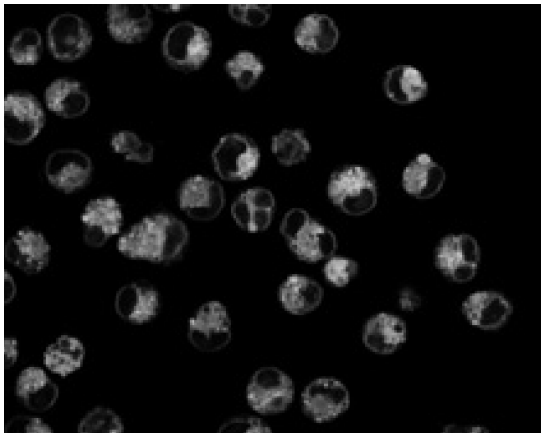

Figure II.6.2.2
Ba/F3 control cells, non-treated, also have high mitochondrial membrane potential (Ψmt), normal for live cells, in proportion of more than 90%, when imaged by laser confocal microscopy with the help of JC-1. The rest of almost 10% of cells are usually apoptotic. Image shown is representative of many acquired from three independent experiments (100x).

In both cases more than 90% of the cells are having high mitochondrial membrane potential (Ψmt), normal for live cells. This fact is reflected in the high intensity of red emission of JC-1, the energized mitochondria being clearly distinct in the citosol. On contrary, when Ba/F3 cells were treated with staurosporine 10 μM, a well-known inducer of dissipation in mitochondrial membrane potential for 24 hours, there remained very few cells (less than 10%) alive, with distinct red energized mitochondria (figure II.6.2.3).

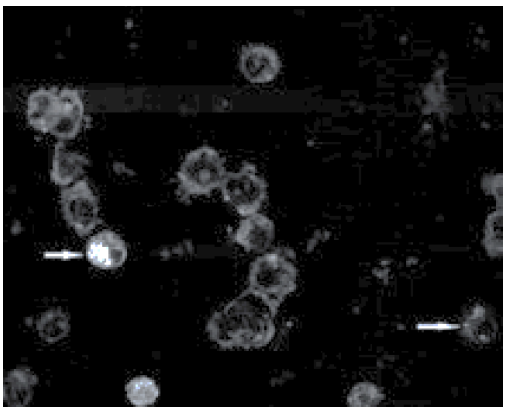

Figure II.6.2.3
Large scale of dispersed green emission of JC-1shown by laser confocal microscopy (100x)

In the presence of staurosporine 10 μM, a well-known inducer of dissipation of mitochondrial membrane potential, for 24 hours, extremely few Ba/F3 cells (less than 10%) are having high mitochondrial membrane potential (Ψmt), normal for live cells (arrows). On contrary, there is evident a large scale of dispersed green emission of JC-1shown by laser

confocal microscopy. Image shown is representative of many acquired from three independent experiments (100x)

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide or CBIC2(3)) is a cationic dye that exhibit potential-dependent accumulation in mitochondria, being a very sensitive marker for mitochondrial membrane potential. The JC-1 dye accumulates in the mitochondria of healthy cells as aggregates, which are fluorescent red in color. If the mitochondrial potential collapses, then the JC-1 dye can no longer accumulate in the mitochondria and remains in the cytoplasm in a monomeric form which fluoresces green. The differential distribution of the red and green forms of the dye is easily analyzed by fluorescence microscopy or laser confocal microscopy (Di Lisa, 1995). JC-1 is far more specific for mitochondrial versus plasma membrane potential, and more consistent in its response to depolarization, than other cationic dyes such as DiOC6(3) and rhodamine 123, giving at the same time very little background (Salvioli, 1997). The ratio of green to red fluorescence of JC-1 is dependent only on the membrane potential and not on other factors such as mitochondrial size, shape, and density that may influence single-component fluorescence signals.

The most widely implemented application of JC-1 is for detection of mitochondrial membrane potential dissipation (Jones, 2002). MPT, the result of the collapse of electrochemical gradient across the mitochondrial membrane, is one of the early events during cellular apoptosis. The mitochondrial voltage-dependent anion channel (VDAC), the physical supposed support for MPT, is increasingly involved in the control of apoptosis (Yuqi, 2009). Noxa, the BH3-only Bcl-2 family protein, was shown to be a key player in p53-induced cell death through the mitochondrial dysfunction. It was showed that the mitochondrial-targeting domain (MTD) of Noxa is a prodeath domain. Peptide containing MTD causes massive cell death in vitro through cytosolic calcium increase; it is released from the mitochondria by opening the mitochondrial permeability transition pore. MTD peptide-induced cell death can be inhibited by calcium chelator BAPTA-AM (Seo, 2009).

Single-channel currents were recorded from inner mitochondrial membranes of HepG2 hepatic cells and of normal rat liver cells by means of patch-clamp techniques. Voltages of -40 mV and below closed the channels usually with a delay of about 2 minutes. Increasing Ca2+ concentrations activated the channels, whereas cyclosporin A (100 nM) blocked. Taken together the results indicate that the currents were recorded from the mitochondrial permeability transition pore (MPTP) (Loupatatzis et al., 2002). Ca2+ stimulates mitochondrial energy metabolism during spleen lymphocyte activation in response to the ascitic Walker 256 tumor in rats. Intracellular Ca2+ concentrations, phosphorylated protein kinase C (pPKC) levels, Bcl-2 protein contents, interleukin-2 (IL-2) levels, mitochondrial uncoupling protein-2 (UCP-2) contents and reactive oxygen species (ROS) were significantly elevated in these activated lymphocytes. Mitochondria of activated lymphocytes exhibited high free Ca2+ concentrations in the matrix and enhanced oligo-mycin-sensitive oxygen consumption, indicating an increased rate of oxidative phosphorylation. The production of ROS was largely decreased by diphenylene iodinium in the activated lymphocytes, suggesting that NADPH oxidase is the prevalent source of these species. Accumulation of UCP-2 and the anti-apoptotic protein Bcl-2 is probably important to prevent mitochondrial dysfunction and cell death elicited by the sustained high levels of intracellular Ca2+ and ROS and may explain the observed higher resistance from activated lymphocytes against the opening of the mitochondrial membrane permeability pore (MPT). All these changes were blocked by pretreatment of the rats with verapamil, an Ltype Ca2+ channel antagonist. These data demonstrate a central role of Ca2+ in the control of mitochondrial bioenergetics in spleen lymphocytes during the immune response to cancer (Degasperi, 2006).

On the other hand, other data suggest a little bit different mechanism of mitochondria-triggered paraptotic cell death, induced by cytosolic Ca2+ overload through receptor-operated channel (vanilloid receptor subtype 1, VR1), in Jurkat cells (Jambrina, 2003). Ca2+ uptake through the VR1 channel, but not capacitative Ca2+ influx stimulated by the muscarinic type 1 receptor, induced sustained intracellular (Ca2+) rises, exposure of phosphatidylserine, and cell death. Ca2+ influx was necessary and sufficient to induce mitochondrial damage, as assessed by opening of the permeability transition pore and collapse of the mitochondrial membrane potential. Ca2+-induced cell death was inhibited by ruthenium red, protonophore carbonyl cyanide m-chlorophenylhydrazone, or cyclosporin A treatment, as well as by Bcl-2 expression, indicating that this process requires mitochondrial calcium uptake and permeability transition pore opening. Cell death occurred without caspase activation, oligonucleosomal/50-kilobase pair DNA cleavage, or release of cytochrome c or apoptosis inducer factor from mitochondria, but it required oxidative/nitrative stress. Thus, Ca2+ influx might triggers a distinct program of mitochondrial dysfunction leading to paraptotic cell death, which does not fulfill the criteria for either apoptosis or necrosis.

One of the mechanisms well known to induce apoptosis is the administration of ionomycin, a ionophore for Ca2+, which results in strong and sustained growth of citosolic calcium, (Ca2+)i, e.g. in the case of thymocytes (Jambrina, 2003). On the other hand, there are extremely few data concerning the involvement of Ca2+ fluxes in the apoptosis of the pro-B cells, and especially of type Ba/F3. Thus, we aimed the characterization of ionomycin-induced effects on Ba/F3 cells in vitro, using laser confocal microscopy. Our obtained data show that cytosolic Ca2+ increased in Ba/F3 cells by 1 μM ionomycin, a well-known inducer of dissipation of mitochondrial membrane potential, in the presence of 1 mM Ca2+ for 24 hours did not induced significant effects on the mitochondrial membrane potential as compared with control cells as has been shown through the help of the JC-1 fluorescent dye. The same effects were also associated by the higher concentrations of ionomycin, e.g. 10 μM (data not shown). These results are in contrast with that induced by the treatment with staurosporine 10 μM, also a well-known inducer of collapsing of mitochondrial membrane potential.

In concusion, Ba/F3 cells, a murine early pro-B cells type, are resistant toward the collapsing of mitochondrial membrane potential induced by cytosolic Ca2+ overload, as shown by the use of JC-1 fluorescent dye and laser confocal microscopy.

II.6.3 Genetic polymorphisms of TNFα and IL1a and oral potentially malignant disorders (OPMDS)

Interleukin (IL-1) and tumor necrosis factor (TNF-α) are inflammatory cytokines that play an important role in periodontitis, and their genetic variations have been suggested to be associated with increased risk of periodontitis. Oral carcinogenesis represents a multi-stage process which encompasses several genetic and molecular changes that promote the progression of oral potentially malignant disorders (OPMDs) to oral squamous cell carcinomas (OSCCs). A better understanding of critical pathways governing the progression of OMPDs to OSCCs is critical to improve oncologic outcomes in the future. Previous studies have identified an important role of tumor necrosis factor α (TNFα) and TNF receptor 1 (TNFR1) in the invasiveness of oral cancer cell lines.

The development of OSCCs may be preceded by oral potentially malignant disorders (OPMDs) which represent a subset of conditions that possess an increased risk of progression to cancer (Warnakulasuriya, 2019).

Epidemiologic and clinical data continue to support the role of chronic inflammation in carcinogenesis. As a potent regulator of transcription and cell survival, TNFa has been implicated in the progression of multiple human cancers through promotion of tumor growth,

angiogenesis, invasion and metastasis. The results from our human biopsy samples demonstrated a progressive-increasein TNFa and TNFR1 expression as well as increased recruitment of CD45+ inflammatory cells from non-progressing OPMD samples to progressing OPMD samples, highlighting the crucial role of TNFa in the development of a pro-invasive environment (Chadwick, 2021).

Experimental studies of Glogauer et al. (2015) demonstrate that TNFα promotes tumor invasion and growth as well as expression of pro-inflammatory cytokines in an OSCC cell line which suggests that these findings represent a novel mechanism linking oral inflammation and malignant transformation. They proposed model for TNFa-induced malignant transformation in the setting of an oral potentially malignant disorder.

Intrinsic and extrinsic factors induce oral premalignant disorders resulting in a cyclic signal process between recruited neutrophils and keratinocytes to establish a tumor-permissive environment via the TNFa/TNFR1 signal pathway. TNFR1 activation of keratinocytes enhances invadopodia development and matrix degradation thereby facilitating invasion. Pro-inflammatory cytokines released by keratinocytes recruit and activate neutrophils which assist with matrix re-modelling and further activation of nearby keratinocytes (Chadwick, 2021).

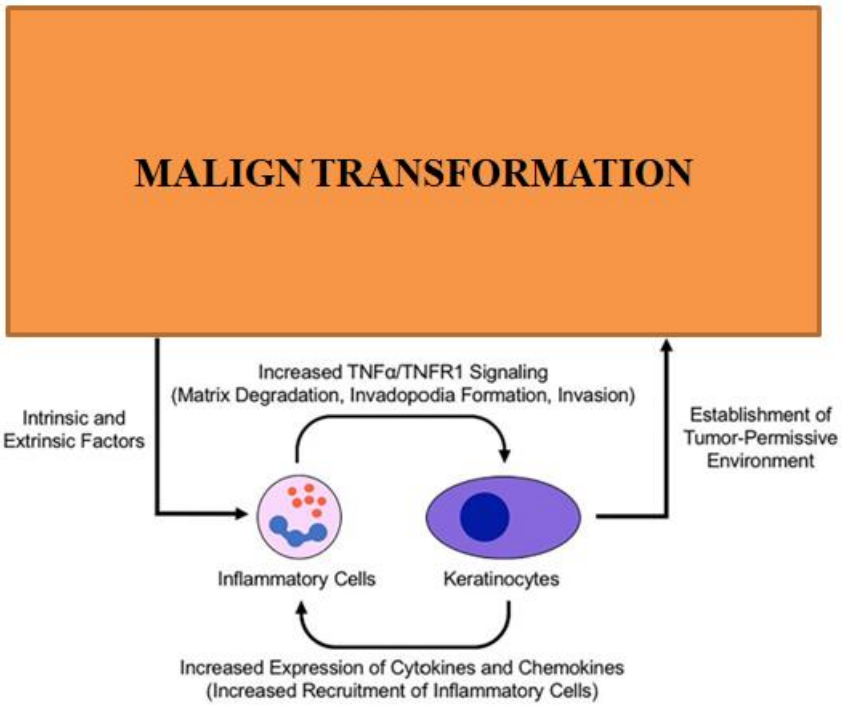

Figure II.6.3.1
TNFa Signaling Is Increased in Progressing Oral Potentially Malignant Disorders
Adapted from Chadwick, 2021

Virulent bacteria could cause gingival fibroblasts apoptosis through lipopolysaccharide release during generalized aggressive periodontitis (GAgP) development and evolution. We showed that treatment with lipopolysaccharide (LPS, 1 μg/mL) for 30 days induced the decrease in the number of cultured rat gingival fibroblasts as compared to control group, which received no treatment. GAgP is considered to have also a genetic etiology, so the aim of our study was to evaluate if some polymorphisms of tumor necrosis factor-alpha (*TNFA*) and interleukin 1A (*IL-1A*) genes are associated with GAgP in a sample of Romanian population. We selected a group of 32 subjects (22 cases and 10 controls) for studying the *TNFA* (-857) polymorphism and 97 subjects (66 cases and 31 controls) for *IL-1A* (-889)

polymorphism. The single nucleotide polymorphisms were genotyped by real-time polymerase chain reaction for all subjects. The genotype and allelic distribution tended to be equally between the cases and the controls group. Similar results were obtained for the dominant and recessive model. The difference between the two groups did not reach statistical significance for neither of the two studied polymorphisms (p=0.76 for *TNFA* (-857) and p=0.84 for *IL-1A* (-889)). The data suggest that *TNFA* (-857) C/T and *IL-1A* (-889) C/T polymorphisms are not associated with susceptibility to GAgP in this Romanian population, potentially because of the small sample size. This is the first such study for Romanian north-eastern population.

Most of the genetic research in oral disease has focused on gene polymorphisms that play a role in the immune response, tissue destructive process, or metabolic mechanism. In some situations, genetic polymorphisms could cause a change in the protein or its expressions, possibly resulting in alterations in innate and adaptive immunity and may thus be deterministic in disease progression (Karthikeyan, 2000). It is thought that single nucleotide polymorphism (SNP) analyses will contribute to the identification of multiple genes associated with periodontitis as genetic markers and risk factors. Periodontal disease is considered a complex disease associated with multiple genetic factors and oral environmental factors. It is also regarded as a multifactorial condition that occurs because of interplay between environmental, behavioral, microbial and genetic factors. Genetic studies revealed the polygenic nature of periodontitis. Genetic polymorphism in cytokine genes is regarded as a promising factor in inducing periodontal disease (Khosropanah, 2013). Apoptosis in gingival fibroblasts might be induced by lipopolysaccharide (LPS) (Takeuchi, 2011). *Porphyromonas gingivalis* is an oral bacterium that causes pathology in a number of dental infections that are associated with increased fibroblast cell death. Studies demonstrated that *P. gingivalis* stimulates cell death by apoptosis rather than necrosis. Some studies showed that apoptosis was induced independent of proteolytic activity and was also independent of caspase activity because a pan-caspase inhibitor, ZVAD-fmk, had little effect. Moreover *P. gingivalis* down-regulate caspase-3 mRNA levels and caspase-3'activity.

The consequence of this down-regulation was a significant reduction in tumor necrosis factor-alpha (TNFA)-induced apoptosis, which is caspase-3-dependent. Immunofluorescence and immunoblot analysis revealed *P. gingivalis* induced translocation of apoptosis-inducing factor (AIF) from the cytoplasm to the nucleus. siRNA studies were undertaken and demonstrated that *P. gingivalis* stimulated cell death was significantly reduced when AIF was silenced (p<0.05). Treatment of human gingival fibroblasts with H-89, a protein kinase A inhibitor that blocks AIF activa- tion also reduced *P. gingivalis*-induced apoptosis (p<0.05). These results indicate that *P. gingivalis* causes fibroblast apoptosis through a pathway that involves protein kinase A and AIF, which is not dependent upon bacterial proteolytic activity and is also independent of the classic apoptotic pathways involving caspase-3 (Desta, 2007). TNFA and interleukin-1alpha (IL-1A) are pro-inflammatory cytokines that participate in the establishment of inflammatory lesions in periodontitis.

Recent reports have indicated that allelic variation of cytokines genes and factors regulating their expression may influence the clinical outcome, susceptibility and progression of periodontal disease. Dys-regulation of cytokine gene expression may be responsible for the repeated cycles of tissue inflammation observed in these disorders (Tai et al., 2002). The above-mentioned studies already examined the association between periodontitis and single nucleotide polymorphisms (SNPs) that affect cytokines productivity. Such reports on *TNFA* and *IL-1A* and periodontitis suggested a correlation between the high level of these cytokines production and the variant alleles of *TNFA* and *IL-1A* SNPs (Takeuchi, 2011, Desta, 2007, Tai et al., 2002). Therefore, we decided to study these SNPs in our population and their relation to a severe form of periodontitis, generalized aggressive periodontitis (GAgP).

GAgP is characterized by a rapid destruction of the periodontal tissues, which affects in general young people without any systemic disorders. The objective of the present study was to evaluate the association between *TNFA* (-857) and *IL-1A* (-889) gene polymorphisms and GAgP.

Materials and Methods

Fibroblast cultures and LPS treatment

Gingival fibroblasts were achieved as previously described (6) from 6-week-old male rats, 150–170 g body weight, from gingival explants, and grown up in specific culture medium, consisting of DMEM (Dulbecco's Modified Eagle Medium), 10% fetal bovine serum (FBS), 100 U/mL Penicillin and 100 mg/mL Streptomycin in an atmosphere containing 5% CO2 at 370C. Medium was supplemented with LPS treatment (1 μg/mL; LPS from *Escherichia coli* 055:B5; Sigma-Aldrich) for 30 days in the case of treated cells. The control group received no treatment. The grown cells were used after the third passage at least. For all batches, the initial number of cells was 100000/flask. Cell viability was calculated as the number of viable cells divided by the total number of cells within the grids on the hemo-cytometer. We prepared a 0.4% solution of Trypan blue in phosphate-buffered saline, pH 7.2 to 7.3. 0.1 mL of Trypan blue stock solution was added to 1 mL of cells suspension. The normal and treated fibroblasts were separated with Trypsin-EDTA standard solution. The formula used was: % viable cells = (1.00 – (number of blue cells/number of total cells)) × 100. To calculate the cell viability for 30 days, we made the sum of all partial calculations when we changed the medium and transferred the cells through passages (seven experiments). Finally, we used also the Nikon Eclipse TE300 and 10× lens to morphologically quantify the cells in culture. The study protocol was approved by the Ethic Research Committee of "Gr. T. Popa" University of Medicine and Pharmacy, Iassy, Romania.

Human subjects and clinical assessments

Thirty-two subjects were selected for the *TNFA* polymorphism study, including 22 with GAgP and 10 without periodontal disease from the ones who sought dental treatment in a private clinic. For the *IL-1A* polymorphism study, we selected a total of 97 adult subjects, 66 for the cases group and 31 for the control group under the same conditions. Informed consent was obtained from all individuals. The study protocol was approved by the Ethic Research Committee of "Gr. T. Popa" University of Medicine and Pharmacy, Iassy, Romania. The clinical criterion was considered to be interproximal attachment loss affecting at least three permanent teeth other than a first molars and incisors. The clinical investigation included also smoking status, plaque and bleeding indexes, pocket depth and the presence of dental mobility (Barnea et al., 2013). Clinical measurements were performed at six sites/tooth and included probing depth (PD), clinical attachment level (CAL), bleeding on probing (BOP), meaning more than 30% of teeth with PD and/or CAL >5 mm or more than 60% of teeth with PD and/or CAL >4 mm. The subjects that did not present loss of gingival attachment for more than one tooth, and the periodontal probe measurement was not deeper than 3 mm, did not present gingival bleedings and bone resorption or a history of periodontal disease, were considered healthy from periodontal point of view and included in the control group, meaning no sites with PD and/or CAL >3 mm and no more than 10% of sites with BOP. Exclusion criteria included mental disorders, lack of judgment, minors, pregnant woman, other ethnical groups and immigrants. We also excluded the subjects with diabetes or the ones having antibiotic treatment in the last six months.

Saliva samples and DNA extraction

Saliva samples may serve as the best alternative sampling for extraction of genomic DNA for its high concentration and acceptable purity (Mohd Rashdan, 2014). Therefore, noninvasive saliva samples (1 mL) were collected from all the subjects, both cases and

control groups, in sterile tubes and frozen until processing. Genomic DNA was extracted out of the saliva samples using the kit Charge Switch gDNA Buccal Cell Kits (Invitrogen). Lyses, binding with magnetic beads, washing, eluting and quantifying DNA were processed accordingly to the manufacturer's instructions. Then, the DNA was quantified using the spectrophotometer NanoDrop 2000 and frozen at -200C until the samples were processed. Disease biomarkers in saliva are used often as a diagnostic tool to screen oral and systemic health.

Genotype determination

Gene polymorphisms were analyzed by the real-time polymerase chain reaction (RT-PCR) technique. The two genotypes were identified by Tagman Genotyping Assays (Invitrogen, Applied Biosystems) according to the TaqMan protocol in 96 well plates, in order to detect the SNPs C>T (-857) rs 1799724 for *TNFA* gene and C>T (-889) rs 1800587 for *IL1A* gene. We took 4 μL of each sample and we add 12.5 μL TaqMan Genotyping Master Mix, 1.25 μL SNP Genotyping Assay (specific for each SNP), 11.25 μL DNase-free water and the DNA samples. We obtained a PCR mix of 25 μL in each microtube. Then, we used a LightCycler 480 (Roche) thermocycler to analyze our 32 and respectively 97 samples at the following parameters: 940C for 5 minutes, followed by 35 cycles – 940C for 30 s, 550C for 30 s and 720C for 30 s, and a final incubation at 720C by 7 minutes followed by a cooling to 40C. A no template control (NTC) tube was used as quality control of the assay for each genotype. Since allele B (rare) represents the hypothesis of the correlation with the pathogenesis of GAgP, we grouped genotypes according to the presence or the absence of allele B, in AA (wild type), AB (heterozygous) and BB (mutant). Mx Pro software (Mx3005P, Stratagene, Agilent Technologies) was used for processing the data obtained from the DNA samples and generate the genotyping results.

Statistical analyses

The frequency of alleles and the distribution of the genotype were compared among groups by *chi*-square test and Fisher's exact test, and odds ratios and 95% confidence intervals were also determined. A 5% significance level was set for all the analyses performed.

Results

Lipopolysaccharide (LPS) fibroblasts treatment

The treatment with LPS (1 μg/mL) for 30 days reduced the total number of viable fibroblasts (assayed by Trypan blue technique) as compared to control group, which received no treatment. That means a 79.29±9.81% total decreasing of LPS treated cultured fibroblasts after 30 days (n=7 experiments). Figure 1 is showing the different density of cultured treated fibroblasts and the morphological appearance of apoptotic ones (evidenced as A →).

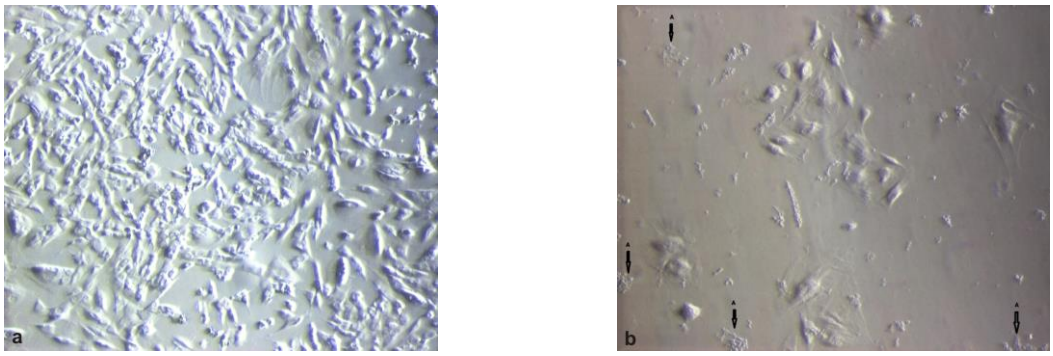

Figure II.6.3.2

The treatment with LPS (1 μg/mL) induced the decrease in the number of cultured fibroblasts as well as the increase of apoptosis (A →) (b) as compared to control group (a), without treatment (10×)

Genotype distribution

In what concerns the *IL-1A* (-889) genotype distribution, the frequency of homozygous for common allele (AA) was 9.37% for the patients with GAgP and 6.45% for healthy subjects, whereas the frequency of heterozygous (AB) was 25% for the patients with GAgP and 29.03% for healthy subjects. The homozygous for rare allele (BB) was also detected in similar values for the two groups, 65.62% for the patients with GAgP, respectively 64.51% for the healthy subjects. The data were not statistical significant ($\lambda 2$=0.34, DF=2, p=0.84). Three samples from the cases group and one from the control group could not be classified (Figure 2). For *TNFA* (-857) genotype distribution, the wild-type (CC) 66.66% was present in the study group and 62.5% in the control group, the heterozygous (CT) was present 28.57% in the study group and 37.5% in the control group, whereas the mutant (TT) was present only in the study group but only for 4.76% and has not been detected in the control group. The statistical analysis did not show significant results ($\lambda 2$=0.54, DF=2, p=0.76). Two samples from the control group and one from the study group could not be classified (Figure 3).

We also constructed a dominant model (negative genotype CC, positive genotype CT+TT) and a recessive model of distribution (negative genotype CC+CT, positive genotype TT). *TNFA* (-857) presented in the dominant model a prevalence of the negative genotype higher for the cases group (66.66%) than for the controls (62.5%), but for the positive genotype the prevalence was higher for the controls (37.5%) as compared to cases (33.33%). The statistical analyses of the contingency tables were not statistically significant ($\lambda 2$=0.04, DF=1, p=0.83). The negative genotype of the recessive model for *TNFA* (-857) was more prevalent in controls *vs.* cases (100% *vs.* 95.23%). The positive genotype of this model was absent in controls group, being present exclusively in cases group, which might show a trend for association with the susceptibility to GAgP, but in a small percentage (4.77%). The results were still not statistically significant ($\lambda 2$=0.39, DF=1, p=0.52). For the second SNP, *IL-1A* (-889), in the dominant model the negative genotype was predominant in the cases group (9.37% *vs.* 6.45% in controls group) and the positive genotype was predominant in the controls (93.55% *vs.* 90.63% in cases group). It did not reach statistical significance either ($\lambda 2$=0.23, DF=1, p=0.63). In what concerns the recessive model, the negative genotype presented similar results for both groups (34.37% in cases *vs.* 35.48% in controls), as well as the positive genotype (65.63% in cases *vs.* 64.52% in controls), the difference being too low to be significant ($\lambda 2$=0.01, DF=1, p=0.91).

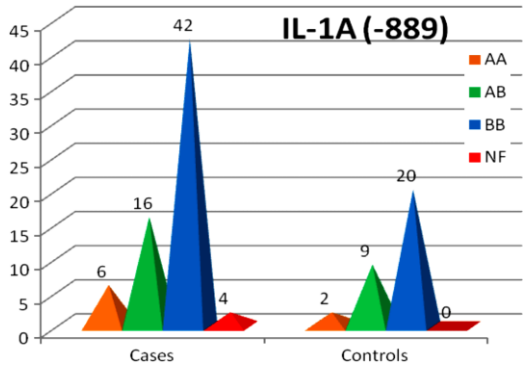

Figure II.6.3.3
Diagram of the genotype distribution for IL-1A (-889) (AA: Wild-type, AB: Heterozygous, BB:Mutant, NF: Not amplified).

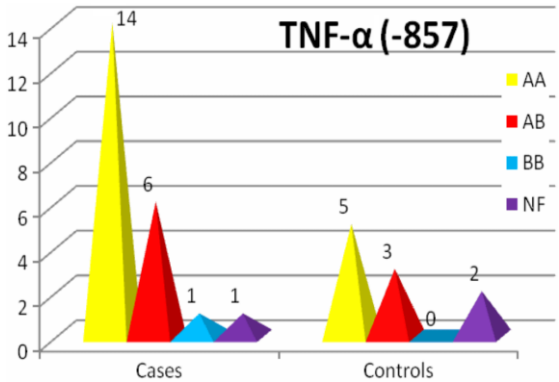

Figure II.6.3.4
Diagram of the genotype distribution for TNFA (-857) (AA: Wild-type, AB: Heterozygous, BB:Mutant, NF: Not amplified).

Allele discrimination

For the *TNFA* (-857) SNP, the frequency of allele C (common) was 80.95% in the cases groups *vs.* 81.25% in controls group. Very close values were observed also for allele T (rare), 19.05% for cases *vs.* 18.75% for controls, and not statistically significant ($\lambda 2$=0.001, DF=1, *p*=0.97). The *IL-1A* (-889) wild-type allele C (21.87% in cases *vs.* 20.96% in controls) and mutant T (78.13% in cases *vs.* 79.04% in controls) were almost equally distributed among the two groups accordingly to *chi*-square test ($\lambda 2$=0.02, DF=1, *p*=0.88). Statistical analyses revealed no significant differences in genotype and allele distributions for either gene between the two groups. No associations were observed between GAgP and *TNFA* (-857) and *IL-1A* (-889) gene polymorphisms in Romanian patients.

Discussion

Although bacteria are essential for the initiation of any form of periodontitis, the quality and types of bacteria have not been sufficient to explain the differences in disease severity. Therefore, it is considered now that some genetic variations (SNPs) commonly found in our population can represent factors which can amplify the inflammatory processes and make individuals more susceptible to an increased severity of periodontitis (Kornman, 1998). Since certain cytokines (TNF-α, IL-1α) are key regulators of the inflammatory response and are important in periodontitis, we investigated the relationship between some genetic variations associated with cytokine production in generalized aggressive periodontitis. A single genetic variation may play only a moderate or limited role in common diseases, but it may have important interactions with other genetic variations or environmental factors (Karthikeyan, 2014).

Not only gene–gene interactions, but also gene–environment interactions form a complex network in which the disease can initiate and progress. Using a decision tree analysis, *TNF*-857 and *IL-1A*-889 SNPs were identified as discriminators between periodontitis and non-periodontitis (Laine, 2013). The cytokine TNF-α has been found at high levels in gingival crevicular fluid and gingival tissues from periodontitis lesions (Engebretson, 1999). Variability in the promoter and coding regions of the *TNFA* gene may modulate the magnitude of its secretory response (Aguillón, 2002). In 2003, it was evidenced that *TNFA* (-857) SNP variant allele carrier frequency of Japanese subjects (who carried at least one variant allele among severe periodontitis patients) was significantly higher than in healthy subjects (Soga, 2003). Many investigators have, however, demonstrated that IL-1 activates the degradation of the extracellular matrix and bone of the periodontal tissue. The gingival fluid levels of IL-1α have been repeatedly associated with periodontitis. In addition, IL-1 is a strong enhancer of tissue levels of PGE2 and TNFA (6). SNPs from regulatory regions (promoter region), like *IL-1A* (-889), can cause changes in gene expression and are essential for the regulation of the transcription of the coding region. Rallele of *IL-1A* (-889) will result in up-regulating of protein production (Barnea, 2013).

It was reported an increased composite genotype of the R-alleles of the *IL-1A*, *IL-1B* and *IL-1RN* genes in non-smoking patients in whom *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* could not be detected (Laine, 2001). These results suggest that *IL-1* gene polymorphisms may play a role in the absence of other (putative) risk factors (Meisel, 2002). However, the prevalence of genotypepositive *IL-1A* (-889) in different ethnic groups and their correlation to clinical manifestations of GAgP had displayed contradictory results. The carriage rate of R-allele of polymorphic *IL-1A* (-889) varies from 34% to 64% for patients and 35% to 60% for controls for Caucasian subjects (Lindhe J, 2008). For Romanian population, R-allele of *IL-1A* (-889) was almost equal in frequency in patients group (78.31%) and controls group (79.04%). The carriage rate for *TNFA* (-857) was also similar between the two groups for the T allele, 21.878% for cases groups and 20.96% for controls.

Regarding the CC, CT and TT genotypes, similar distributions were observed among the groups as well. Our data indicated that for *TNFA* SNP, CC was more prevalent in cases (66.66% *vs.* 62.5%) than controls, CT was more frequent in controls (37.5% *vs.* 28.57%) than in cases group and TT was absent in controls, being evidenced only in cases group (4.76%). Therefore, no significant differences among groups were found. The sample size for this SNP was small (32), thus careful interpretation of the data is necessary. Our data indicated that *IL-1A* for CC genotype was more prevalent in cases (9.37%) as compared to controls (6.45%), CT genotype was more frequent in controls (29.03% *vs.* 25%) than in cases and TT genotype was almost equal distributed between the two groups (65.62% in cases *vs.* 64.51% in controls). Moreover, none of these differences was significant. Lack of association between genotypes and clinical status may be due to small sample size (97), particularly for alleles of low prevalence (De Menezes, 2008).

A lack of significant association was observed for the dominant, as well as for the recessive model. Very few studies reported some correlation between these polymorphisms and periodontitis. Other *TNFA* and *IL-1A* SNPs were also investigated in relation to periodontitis. *IL-1A* and *TNFA* polymorphisms seem to be equal distributed in the Romanian population between cases and controls, fact that could not permit any conclusions regarding its effect on GAgP. These results are in concordance with other studies of the same polymorphisms, but including other population groups (Imamura, 2008). The increased prevalence of periodontitis in young smokers was evidenced since 1993 (Haber, 1993). Also, some studies confirmed the importance of smoking as a factor in severe loss periodontal attachment in AgP (Mullaly, 1999). In our study, smoking could not be statistically correlated with GAgP.

The frequency of genetic polymorphisms may vary considerably among distinct ethnic groups, so the application of such markers for diagnosis and prognosis of periodontitis should be examined in different populations. The inconsistent results observed in the literature could be attributed to several factors related to the definition of disease, population heterogeneity, environmental and confounding risk factors (Kinane, 2000). Finally, the genetic basis for periodontitis may not be related to a single genetic variant, but may be influenced by multiple genes acting synergistically with environmental factors to increase or decrease the likelihood of developing a disease (Wilson, 1993).

Conclusions

First of all, we showed that treatment with lipopolysaccharide (1 μg/mL) for 30 days induced the decrease in the number of cultured rat gingival fibroblasts and the increase of apoptotic index as compared to control group, which received no treatment. Secondly, the data suggest that *TNFA* (-857) C/T and *IL-1A* (-889) C/T polymorphisms are not associated with susceptibility to generalized aggressive periodontitis in this northeastern Romanian population, potentially because of the small sample size. We need more samples in order to reduce the effects of sampling variation and to define reliably the association between *TNFA* and *IL-1A* polymorphisms and generalized aggressive periodontitis. Further functional analysis is needed to elucidate the molecular mechanism of periodontitis. Combining different studies and research methods may help identify new research targets, this being the quintessence of personalized medicine of the future. With nowadays advances in technology and growth in genetic knowledge, a worldwide database could be prepared in the near future with a summary of various genomic markers and their clinical implications in various types of periodontitis, allowing this way to screen susceptible individuals and to develop new therapeutic strategies.

Acknowledgments The research was financed by European Social Fund, project POSDRU/107/1.5/S/78702.

II.6.4. Pro-B lymphocyte apoptosis induced by the combination of Cytosporone b (nur77 agonist) and Cyclosporine a in the presence of gingival fibroblasts could be slightly reduced by 13-cis retinoic acid

Inflammation is closely related to oral squamous cell carcinoma (OSCC) but its mechanism is still obscure. Toll-like receptor 2 (TLR2) plays an important role in oral chronic inflammatory diseases, but the role of TLR2 in OSCC is unclear. Histological and clinical results both indicated that TLR2 played a protective role in oral tumor genesis. The results of a cytometric bead array (CBA) indicated that TLR2 deficiency resulted in Th1 and Th2 cytokine abnormalities, especially Th2 abnormalities. Immunohistochemistry also showed that TLR2 deficiency increases the number of tongue-infiltrating M2 macrophages. Overall, our results demonstrated that TLR2 plays an important role in the prevention of oral tumor-genesis and affects the levels of Th2 cytokines and tongue-infiltrating M2 macrophages; therefore, it may be used to prevent the development of oral cancer (Bang, 2019}.

During inflammation of the gums, resident cells of the periodontium, gingival fibroblasts, interact with heterogeneous cell populations of the innate and adaptive immune system that play a crucial role in protecting the host from pathogenic infectious agents. There were investigated the effects of chronic inflammation, by exposing peripheral blood mononuclear cells, peripheral blood lymphocyte cultures, and gingival fibroblasts peripheral blood mononuclear cells co-cultures to Toll-like receptor 2 (TLR2) and TLR4 activators for 21 days and assessed whether this influenced leukocyte retention, survival, and proliferation. TLR2 agonists doubled the T cell proliferation, likely of a selective population, given finally the net decrease of T cells (Moonen, 2019).

Gingival fibroblasts can participate in the immune response and play an immune-regulatory role. Human gingival fibroblasts were stimulated by the Thl cytokine, IFN-gamma, and lipopolysaccharide (LPS), thus Thl stimuli separately or in combination, or by Th2 cytokines, IL-4 and IL-13, separately or in combination (Th2 stimuli). The results showed that gingival fibroblasts can be polarized into functionally distinct subtypes, immune-activating but tissue-destructive gingival fibroblasts 1 or tissue-reparative gingival fibroblasts 2, in response to Th1 and Th2 stimuli, respectively (Jang, 2019).

In periodontitis gingival fibroblasts present in the bone-lining mucosa have the capacity to activate the formation of osteoclasts, but little is known about which local immune cells (co-)mediate this process. Lymphocyte retention is likely mediated by lymphocyte function-associated antigen-1 expression, which was significantly higher in gingival fibroblasts-peripheral blood lymphocytes cultures compared to gingival fibroblasts - monocyte cultures. When assessing inflammatory cytokine expression, high tumor necrosis alpha expression was only observed in the gingival fibroblasts-peripheral blood mononuclear cell cultures, indicating that this tripartite presence of gingival fibroblasts, monocytes, and lymphocytes is required for such an induction (Moonen, 2018).

In vitro, mouse gingival fibroblasts proliferated and produced large amounts of anti-inflammatory cytokines and tissue inhibitor of metalloproteinase-1 (Timp-1). Gingival fibroblasts deposited on the adventitia of abdominal aorta survived, proliferated, and organized as a layer structure. Furthermore, gingival fibroblasts locally produced IL-10, TGF-β, and Timp-1. In a mouse elastase-induced abdominal aortic aneurysm model, gingival fibroblasts prevented both macrophage and lymphocyte accumulations, matrix degradation, and aneurysm growth. In an angiotensin II/antiTGF-beta model of aneurysm rupture, GF cell-based treatment limited the extent of aortic dissection, prevented abdominal aortic rupture, and increased survival. Thus, gingival fibroblasts cell-based therapy is a promising approach to inhibit aneurysm progression and rupture through local production of Timp-1 (Giraud, 2017).

The aim of this study was to explore the relationships between the gingival fibroblasts and pro-B lymphocytes apoptosis in vitro because the current medical literature is lacking such data. Concrete we investigated the effects of gingival fibroblasts on the apoptosis of Nur77 receptors activated pro-lymphocytes in vitro, also in the presence of some retinoid derivatives. Gingival fibroblasts were obtained from male rats. Pro-B cells of murine type in cultures were used for actual experiments. The apoptosis of pro-B cells was induced using a combination of cytosporone B (50 μM) and cyclosporine A (1 μM). The apoptotic effects were revealed using the classical flow cytometry techniques. We clearly showed that pro-B lymphocyte apoptosis induced by the combination of cytosporone B (Nur77 agonist) and cyclosporine A in the presence of gingival fibroblasts could be slightly reduced by 13-cis retinoic acid. The inhibitory effects of 13-cis retinoic acid were powerful than those of tazarotenic acid. The effects of 13-cis retinoic acid and tazarotenic acid were evaluated toward the effects of the combination of cytosporone B and cyclosporine.

Material and methods

Gingival fibroblasts were obtained as previously described from 6-week-old male rats, 150–170 g body weight, from gingival explants, and grown up in specific culture medium, consisting of DMEM (Dulbecco's Modified Eagle Medium), 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in an atmosphere containing 5% CO2 at 37^{o}C. The apoptosis of pro-B cells was induced using a combination of cytosporone B (50 μM) and cyclosporine A (1 μM). 13-cis retinoic acid and tazarotenic acid were administered as treatment (1 μM) for 24 h, the same time as of cytosporone B and cyclosporine A, the inductors of apoptosis in our experiments. The absolute apoptosis control was represented by valinomycin (1 μM). Cytosporone B is the only known activator of Nur77 receptors. 13-cis retinoic acid represents the principal activator of RARβ and RARγ. On the other hand, tazarotenic acid is considered to be relatively selective and a potent agonist for RARβ and RARγ and less for RARα. The apoptotic effects, induced through chemical triggers, were revealed using the classical flow cytometry techniques. The control group received no treatment. The experimental design involving rats were previously approved by the Ethics Committee of the Grigore T. Popa University of Medicine and Pharmacy - Iași.

Results and discussions

When we statistically analyzed the experimental data we obtained throughout our experiments, we clearly showed that pro-B lymphocyte apoptosis induced by the combination of cytosporone B and cyclosporine A in the presence of gingival fibroblasts could be slightly reduced by 13-cis retinoic acid (Fig. II.6.4.1). The inhibitory effects of 13-cis retinoic acid were powerful than those of tazarotenic acid (Fig. II.6.4.2). The effects of 13- cis retinoic acid and tazarotenic acid were evaluated toward the effects of the combination of cytosporone B and cyclosporine A in the above mentioned conditions (Fig. II.6.4.3). All the above obtained results are to be compared to control group (data not shown), in which the natural and culture conditions-induced apoptotic proportion for pro-B lymphocytes is 10.6±1.81% for 24 hours. The adding of supplemental DMEM medium did not significantly alter the proportion of natural apoptotic cells.

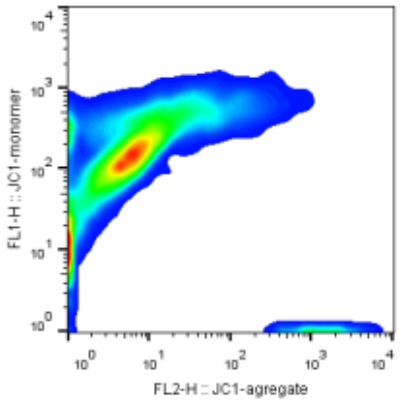

Figure II.6.4.1
The proportion of apoptotic pro-B lymphocytes in the presence of fibroblasts and 1 µM 13-cis retinoic acid

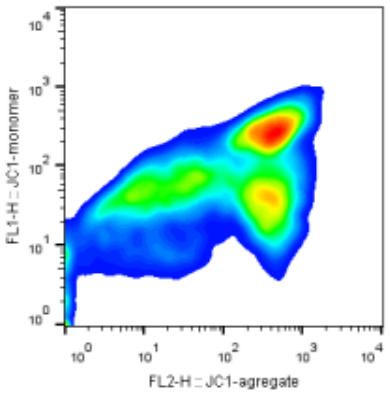

Figure II.6.4.2
Apoptotic pro-B lymphocytes in the presence of fibroblasts and 1 µM tazarotenic acid

In contrast, the administration of cytosporone B (50 µM) and cyclosporine A (1 µM) for 24 hours trigger pro-B lymphocytes apoptosis in a proportion of 78.46±5.29%.

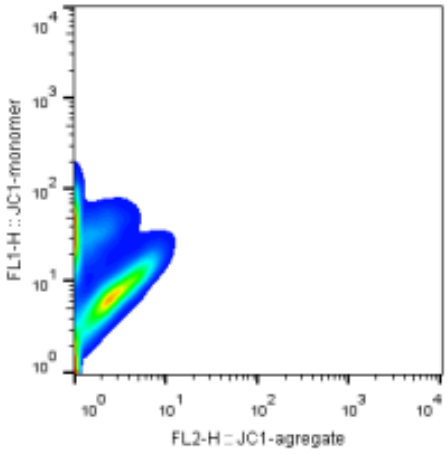

Figure II.6.4.3
The treatment for 24 hours with cytosporone B (50 µM) and cyclosporine A (1 µM) generated an enhanced rate of apoptotic pro-B lymphocytes

We explored the relationships between the gingival fibroblasts and pro-B lymphocytes apoptosis because the current medical literature is lacking such data. MSC-like populations derived from induced pluripotent stem cells (iPSC-MSC) serve as an alternative stem cell source due to their high proliferative capacity. In this study, we assessed the immune-modulatory potential of iPSC-MSC generated from periodontal ligament (PDL) and gingival (GF) tissue. The iPSC-MSC lines exhibited a similar level of suppression of mitogen-stimulated peripheral blood mononuclear cells (PBMNC) proliferation compared to their respective parental fibroblast populations in vitro.

Moreover, iPSC-MSC demonstrated the ability to suppress T-cells effector cells, Th1/Th2/Th17 populations, and increase levels of Treg cells. In order to investigate the mechanisms involved, expression of common MSC-derived soluble factors known to suppress lymphocyte proliferation were assessed in iPSC-MSC cultured with PBMNC with direct cell-cell contact or separated in transwells. Real-time PCR analysis of factors known to be involved in MSC mediated immune regulation, found a general trend of elevated IDO1

and IL6 transcript levels in iPSC-MSC lines and their respective primary cells co-cultured with activated PBMNC, with a wide range of gene expression levels between the different mesenchymal cell types. The results suggest that different iPSCMSC may be useful as a potential alternative source of cells for future clinical use in therapeutic applications because of their potent immunosuppressive properties (Ng, 2016).

There was used immunohistochemistry to quantify and compare the expression of Toll-like receptor 2 (TLR2) and cluster of differentiation 14 (CD14) in gingival tissues of both healthy individuals and patients with chronic periodontitis. There was also correlated the expression of TLR2 and CD14 with the histological grades of chronic periodontitis. There were examined 30 gingival specimens from chronic periodontitis patients and 10 from healthy individuals. Tissues from both groups were immuno-stained with antibodies against TLR2 and CD14. TLR2 and CD14 were expressed by endothelial cells, fibroblasts, lymphocytes and plasma cells. The immuno-histochemical expression of TLR2 and CD14 was significantly greater in inflammatory cells of the chronic periodontitis group than in healthy individuals. Expression of these molecules was greater in the inflammatory cells of connective tissue adjacent to pocket epithelium in both groups. The expression of TLR2 and CD14 was greatest in the periodontitis group that was classified as severe grade, followed by moderate and mild grades, which suggest a role of TLR2 and CD14 in the pathogenesis of chronic periodontitis.

The positive correlation of TLR2 and CD14 expression levels with the severity grades of chronic periodontitis suggests that they are correlated also with disease severity; therefore, they may be useful for predicting disease progression. The findings are consistent with the possibility that CD14 acts as a co-receptor for TLR2 (Sumedha, 2014).

Lipopolysaccharide signaling induced in host cells involves Toll-like receptor 4 (TLR4) accessory molecules, including LPSbinding protein (LBP), cluster of differentiation 14 (CD14) and lymphocyte antigen 96 (MD-2). However, expression of these innate defense molecules in various compartments of the human periodontium is unclear. The aim of this study was to investigate the expression profile of TLR4 in human gingiva. TLR4 immune-reactivity was found in healthy gingival epithelium and periodontitis tissue, and appeared to be lower in junctional epithelium. Fibroblasts and inflammatory cells stained more strongly for TLR4 in diseased periodontal tissues. Three TLR4 splicing variants, two MD-2 splicing variants and one CD14 mRNA were expressed by gingival keratinocytes and fibroblasts. Expression of TLR4, CD14 and MD-2 proteins was detected in keratinocytes and fibroblasts in vitro. TLR4 protein from gingival keratinocytes and fibroblasts could be co-immuno-precipitated with CD14 or MD2, suggesting an association between the related molecules in vivo. LBP transcript was detected in gingival biopsies, but not in primary cultures of gingival keratinocytes or fibroblasts. TLR4, CD14 and MD-2, but not LBP, are expressed in human gingival keratinocytes and fibroblasts. The TLR4 expression level in the junctional epithelium appeared to be lowest within the periodontal epithelial barrier (Li, 2014).

CC chemokine ligand 20 (CCL20) is involved in the recruitment of Th17 cells and thus in the exacerbation of periodontal disease, but the effect of simultaneous interleukin (IL)-22 and IL-1 beta stimulation on CCL20 production in human gingival fibroblasts (HGFs) is uncertain. In this study, we investigated the mechanisms of IL-1 betaand/or IL-22-induced CCL20 production in HGFs. A single stimulation of IL-22 could not induce CCL20 production. On the other hand, IL-22 could increase CCL20 production from IL-1 beta-stimulated HGFs in a dose-dependent manner. C-Jun N terminal kinase (JNK) and inhibitor of nuclear factor kappa B (I kappa B)-alpha phosphorylation were increased in IL-1 beta- and IL-22-stimulated HGFs. An inhibitor of nuclear factor (NF)- kappa B decreased IL-1 beta- and IL-22- induced CCL20 production, though an inhibitor of JNK did not modulate CCL20 production. These data suggest that IL-1 beta in cooperation with IL-22 could increase Th17

cell accumulation in periodontally diseased tissues to enhance CCL20 production in HGFs (Hosokawa, 2014).

Conclusions

We clearly showed that pro-B lymphocyte apoptosis induced by the combination of cytosporone B (Nur77 agonist) and cyclosporine A in the presence of gingival fibroblasts could be slightly reduced by 13-cis retinoic acid. The inhibitory effects of 13-cis retinoic acid were powerful than those of tazarotenic acid. The effects of 13-cis retinoic acid and tazarotenic acid were evaluated toward the effects of the combination of cytosporone B and cyclosporine A. The intrinsic pathways of retinoid derivatives remain to be established by further experiments.

II.7. Experimental and theoretical aspects about capecitabine and his end product, the "old 5-fluorouracil"

Capecitabine, an oral pro-drug that is metabolized to 5-FU, has been used in clinical practice for more than 20 years, being part of the therapeutic standard for digestive and breast cancers. The use of Capecitabine has been evaluated in many trials including cases diagnosed in recurrent or metastatic settings. Induction regimens or a combination with radiation therapy were evaluated in head and neck cancers, but 5-FU still remained the fluoro-pyrimidine used as a part of the current therapeutic standard. Quantifications of levels or ratios for enzymes are involved in the Capecitabine metabolism to 5-FU but are also involved in its conversion and elimination that may lead to discontinuation, dose reduction or escalation of treatment in order to obtain the best therapeutic ratio.

These strategies based on biomarkers may be relevant in the context of the implementation of precision oncology. In particular for head and neck cancers, the identification of biomarkers to select possible cases of severe toxicity requiring discontinuation of treatment, including "multi-omics" approaches, evaluate not only serological biomarkers, but also miRNAs, imaging and radiomics which will ensure capecitabine a role in both induction and concomitant or even adjuvant and palliative settings. An approach including routine testing of dihydropyrimidine dehydrogenase (DPD) or even the thymidine phosphorylase (TP)/DPD ratio and the inclusion of miRNAs, imaging and radiomics parameters in multi-omics models will help implement "precision chemotherapy" in HNC, a concept supported by the importance of avoiding interruptions or treatment delays in this type of cancer.

The chemo-sensitivity and prognostic features of HPV-OPC cancers open new horizons for the use of Capecitabine in heavily pretreated metastatic cases. Vorinostat and lapatinib are agents that can be associated with capecitabine in future clinical trials to increase the therapeutic ratio. Capecitabine is a fluoropyrimidine–carbamate, being included in the therapeutic protocol of metastatic breast cancer and colorectal cancer in combination with other agents or in concurrent treatment with radiotherapy. The principle behind the replacement of fluorouracil with Capecitabine focuses on the transformation by enzymes in tumors of this oral pro-drug in 5-FU. Thymidine phosphorylase (TP) is identified in higher amounts at the tumor level; thus the conversion of Capecitabine to 5-fluorouracil occurs mainly at the tumor level, resulting in a low concentration of the agent in plasma or normal tissues. In normal and tumor cells, fluorouracil is metabolized by 5-fluoro-20 -deoxyuridine 50 -monophosphate (FdUMP) and 5-fluorouridine tri-phosphate (FUTP). FdUMP is involved in blocking the synthesis of a thymidine triphosphate promoter, with the final consequence of blocking the DNA site. Thus, the reduction of FdUMP levels has the inhibition of cell division as a direct consequence. FUTP can be replaced by uridine tri-phosphate (UTP) during RNA synthesis, resulting in fraudulent RNA (Thomas, 1998, Walko, 2005).

II.7.1. Metabolism of Capecitabine

Capecitabine is selectively converted from 5′-DFUR to 5-FU in cancer cells by thymidine phosphorylase (TP). The addition of 5-NU, which is an inhibitor of TP, suppressed the increase in plasma concentration of 5-FU, indicating that 5′-DFUR is converted to 5-FU in the blood. In the absence of TP inhibitor, capecitabine can be converted to 5-FU even in the blood

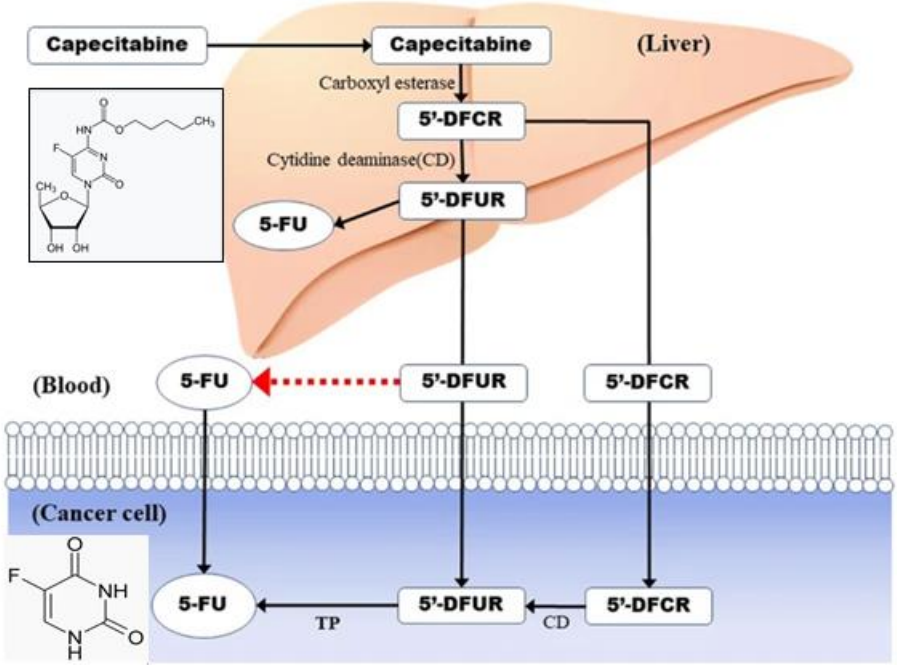

Figure II.7.1
Metabolism of capecitabine after Yoshida, 2020

II.7.2 Capecitabine–From Pharmaco-Dynamics to Biomarkers of Response

An enzyme cascade consisting of three enzymes converts the drug Capecitabine into 5-FU with the effect of inhibiting thymidylate synthesis and finally DNA synthesis. Two enzymes (carboxyl-esterase and cytidine-deaminase) are located in the liver tissue, and the final stage of drug metabolism is totally dependent on the presence of Thymidine phosphorylase (TP). TP is identified in a higher amount in the tumor and is a factor associated with angiogenesis and tumor growth and is possibly linked to an unfavorable prognosis. Dihydropyrimidine dehydrogenase (DPD) is the enzyme responsible for converting 5-FU to α-fluoro-β-alanine (FBAL), metabolites that can be eliminated, thus limiting the toxicity associated with chemotherapy.

Some 60–90% of Capecitabine is converted by this enzyme, while about 10% of the amount of chemotherapeutic is excreted unchanged in the urine. It is estimated that up to 96% of capecitabine is found in the form of FBAL in the urine. Overexpressed or reduced DPD may affect the plasma concentration of the drug and the toxicities associated with Capecitabine. The absence of DPD can be potentially fatal due to toxicity associated with the accumulation of large amounts of drugs that cannot be metabolized, but it is very rare (Nishida, 2003, Van Cutsem, 2004, Venturini, 2002). The prognostic value of this enzyme has been identified in association with first-line therapy with Capecitabine in breast cancer and with βIII-tubulin levels and is associated with outcome in cases involving Capecitabine and taxanes in first-line chemotherapy.

Thymidylate synthase (TS), DPD and TP were evaluated as potential biomarkers in stage III colon cancer patients who received adjuvant chemotherapy in the XELOX protocol or with 5-FU/Leucovorin bolus. A lower tumor level of DPD and a higher TP/DPD ratio have been associated with XELOX regimen efficacy in terms of DFS. In the case of 5-FU/Leucovorin, none of the enzymes appear to have biomarker value.

By genotyping the 50 and 3' ends, the expression of TS from patients' peripheral blood before the chemotherapy treatment and by analyzing TS, TP and DPD genes in RNA extracted from a paraffin-embedded tumor, a translational study aimed to evaluate the response to a Capecitabine chemotherapy regimen and also to identify the treatment associated toxicities in oral squamous cell carcinoma. The analysis only identified the correlation of relative TP tumor expression with anemia; 38 single genes but also metagenes including cytotoxic cell metagenes has been associated with the benefit of capecitabine adjuvant treatment in TNBC. Both the genes involved in antitumor immunity and those related to the metabolism of Capecitabine to 5-FU appear to be involved in the response of this aggressive type of breast cancer to adjuvant chemotherapy with Capecitabine (Zhao, Schmoll, 2012, Garcia, 2007, Asleh, 2020).

A prospective study included patients diagnosed with colorectal cancer and liver metastases who were treated with oxaliplatin plus 5-fluorouracil or Capecitabine in the first line. The study aimed to identify biomarkers associated with PFS and OS, both serological and imagistic biomarkers extracted from magnetic resonance imaging (MRI) being evaluated. The study included 20 cases, and the results demonstrated the ability of chemotherapy to significantly reduce the number of circulating tumor cells. The circulating concentrations of angio-genetic factors were also reduced until they were comparable to pretreatment values. These results were recorded during the second cycle of chemotherapy. No variation of imaging biomarkers was associated with PFS or OS. The infusion/permeability parameter MRI, K-trans, was increased during treatment. The correlation of higher and increasing values of K-trans with OS may be the basis for the use of this imaging parameter as a biomarker for the choice of anti-agiogenic therapy in the protocol that includes chemotherapy based on 5-FU or Capecitabine. The NALA Phase III trial looked at the benefit of the combination of Neratinib plus Capecitabine compared to the combination of lapatinib-capecitabine in previously treated metastatic breast cancer patients. HER2 positive cases were associated with a greater benefit in the Neratib–capecitabine combination. PIK3CA mutations were associated in both treatment arms with a shorter PFS. Circulating levels of C-C motif chemokine ligand 5 (CCL5) have been associated with an unfavorable prognosis in pancreatic cancer treated with Capecitabine, the result being associated with a possible CCL5-induced immunosuppressive tumor microenvironment (Mahmood, Saura, 2021).

Dose adjustment of fluoro-pyrimidine based on testing for DPD in head and neck cancers was proposed in a prospective study of 65 patients to reduce the rate of toxicity. Monitoring of uracil/UH2 plasma levels was used as a surrogate marker for the enzyme level, and 5-FU doses were reduced depending on the DPD deficiency adapted to clinical parameters. DPD deficiency was mild to mark in 20–28% of cases, with dose reduction ranging from 10% to 100%. In this group, only 9% of patients experienced severe side effects compared to 22% in the group in which no DPD deficiency was tested. There was no need to postpone chemotherapy in the group in which the dose was tested /adjusted. Given the importance of both reducing the rate of severe toxicity and avoiding delayed chemotherapy in head and neck cancers, the authors advocate for routine testing of DPD levels in this group of patients treated with fluoro-pyrimidines. Adjusting the dose of fluoro-pyrimidine according to the DPD level, whether it is a reduction or an escalation, is part of the treatment personalization strategy proposed by Chamorey, the correction of the dose of fluoro-pyrimidine will be directly proportional to the DPD level. High intrinsic lymphocytic DPD

activity was assessed as a possible biomarker of PFS, OS and response rate. A cut-off at 0.30 nmol/min/mg protein for DPD activity was identified as significant to discriminate patient outcomes. DPD deficiency is associated with 150 toxic deaths associated with the fluoropyrimidine-based regimen in France annually. In this context, noting a proportion of 50% of 76,200 patients receiving treatment based on this class of agents. Of these, 62%, 26% and 12% representing digestive, breast and neck cancer, respectively, Barin-Le Guellec proposed routine DPD testing for all patients receiving 5-FU or Capecitabine (Chamorey, 2020, Yang, 2011, Guellec, 2020).

II.7.3. Capecitabine in oral cancers—a 5-FU Equivalent Substitute?

With a net benefit of 8% compared to concurrent chemotherapy, chemo–radiation has become a therapeutic standard in locally advanced HNC cases. However, for bulky disease, induction chemotherapy followed by concurrent chemo–radiation is an option often chosen by clinicians, although data on induction chemotherapy are still controversial.

A triple combination in the TPF regimen (Docetaxel, Cisplatin, 5-Fluorouracil) is considered the standard induction regimen, demonstrating benefits over mono-therapy or over platinum doublet. However, the TPF regimen is associated with high rates of toxicity, with 31% of patients having quality of life (QOL) affected but an 86% overall response rate and a 3-year survival rate of 65.1%, justifying the use of this regimen in clinical practice. The mTPF is a modified regimen with a favorable toxicity profile, being usable in patients aged >70 years but not eligible for the standard regimen. Therapeutic regimens including platinum salts, 5-fluorouracil and taxanes are among the therapeutic options in recurrent or metastatic disease. EXTREME regimen (fluorouracil/platinum/cetuximab) combines monoclonal antibodies with chemotherapy. Until first-line immunotherapy was validated as a standard regimen, it was considered the optimal treatment for this category of patients (Haddad, 2013, Albers, 2017, Fayette, 2016, Guigay, 2021).

A Phase II study evaluated the efficacy and tolerability of 1,250 mg/m2 of Capecitabine twice a day as palliative mono-therapy for 1–14 days every 21 days for recurrent or metastatic HNC previously treated with platinum salts. The protocol provides for the administration of at least two cycles, and the overall response rate was 24.2%. The toxicity rate was a maximum of 12.5% (asthenia), 10% for dysphagia, erythrodysesthesia mucositis and 7.5% for diarrhea. The results advocated the inclusion of capecitabine in the palliative treatment of HNC previously treated with platinum salts. Capecitabine monotherapy has shown benefit in recurrent/metastatic nasopharyngeal carcinoma (NPC), according to a study that included 49 patients, 48 of whom were previously treated with platinum-based chemotherapy. With a median follow-up of 10 months, overall survival (OS) at one and two years was 54% and 26%, respectively, and patients who were treated for local–regional recurrences as well as those with hand–foot syndrome had better OS. Péron et al. demonstrated the benefit and feasibility of treatment with Capecitabine, and in heavily pretreated frail HNC patients, fatigue, mucositis and hand–foot syndrome were the most commonly reported toxicities (Martinez-Trufero, 2010, Chua, 2008, Péron, 2012).

Won, 2011, evaluated in a Phase II study the efficacy and toxicity of chemotherapy combined with Capecitabine and Cisplatin (Capecitabine 1250 mg/m2 twice daily for the first 14 days of a 3-week repeat cycle and Cisplatin 60 mg/m2 IV day 1) in recurrent or metastatic cases of head and neck squamous cell carcinoma (HNSCC). With an overall survival at 1 year and a survival rate of 10.3 months, respectively, 43.3% of the reported acute grade 3 or 4 toxicities included neutropenia (14.6%), anemia (1.5%), fatigue (4.4%), anorexia (8.8%), diarrhea (4.4%), stomatitis (3.6%) and hand syndrome (1.5%). Moreover, the study did not report toxic deaths related to treatment, and the authors consider the regimen acceptable as a toxicity profile and with a satisfactory therapeutic response (Won, 2011).

Patients diagnosed with metastatic oro-pharyngeal cancers associated with human papilloma virus (HPV-OPC) infection have a median overall survival (OS) of over 2 years, being considered eligible to receive multiple palliative therapies. The increased chemo sensitivity of this particular subclass of head and neck cancers justifies the proposal by Fazer and colleagues to use Capecitabine with possible benefits for heavily pretreated HPV-OPC patients. The average duration of treatment with Capecitabine was 9 months in a small group of seven patients, four of them having a partial response, one case showing stationary disease and two patients being diagnosed with progressive disease. It is worth mentioning one case that continued chemotherapy with Capecitabine 33 months after the initiation of treatment. The patient selection group was heterogeneous both in terms of initial treatment and metastatic sites. Among the palliative treatments, we mention radiotherapy and ablation of liver metastases, but also biological therapy with Cetuximab, immunotherapy with Nivolumab and Pembrolizumab, as well as multiple chemotherapy protocols including agents such as Cisplatin, Gemcitabine and Pemetrexed. An average time from the diagnosis of metastatic disease to initiation of treatment with Capecitabine of 21 months and a median treatment of 9 months with Capecitabine-based chemotherapy discontinued for reasons of toxicity or disease progression justifies the authors' proposal of using Capecitabine in heavily pretreated metastatic HPV-OPC cases (Fakhry, 2014, Fazer, 2020).

Capecitabine in combination with Lapatib has also demonstrated equivalence with the EXTREME regimen in the first line of treatment for metastatic head and neck cancer, other than nasopharyngeal carcinoma, evaluated in patients having an ECOG performance index of 0 to 2, the toxicity profile of the combination being considered favorable. A Capecitabine dose of 1000 mg/m^2) twice daily and Lapatinib at a dose of 1250 mg daily with an administration of Capecitabine for 14 days of each 21-day protocol included four cycles of chemotherapy associating Lapatinib daily until disease progression (Weiss, 2016). Histone de-acetylase-inhibitor vorinostat was tested in combination with Capecitabine in head and neck cancers, considering the in vivo and in vitro data supporting the activity of vorinostat in combination with deoxy-5-fluorouridine (50 -DFUR) and the potential of vorinostat to up-regulate TP. The synergistic anti-proliferative result of Capecitabine and vorinostat justifies the proposal of Di Gennaro and collaborators to implement clinical trials to support this treatment, the hypothesis formulated more than 10 years ago. Wisniewska-Jarosinska et al. mentioned both an effect of free radicals and an increase in the G0/G1 cell population and reduction of the populations in the S phase as factors that support the cyto- and genotoxic effects in head and neck cancer cells and the protection of healthy cells associated with chemotherapy based on capecitabine (Di Gennaro, 2010, Wisniewska-Jarosinska, 2011).

Evaluated in a Phase I trial, vorinostat in a maximum tolerated dose (MTD) of 300 mg was administered in cases of Stage III, IVa, IVb HNSCC cancers, including larynx, hypo-pharynx, naso-pharynx, and oropharynx, both HPV positive and negative cases, concurrent with standard chemo-radiotherapy. The complete response rate of 96.2% and the favorable toxicity profile, including especially cases of hematological toxicity, justify the testing of vorinostat in Phase II and III trials (Teknos, 2019).

A series of four drug release formulations based on 5-fluorouracil encapsulated into a chitosan-based matrix were prepared by in situ hydrogelation with 3,7-dimethyl-2,6-octadienal. The formulations were investigated from structural and morphological aspects by FTIR spectroscopy, polarized light microscopy and scanning electron microscopy. It was established that 5-fluorouracil was anchored into the matrix as crystals, whose dimension varied as a function of the crosslinking density. The in vitro drug release simulated into a media mimicking the physiological environment revealed a progressive release of the 5-fluorouracil, in close interdependence with the crosslinking density. In the context of Pharmacokinetics behavioral analysis, a new mathematical procedure for describing drug

release dynamics in polymer-drug complex system is proposed. Assuming that the dynamics of polymer-drug system's structural units take place on continuous and non-differentiable curves (multi-fractal curves), we show that in a one-dimensional hydrodynamic formalism of multi-fractal variables the drug release mechanism (Fickian diffusion, non-Fickian diffusion, etc) are given through synchronous dynamics at a differentiable and non-differentiable scale resolutions. Finally, the model is confirmed by the empirical data.

Chitosan based formulations are of increasing interest in the drug delivery field, due to its intrinsic properties such as biocompatibility and biodegradability, which recommend it for in vivo applications (Prausnitz, 2008, Patel, 2013, Jacob, 2018). In order to further improve the chitosan ability to anchor large amounts of drugs and to release them in a controlled manner, many attempts were pursued consisting mainly in chitosan crosslinking with various agents. In this line of thoughts, an eco-friendly method was developed by crosslinking chitosan with eco-friendly mono-aldehydes (Ailincai, 2010, Iftime, 2017, Marin, 2018, Olaru, 2018, Bejan, 2018). The method proved to be a successful one, providing hydrogels whose properties can be simple controlled by the nature of the aldehyde. Thus, by using the natural aldehyde: 3,7-dimethyl-2,6-octadienal, also known under the commercial name: citral, hydrogels, with excellent biocompatibility and biodegradability which were further developed as matrix for drug delivery systems, were obtained. On the other hand, the homogeneity assumption in its various forms (homogenous kinetic space, law of mass etc.) has become almost dogmatic in Pharmacokinetics (PK).The functionality of such a hypothesis allowed the development of a class of differentiable models in the description of dynamics of biological systems (i.e. "compartmental" analysis) and mainly, of drug release dynamics in such systems. However, biological systems are nowadays understood as inherently non-differential (fractal). Specifically, the microenvironments where any drug molecules with membrane interface, metabolic enzymes or pharmacological receptors are unanimously recognized as unstirred, space-restricted, heterogeneous and geometrically fractal. It is thus necessary to define a new class of models, this time non-differentiable, in describing biological system dynamics and particularly drug release dynamics in such systems.

Usually, such an approach, known as Fractal Pharmacokinetics, implies the use of fractional calculus, expanding on the notion of dimension etc. As such, it is possible in the context of "compartmental analysis" (Pereira, 2010), to describe diffusion in dense objects (Lemehaute, 1983), dynamics in polymeric networks (Barkai, 1998), diffusion in porous and fractal media, kinetics in viscoelastic media etc. More recently, "compartmental analysis" through PK allowed the modeling of processes such as drug dissolution, absorption, distribution, whole disposition, kinetics with bio-molecular reactions etc. In this paper, in the context of "compartmental analysis", a new method for describing drug release dynamics in complex systems (evidently discarding to fractional derivative and other standard "procedures" used in PK), considering that drug release dynamics can be described through continuous but non-differentiable curves (multi-fractal curves) is proposed. Then, instead of "working" with a single variable described by a strict, non-differentiable function, it is possible to "operate" only with approximations of these mathematical functions, obtained by averaging them on different scale resolutions. As a consequence, any variable purposed to describe drug release processes will still perform as the limit of a family of mathematical functions, this being non-differentiable for null scale resolutions and differentiable otherwise. Finally, the theoretical model is confirmed by the empirical date related to the 5-fluorouracil release from chitosan-based matrix.

Materials and methods

Materials 3,7-dimethyl-2,6-octadienal (95%), chitosan (243 kDa, DA: 87%), 5 fluorouracil and phosphate buffer solution with a pH = 7.4, have been purchased from Aldrich and used as received.

Formulation preparation The formulations were prepared by in situ hydrogelation of chitosan with 3,7-dimethyl-2,6- octadienal in the presence of 5-fluorouracil (Ailincai, 2018). Shortly, a 2% solution of 3,7-dimethyl-2,6-octadienal mixed with 5-fluorouracil was slowly dropped into a 3% solution of chitosan dissolved in aqueous acetic acid (1%), produced by Aldrich. The amount of drug and chitosan were kept constant, while the quantity of 3,7-dimethyl-2,6-octadienal was varied to reach different molar ratios of the amine/aldehyde groups, from 1/1 to 4/1, and thus to obtain hydrogels with different crosslinking density (Marin, 2017). The hydrogelation time increased as the amount of aldehyde decreased. Thus, it instantly occurred for a 1/1 molar ratio of amine/ aldehyde functional groups and proceeded slowly, during 24 h for a 4/1 molar ratio. Finally, the obtained hydrogels were lyophilized and submitted to analysis. The formulations were coded U1, U2, U3, U4, the number corresponding to the molar ratio of amino/aldehyde groups.

Methods

The gelation time was determined when visually the reaction mixture was transformed from viscous to rubbery state. The xerogels have been obtained by lyophilization from the corresponding hydrogels, using a Labconco FreeZone Freeze Dry System, (FreeZoner2.5 Liter Freeze Dry Systems) equipment for 24h at -50^{o}C and 0.04 mbar. The formulations were characterized by FTIR spectroscopy, on a FTIR Bruker Vertex 70 Spectrophotometer. The xerogels morphology was investigated with a field emission Scanning Electron Microscope SEM EDAX – Quanta 200 at accelerated electron energy of 20 keV.

The release kinetics from the developed drug delivery systems has been monitored by registering the absorbance at 265 nm from the supernatant in which the release was done, after which the concentration was calculated using the Beer-Lambert law. The UV-vis spectra of the supernatant were registered on a Horiba Spectrophotometer, and the absorbance was fitted on a prior drawn calibration curve. The calibration curve for the 5-fluorouracil was traced using the absorption maximum from its spectrum, at 265 nm.

Results and discussions

The presence of the 5-fluorouracil into formulations was evidenced by polarized light microscopy (Figure II.7.2.1), which revealed the clear segregation of the drug into the hydrogels with high crosslinking density (U1, U2), while for the hydrogel formulations with lower crosslinking density (U4) a bi-refringent, granular texture was observed, characteristic for sub-micrometric dimensions of the crystals, which fall under the detection limit of the equipment.

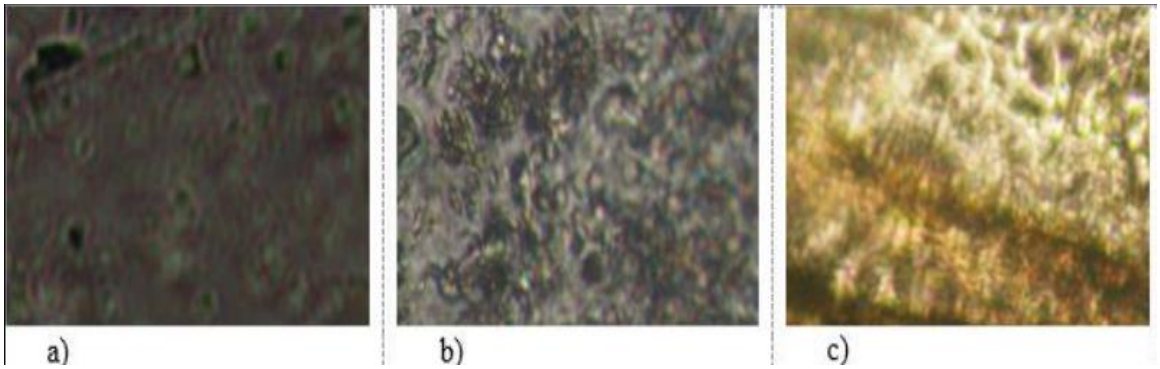

Figure II.7.2.1

Representative POM images of the formulations a) U1; b) U2; c) U4

Further, the formulation morphology was assesed by scanning electron microscopy. As can be seen in Figure II.7.2.2, they have a porous morphology, with evident drug crystals encapsulated into the pore walls. The diameter of the drug crystals decreased as the crosslinking degree was diminished, in line with the polarized light microscopy observation, as also observed for other chitosan based formulations.

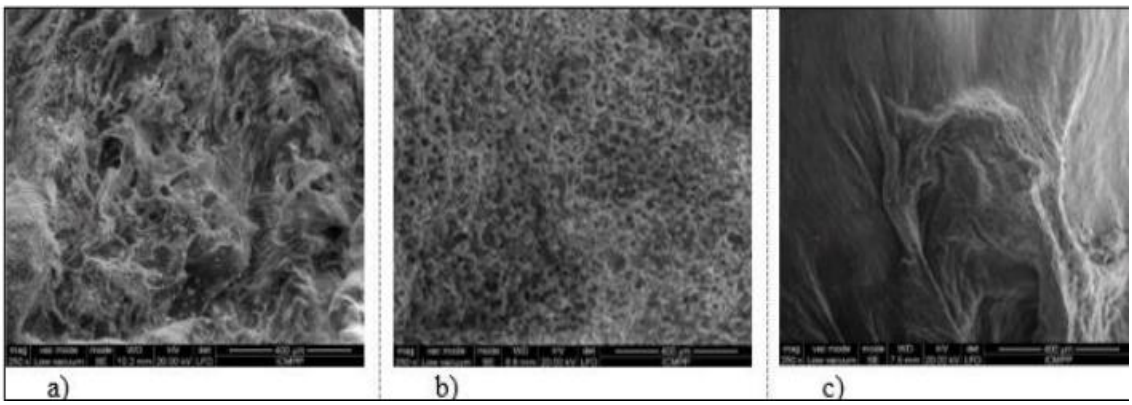

Figure II.7.2.2
Representative SEM images of the formulations: a) U1; b) U3; c) U4

The in vitro drug release of the 5-fluorouracil showed a different trend, depending by the crosslinking density. Thus, the formulation U1 with the highest crosslinking density exhibited the fastest release rate, reaching almost 100% drug released in less than 24 h. The release rate slow down as the crosslinking degree decreased along with the total percent of the drug released, reaching in the case of the formulation U4 around 75 % drug release in less than 24 h (Figure II.7.2.3). This correlated very well with the size of the drug into formulations; as the drug crystals size decreased, the release rate diminished, in agreement with the stronger anchoring of the drug into the chitosan based matrix.

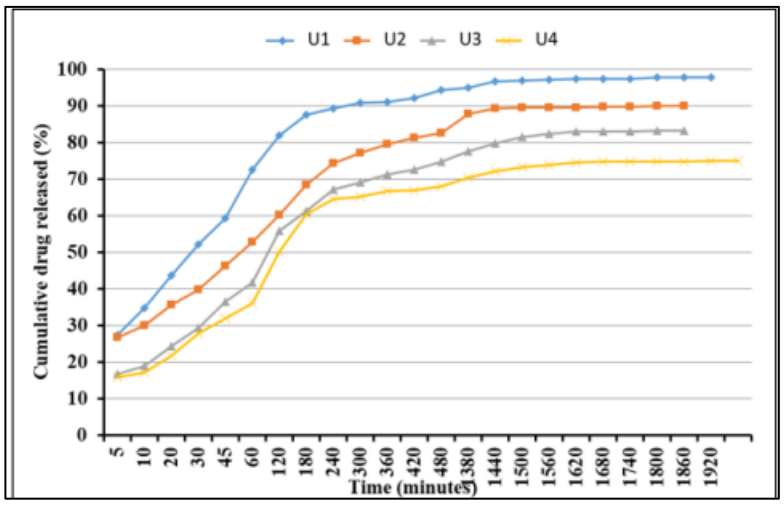

Figure II.7.2.3
The 5-fluorouracil release from the formulations (blue: U1; orange: U2; grey: U3, yellow: U4)

Theoretical model Let it be considered the one-dimensional multifractal hydrodynamic-type equations (Merches, 2016), in the form:

$$\partial_t V_D + V_D \partial_x V_D = -\partial_x \left[-2\lambda (dt)^{\left[\frac{4}{f(\alpha)}\right]-2} \frac{\partial_x \partial_x \sqrt{\rho}}{\sqrt{\rho}} \right] \quad (1)$$

$$\partial_t \rho + \partial_x (\rho V_D) = 0 \quad (2)$$

In the above – written relations, x is the fractal spatial coordinate, t is the non – fractal time having the role of an affine parameter of the motion curves, VD is the differential velocity independent on the scale resolution dt, $f(\alpha)$ is the singularity spectrum of order α and $\sqrt{\rho}$ is the states function amplitude. These equations for the initial and boundary conditions:

$$V_D(x, t=0) = V_0, \qquad \rho(x, t=0) = \frac{1}{\sqrt{\pi}\alpha} exp\left[-\left(\frac{x}{\alpha}\right)^2 \right] \quad (3)$$

$$V_D(x = V_0 t) = V_0, \qquad \rho(x = -\infty, t) = \rho(x = +\infty, t) = 0 \quad (4)$$

$V0$ the initial velocity and α the parameter of Gaussian distribution of positions, using the mathematical procedures from (Mandelbrot, 1982, Nichita2019, Merche, 2016, 2017), admit the solution:

$$V_D(x, t, \sigma, \alpha) = \frac{V_0 \alpha^2 + \left(\frac{\sigma}{\alpha}\right)^2 xt}{\alpha^2 + \left(\frac{\sigma}{\alpha}\right)^2 t^2} \quad (5)$$

$$\rho(x, t, \sigma, \alpha) = \frac{\pi^{-1/2}}{\left(\alpha^2 + \left(\frac{\sigma}{\alpha}\right)^2 t^2\right)^{1/2}} \exp\left[-\frac{(x - V_0 t)^2}{\alpha^2 + \left(\frac{\sigma}{\alpha}\right)^2 t^2} \right] \quad (6)$$

with

$$\sigma = \lambda (dt)^{\left[\frac{2}{f(\alpha)}\right]-1} \quad (7)$$

the multi-fractal degree. From here, the non-differentiable velocity VF takes the form:

$$V_F(x,t,\sigma,\alpha) = \sigma \frac{(x - V_0 t)}{\alpha^2 + \left(\frac{\sigma}{\alpha}\right)^2 t^2} \tag{8}$$

Introducing the non-dimensional variables:

$$\xi = \frac{x}{V_0 \tau_0}, \qquad \eta = \frac{t}{\tau_0} \tag{9}$$

and non-dimensional parameters

$$\mu = \frac{\sigma \tau_0}{\alpha^2}, \qquad \phi = \frac{\alpha}{V_0 \tau_0} \tag{10}$$

with τ_0 the specific time, (5), (6) and (8) become:

$$V \equiv V_D(\xi,\eta,\mu) = \frac{V_D(x,t,\sigma,\alpha)}{V_0} = \frac{1 + \mu^2 \xi \eta}{1 + \mu^2 \eta^2} \tag{11}$$

$$\rho(\xi,\eta,\mu,\phi) = \pi^{\frac{1}{2}} \alpha \rho(x,t,\sigma,\alpha) = (1 + \mu^2 \eta^2)^{-\frac{1}{2}} exp\left[-\frac{(\xi - \eta)^2}{\phi^2 (1 + \mu^2 \eta^2)}\right] \tag{12}$$

$$U \equiv V_F(\xi,\eta,\mu) = \frac{V_F(x,t,\sigma,\alpha)}{V_0} = \mu \frac{(\xi - \eta)}{1 + \mu^2 \eta^2} \tag{13}$$

Now taking out the quadratic term in η between (11) and (13), it results that for $\xi = const.$ the ratio $V\ U$ is homographic dependent of ξ by the form:

$$\frac{U}{V} = \frac{\mu(\xi - \eta)}{1 + \mu^2 \xi \eta} \tag{14}$$

From here, the condition (dynamical simultaneity):

$$d\left(\frac{U}{V}\right) = 0 \Leftrightarrow V = const U \tag{15}$$

(i.e the extension of the first principle of Newton to any scale resolution, or equivalently, synchronizations" of drug release dynamics at differentiable scale with drug release dynamics at non-differentiable scale), implies correlations between phase and amplitude of the shape function, by the form:

$$\ln \rho = \rho_0 exp[const(s - s_0)] \tag{16}$$

$\rho 0$ and $s0$ are integration constants. Thus, it is stated that various "mechanisms" involved in the drug release process can be mimed through period doubling, quasi-periodicity, intermittences etc. (Agop, 2020). Because through the restriction (15) given, for example, by $V = -U$, the multi-fractal type conservation laws (1) and (2) take the form of the multi-fractal type "diffusion" equation:

$$\partial_t \rho = \lambda (dt)^{\left[\frac{2}{f(\alpha)}\right]-1} \partial_l \partial^l \rho = \sigma \partial_l \partial^l \rho \tag{17}$$

It results that these "mechanisms" "manifest"/are "perceived" as diffusions at various scale resolutions in a multi-fractal space (fickian-type diffusion, non-fickian-type diffusion etc.) To expand on this hypothesis, we approach on investigating the following scenario: the one-dimensional drug diffusion of multi-fractal type from a controlled-release polymeric system with the form of a plane shut, of thickness δ. If drug release of multi-fractal type

occurs under perfect sink condition, the following initial and boundary conditions can be assumed:

$$\begin{aligned} t = 0, \quad & -\frac{\alpha}{2} < x < \frac{\alpha}{2}, \quad \rho = \rho_0 \\ t > 0, \quad & x = \pm\frac{\alpha}{2}, \quad \rho = \rho_1 \end{aligned} \tag{18}$$

$\rho 0$ is the initial drug states density of the multi-fractal type in the "device" of multi-fractal type and $\rho 1$ is the drug states density at the "polymer-fluid" interface of multi-fractal type. This solution equation under these conditions can take the following form (for details in the classical case see (Ailincai, 2020). In Figure II.7.2.4 there are represented the

$$f = \frac{\rho_t}{\rho_\infty} = 2\left(\frac{\sigma t}{\delta^2}\right)^{\frac{1}{2}} = \left\{\pi^{-1/2} + \sum_{n=1}^{\infty}(-1)^n erfc\left[\frac{n\delta}{2(\sigma t)^{\frac{1}{2}}}\right]\right\} \tag{19}$$

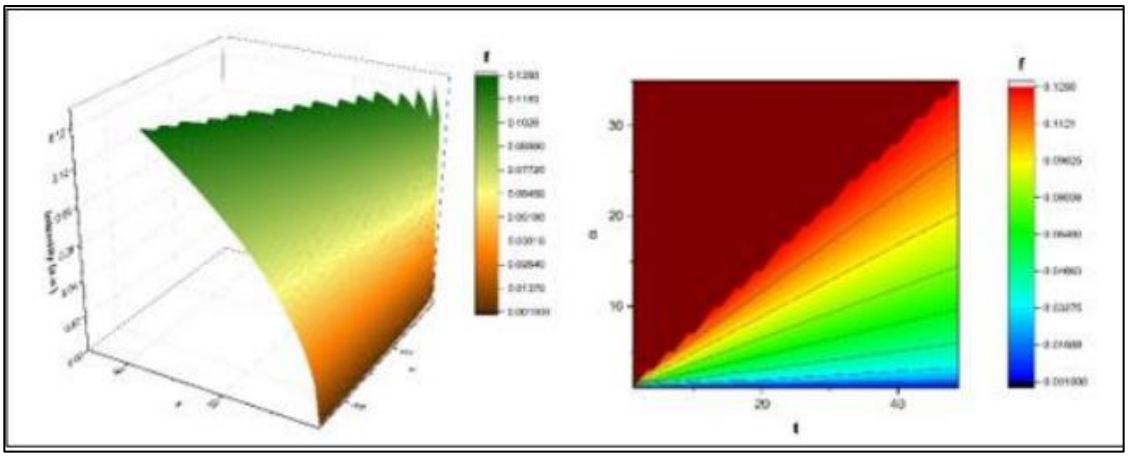

Figure II.7.2.4
3D (left-side) and contour plot (right-side) representations of our multifractal function use for drug release mechanism analysis

An accurate expression can be obtained for small values of t since the second term of (20) disappears and then it becomes:

$$\frac{\rho_t}{\rho_\infty} = 2\left(\frac{\sigma t}{\delta^2}\right)^{\frac{1}{2}} = const(t)^{\frac{1}{2}} \tag{20}$$

In such a context, ρt $\rho\infty$ can be assimilated to the fraction of dissolved drug i.e. Mt $M\infty$ $\equiv \rho t$ $\rho\infty$, where Mt is the amount of drug dissolved in time t and $M\infty$ is the total amount of time dissolved when the pharmaceutical dosage form is exhausted (Cobzeanu, 2017, Ailincai, 2020). A verification of our model is presented in Figure II.7.2.5, for the drug release of 5-fluorouracil release from chitosan-based matrix. The empirical data was fitted with the mathematical function. The figure shows that the model is well equipped to predict the drug release dynamics (Crank, 1965).

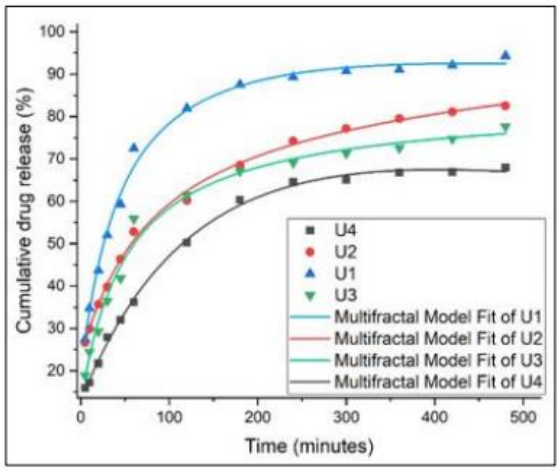

Figure II.7.2.5
Experimental showcase of the 5-fluorouracil release from the formulations fitted by the multifractal theoretical model

A series of drug delivery systems were prepared by encapsulation of the 5-fluorouracil into a hydrogel formed by crosslinking chitosan with 3,7-dimethyl-2,6-octadienal. The hydrogel proved excellent ability to anchor the drug, assuring its prolonged release during 24 h. The release rate has been tuned by varying the crosslinking density, reaching 96 % for a high crosslinking density and a 75 % rate for a lower one. A theoretical model in a multi fractal paradigm was developed for understanding the drug release dynamics, considering that these behaviors are described by continuous but non-differentiable curves. In such a context the irrotational type dynamic of the polymer drug structural units implies the functionality of a multi-fractal type hydrodynamic formalism. For the uni-dimensional case of this multi-fractal type hydrodynamic formalism, we can see that ratio between the differentiable velocity and the non-differentiable one for a certain distance depends in a homographic manner on time. The conditions for the simultaneous dynamics imply the synchronization of the drug release mechanisms at the two scale resolutions, expressed through diffusion functions of multi-fractal type (the diffusion process depends on the scale resolutions). The model is confirmed by experimental data.

Conclusions

Capecitabine will certainly be part of the therapeutic protocols of HNC, both in induction settings in a concurrent approach with radiotherapy, but even in the palliative treatment of recurrent or metastatic disease including HNC re-irradiation. The identification of patient groups that will benefit from Capecitabine more than 5-FU chemotherapy, both in terms of tumor control and the reduction of toxicities will be the object of future studies. An approach including routine testing of DPD or even the TP/DPD ratio and includes miRNAs, imaging and radiomics parameters in multi-omics biomarker models will help us implement a "precision chemotherapy" in HNC. This will help us avoid discontinuing or delaying treatment in this type of cancer. The chemo-sensitivity and prognostic features of HPV-OPC cancers open new horizons for the use of Capecitabine in heavily pretreated metastatic cases. Vorinostat and Lapatinib are agents that can be associated with Capecitabine in future clinical trials to increase the therapeutic ratio.

Acknowledgement

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II.8. New techniques and toxicity in oral cancer - implications for oral health

Most salivary gland tumors arise in the parotid glands and it presents as main therapeutic method surgical intervention followed by radiation in cases of local unfavorable prognosis (Ang, 1994). Postoperative radiotherapy is recommended in stages T3-T4, in incomplete resections with positive margins or perineural invasion bone, in forms with increased aggression or recurrences (Kirkbride, 2001). In such situations adjuvant chemotherapy appears to be effective. In advanced, inoperable, or loco-regional recurrences, local irradiation with heavy particles (neutrons or carbon ions) appears to have superior effects in terms of local control compared with standard photon radiation with no data concerning improvement of overall survival (Douglas, 2003).

Sequences and multimodal treatment steps

Adjuvant radiation therapy should be initiated ideally 4-6 weeks after surgical act. There is still no clear evidence about the role of concomitant chemotherapy. Clinical examination can reveal invasion of local structures such as skin, facial nerve (palsy), or pterygoid muscles (lockjaw) or spread to draining lymph nodes. Fine needle biopsy guided by ultrasound can confirm the presence or malignancy. Modern radiotherapy planning requires a comprehensive knowledge of cross sectional anatomy and the ability to visualize structures in three dimensions.

Formal teaching in anatomy should be part of training, using standard atlases, various online resources, three-dimensional (3D) images, which have become more accessible with picture archiving and conservation systems (PACS). Cross-sectional imaging is performed to assess tumor extension (particularly with deep edges positioned opposite to the para-pharyngeal spaces) and to asses local lymph nodes. CT scanning provides detailed cross-sectional anatomy of the normal organs, as well as 3D tumor information. These images provide density data for radiation dose calculations by conversion of CT Hounsfield units into relative electron densities using calibration curves.

Compton scattering is the main process of tissue interaction for megavoltage beams and is directly proportional to electron density. Hence CT provides ideal density information for dose corrections for tissue inhomogeneity, such as occurs in lung tissue. Clinical studies have shown that 30–80 per cent of patients undergoing radiotherapy benefit from the increased accuracy of target volume delineation with CT scanning compared with conventional simulation.

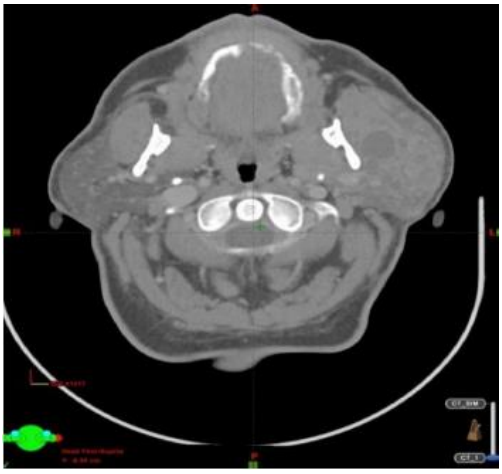

Figure II.8.1
CT scan for a left parotid tumor

MRI examination is preferred to CT because examinations may indicate a greater accuracy of perineural invasion. The scan volume starts from skull base and include all viscera-cranium and cervical lymph areas. Preoperative scanning is compulsory and improve prognosis for defining volumes irradiated in postoperative radiotherapy.

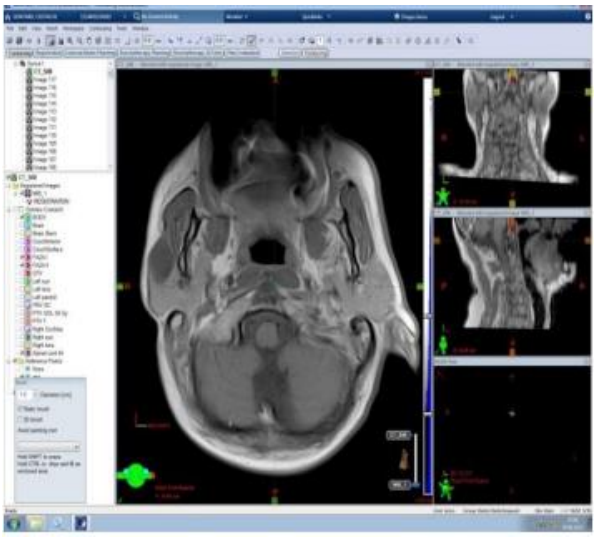

Figure II.8.2
MRI scans of left parotid tumor before excision

CT images fused with preoperative MRI are carefully evaluated to detect lymph nodes or macroscopic residual outstanding. The volume of irradiation is determined by pathological features such as perineural invasion of a major nerve, and eventually after discussions with surgeon and pathologist. The delineation of the clinical target volume will be individualized based on the extent of the disease and surgery. Considering that irradiation is adjuvant to surgery, the GTV cannot be limited even in the presence of residual masses. Gross tumor volume (GTV) is the primary tumor or other tumor mass shown by clinical examination, at examination under anesthetic (EUA) or by imaging. GTV is classified by staging systems such as TNM (UICC), AJCC or FIGO. Tumor size, site and shape may appear to vary depending on the imaging technique used and an optimal imaging method for each particular tumor site must therefore also be specified. A GTV may consist of primary tumor (GTV-T) and/or metastatic lymphadenopathy (GTV-N) or distant metastases (GTV-M). GTV always contains the highest tumor cell density and is absent after complete surgical resection.

Clinical target volume (CTV) contains the GTV when present and/or subclinical microscopic disease that has to be eradicated to cure the tumor. CTV definition is based on histological examination of post mortem or surgical specimens assessing extent of tumor cell spread around the gross GTV, as described by Holland et al. (1985) for breast cancer. The GTV-CTV margin is also derived from biological characteristics of the tumor, local recurrence patterns and experience of the radiation oncologist. A CTV containing a primary tumor may lie in continuity with a nodal GTV/CTV to create a CTV-TN (e.g. tonsilla tumor and ipsi-lateral cervical nodes). Planning target volume (PTV) is used in treatment planning to select appropriate beams to ensure that the prescribed dose is actually delivered to the CTV (Chen, 2007).

CTV60 is made based on loco-regional extension and on the surgical act. Particular attention is given to the deep excision margin which is likely to be close or involved if the facial nerve has been preserved. Para-pharyngeal and infra-temporal fossa spaces must be covered adequately. In general CTV60 medial limit must be tangent to the side of the internal jugular vein, but when the tumor affects the deep lobe of parotid irradiation volume should

include the para-pharyngeal space. CTV60 lateral limit must be just under the skin, without inclusion of postoperative scar. The position of contralateral parotid on the planning CT can be useful guide to the superior and inferior limit of the CTV60. In adenoid cystic carcinomas, the CTV60 must include the entire path of the facial nerve starting from stylo-mastoidian foramen at the skull base. Primary irradiation volume must include ipsi-lateral infra-digastric lymph nodes because inferior pole of the parotid reach on this region (Barrett, 2009).

If there are indications of adjuvant radiation after neck dissection, cervical lymph levels I- V levels must be included in CTV60 volume. There is still no concrete data about bilateral elective neck irradiation. The recommended dose in such cases of positive lymph nodes is 60Gy/30fractions. If prophylactic irradiation areas include nodal irradiation volumes Ib, II and III, an intermediate CTV44 volume can be defined.

As organs at risk (OAR), besides classical (spinal cord, brainstem, lens, optic nerves) should be outlined contralateral parotid, internal ear and cochlea. The contralateral parotid does not receive a higher dose as 28Gy (more than *xerostomia* occurs). The inner ear should be defined as an OAR, as reducing dose to the cochlea may reduce the risk of deafness.

Similar principles can be applied for volume definition for tumors of the submandibular or minor salivary glands. In adenoid cystic carcinomas the nerve innervating the primary tumor site should be include up to the skull base. In adenoid cystic carcinomas of the submandibular gland this should include the lingual nerve (a branch of the mandibular nerve, V3) back to the foramen ovale and the marginal mandibular branch of the facial nerve to the stylomastoid foramen. For tumors arising in or close to the midline (e.g. hard palate), prophylactic lymph node volumes should be outlined bilaterally if lymph nodes are to be included in the CTV.

Parotid gland irradiation depends on the available equipment, staff training and the possibility of initiating therapy in an optimal range. In principle there are using 60Co machines, linear accelerators and neutron therapy and as basic therapy there are three approaches using conventional, 3D conformational irradiation (3D-CRT), and intensity modulated radiation therapy (IMRT) planning.

Conventional therapy uses 2-3 oblique photons beams and is available on older 60Co machines and linear accelerators (energies of 4 or 6MV). In principle, this technique can provide the uniformity of the dose distribution on the CTV without increasing dose to the adjacent critical organs. For this purpose there are involves unilateral anterior and posterior wedged pair fields. A slight inferior angulation of the beams avoids an exit dose through the contralateral eye. An additional third lateral photon beam may provide a more homogenous distribution but will increase dose to the contralateral parotid gland and possibly to the spinal cord.

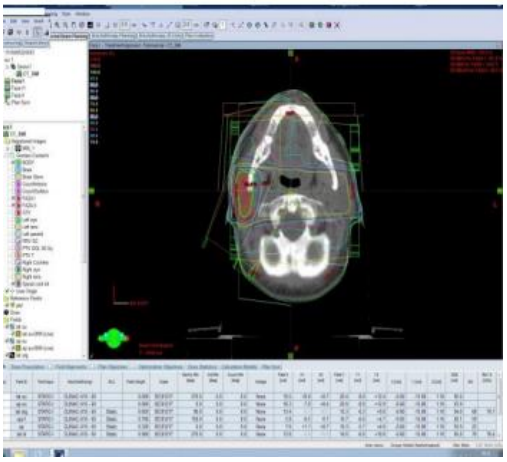

Figure II.8.3
Conformal 3D doze solution for left parotid tumor (incomplete distribution of 95 isodose)

3D conformational radiotherapy (3DCRT) use conformal geometrically shaped beams of uniform intensity. It provides superior coverage and enhanced protection of organs at risk and more normal tissues may be spared with technique (Nutting, 2001). Hotspots in the mandible of > 107% should be avoided in order to reduce the risk of osteo-radionecrosis. Excessive dose in the temporo-mandibular joint should avoid reducing the risk of long-term temporo-mandibular joint dysfunction and lockjaw. The cochlear dose should be kept below 50Gy if possible to minimize the risk of hearing damage.

Intensity modulated radiation therapy (IMRT) is a new technology in radiation oncology that delivers radiation more precisely to the tumor while relatively sparing the surrounding normal tissues. It also introduces new concepts of inverse planning and computer-controlled radiation deposition and normal tissue avoidance in contrast to the conventional trial-and-error approach. IMRT has wide application in most aspects of radiation oncology because of its ability to create multiple targets and multiple avoidance structures, to treat different targets simultaneously to different doses as well as to weight targets and avoidance structures according to their importance. By delivering radiation with greater precision, IMRT has been shown to minimize acute treatment related morbidity, making dose escalation feasible which may ultimately improve local tumor control. IMRT has also introduced a new accelerated fractionation scheme known as SMART (simultaneous modulated accelerated radiation therapy) boost. By shortening the overall treatment time, SMART boost has the potential of improving tumor control in addition to offering patient convenience and cost savings. IMRT techniques employ variable intensity across multiple radiation beams leading to the construction of highly conformal dose distributions. This is achieved by subdividing each radiation beam into smaller radiation beam lets and varying the individual intensities of these beams lets.

The advantages of this technique are improved target volume conformity, particularly in volumes with complex concave shapes, and improved sparing of normal tissues and organs at risk (OARs) resulting in reduced acute and late toxicities. IMRT also has the ability to produce inhomogeneous dose distributions, which allows the simultaneous delivery of different doses per fraction to separate areas within the target volume. This could facilitate localized dose escalation strategies without increasing total treatment time (for example, by using hypo-fractionated regimens), which may have the potential radiobiological benefit of reducing the impact of accelerated repopulation in tumors clonogens. Despite the obvious benefits of IMRT, there are still some disadvantages. The planning and quality assurance (QA) processes required for IMRT are more complex and time-consuming compared with conventional conformal radiotherapy (CRT) techniques, which can have significant impact on departmental resources. However, several commercial systems are now available that allow multiple plan measurement of IMRT plans and facilitate batching of patient QA measurements to improve efficiency. A standard IMRT plan often requires multiple fixed angle radiation beams, which can increase treatment delivery time. This can impact on patient comfort on the treatment couch, reproducibility of treatment position and intra-fraction motion. There are also some concerns that the increased treatment time could have radiobiological implications owing to the possibility of increased tumor cell repair and repopulation during the extra time required to deliver the treatment (Bragg, 2002).

IMRT plans use a larger number of monitor units (MU) compared with conventional CRT plans leading to an increase in the amount of low dose radiation to the rest of the body.

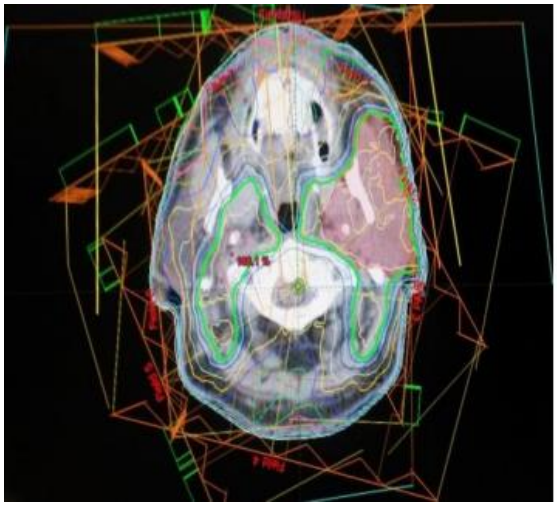

Figure II.8.4
IMRT doze solution for left parotid tumor

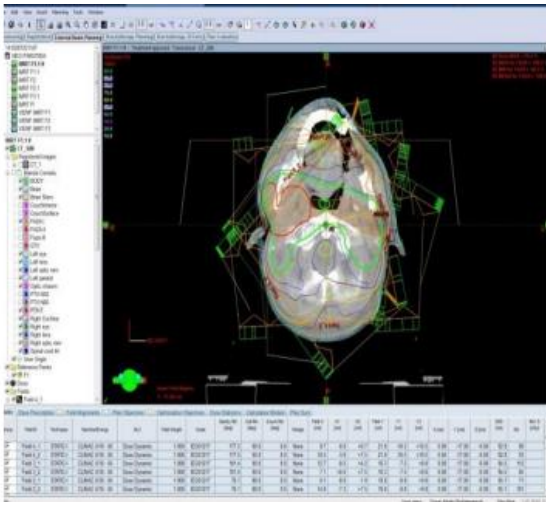

Figure II.8.5
IMRT dose solutions for a right parotid tumor

The increase in MU and subsequent increase in low dose radiation has led to concerns of increased risk of secondary radiation-induced malignancies, which is of particular relevance in pediatric patients or patients with long life expectancies. There are estimates in the literature that the number of MU in an IMRT plan is two to three times higher than a conventional radiotherapy plan with an increase in the incidence of radiation-induced secondary malignancies from 1−1.75% for patients who survive for 10 years or more. More recently, there has been some interest in arc-based or rotational therapies in an attempt to overcome some of the limitations associated with fixed field IMRT.

The basic concept of arc therapy is the delivery of radiation from a continuous rotation of the radiation source and allows the patient to be treated from a full 360° beam angle. Arc therapies have the ability to achieve highly conformal dose distributions and are essentially an alternative form of IMRT. However, a major advantage over fixed gantry IMRT is the improvement in treatment delivery efficiency as a result of the reduction in treatment delivery time and the reduction in MU us age with subsequent reduction of integral radiation dose to the rest of the body. In addition to the subsequent advantages from the shorter treatment delivery time, a further potential benefit is the availability of extra time within a set treatment appointment time slot to employ image-guided radiotherapy (IGRT). IGRT involves the incorporation of imaging before and/or during treatment to enable more precise verification of treatment delivery and allow for adaptive strategies to improve the accuracy of treatment. The main drawback of IGRT is the requirement for more time on the treatment couch and an increase in the total amount of radiation to the patient, especially with daily IGRT imaging schedules. These disadvantages are less of an issue with arc therapies, which have shorter treatment delivery times and fewer MU. There are inherent limitations with these planning studies. Even if the same strict planning objectives and calculation algorithms were used, it is extremely difficult to completely eliminate planner bias especially if multiple planners are involved in the process. Direct comparisons between different studies are not possible because of significant differences in target volume definitions, dose prescription and treatment schedules. Radiation techniques also vary between the studies, for example in the number of fields and arcs used in the fixed field IMRT and VMAT plans, and IMRT technique (SW or SS). As a result, it is not surprising that the results on PTV coverage and OAR sparing can appear conflicting between the studies.

Neutron therapy when they can be accessed is the first choice for inoperable tumors, residual masses or major relapses. Various studies have shown a higher local control rate risk

however of a higher toxicity (Chen, 2007). A study from Heidelberg for advanced, inoperable, recurrent, or incompletely resected adenoid cystic carcinoma compared results of treatment with neutrons, photons, or mixed beam. Sever grade (3 and 4) toxicity was 19% with neutrons, compared to 10% with mixed beam and 4% with photon therapy. The 5-yaer local control was 75% for neutrons and 32% for mixed beams and photons survival was identical (Chen, 2007).

Although multimodal treatments of head and neck cancers have made significant progress, the toxicities associated with irradiation of radiosensitive organs from the proximity of the tumor volume are common. Xerostomia, radiation-induced oral mucositis, radio necrosis of the jaw and dental pathologies are just some of the negative effects of treatment.

Oral mucositis is one of the most common side effects of head and neck cancer patients occurring during radiotherapy or chemo-radiotherapy, and is often the cause for discontinuation of the optimal radiotherapy delivery, and one cause of severely quality of life impairments. Over the last decade, research has proven that oral mucosal toxicity is not only caused by direct lesions, but is also the consequence of biological events involving sub mucous tissues. Late toxicity to the oral mucosa is relatively unknown, being mainly caused by the reduction in salivary flow. The most frequent late radiation effects on mucosal layers of the upper aerodynamic tract are thinning of the epithelium, loss of mucosal flexibility and sub-mucosal damage (Massaro, 2014, Zackrisson, 2003).

Radiation-induced mucositis is an inflammatory reaction of the mucous membrane of the oral and oropharynx and is an inevitable but transient effect in the case of irradiation of the head and neck cancers. The first signs and symptoms of oral mucositis include erythema and edema, burning sensation and increased warm and spicy food sensitivity. In the next stage the irradiated mucosa shows redness, inflammation, begins to decompose, with the formation of white or yellow pseudo membranes as a result of basal layer desquamation. The erythematous areas can turn into desquamation patches and later in painful ulcers that are at increased risk of infection. Radiation-induced mucositis has negative consequences to the nutritional capacity and to the fluids intake being a cause of malnutrition and dehydration (Vissink, 2003).

The response of the acute mucosa to radiotherapy is due to the basal cell, the death of the mucosal epithelium, compromising the ability of the mucosa to regenerate thus leading to thinning of the epithelium and ulcers, but it also affects the endothelium of the blood vessels. The parenchymal component (salivary acini) is also radiosensitive. Serous cells are more radio-sensitive than mucosal cells, so the parotid glands are more sensitive to xerostomia than submandibular or sublingual glands. The first radiation-induced changes include degeneration or destruction of the acinar tissue with subsequent inflammation and significant loss of salivary secretion during the first weeks of treatment. After months of irradiation, fibrosis occurs due to chronic inflammation but also adiposis, loss of vasculature and degeneration of the parenchyma occurred, all of these physiopathological changes ultimately leading to xerostomia. Low concentration of Ca^{2+} caused by xerostomia leads to greater solubility of tooth structure and demineralization impairments. Dysphagia, a consequence of the edema of structures involved in irradiation, is also aggravated by the loss of lubricating capacity with consistency in affecting mastication and swallowing. It is initially observed as progressive loss of salivary secretion and the mouth becomes dry and tender. Characteristics such as age, sex, salivary gland function at the onset of treatment are factors that influence the risk of xerostomia (Gupta, 2015, Sonis, 1998). Osteo-radionectrosis (ORN) of the mandible is one of the most severe chronic side effects of head and neck radiotherapy with significant associated morbidity and subsequent treatment may vary from close observation to radical surgical resection. ORN is a radiation induced fibrosis with histo-pathological formation phases very

similar to chronic wounds, with the activation and regulatory disruption of fibroblast activity substrate (Rivero, 2017).

Materials and methods

Our study included 20 patients diagnosed with oro-pharyngeal cancers and multimodal treated with platinum-based chemotherapy alone or in combination with taxanes or fluorouracil (2-4 cycles) followed by radiotherapy delivered in a total dose of 70Gy/35 fractions to the target volume of the primary tumor, 66Gy/33fractions on the tumor volume of the high-risk cervical lymph nodes and 50Gy/25fractions on the target volume of low-risk supraclavicular lymph nodes.

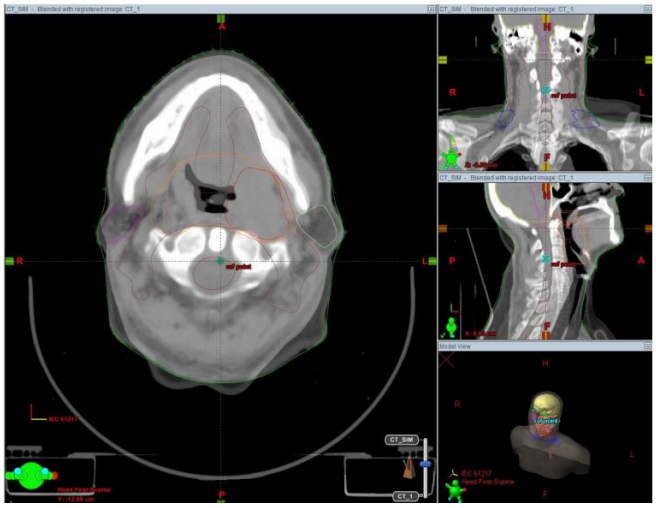

Figure II.8.6

Registration between contrast agent diagnostic imaging and CT simulation for target volume delineation and 3D reconstruction of OARs - image from Varian Eclipse ™ (TPS)

Using Volumetric Intensity Modulated Arc Therapy (VMAT) technique, the mean doses for parotid glands, oral cavity and mandible considered organs at risks (OARs) was evaluated. For radiotherapy planning, a CT simulation was performed and used for delineation of the target volumes and radiosensitive organs. For a more precise delimitation of tumor anatomic borders and to identify the maximum tumor volume prior to administration of the chemotherapy, a rigid co-registration algorithm was used between CT imaging (CT or MRI) and simulation CT images (Fig II.8.6-7). A two arcs algorithm with a 600cGy /minute dose rate was used to create VMAT (RapidArc®) treatment plans (Fig 84, 85).

Figure II.8.6

Isodose curves distribution of oral cavity region - image from Varian Eclipse ™ TPS

Results

The maximum dose received by the left parotid exceeded the mean dose of 26Gy for 14 of the 20 cases. In the case of right parotid, the average dose of 26Gy was exceeded in only 12 cases. In 11 out of 20 cases, both parotids received doses greater than 26Gy. Average mean doses obtained by the left parotid and parotid gland respectively were 3256.30cGy respectively 3326.23cGy. In all cases, the average dose received by the oral cavity exceeded 30Gy with an average of 5458.6cGy and the median Dmean received by mandible was 4260.64cGy. The average volume for the target tumor of the primary tumor that received the maximum prescribed dose was 162.23 cmc (Table XV).

Table II.8.1
Doses received by OARs - 20 patients and the volume of the primary tumor target volume (PTV-T)

Case No.	PTV-T Volume(cmc)	Dmean Left parotid (cGy)	Dmean Right parotidP (cGy)	Dmean Oral cavity (cGy)	Dmean Mandi (cGy)
1	179.63	1979	1947.7	6522	4311
2	118.05	2428	2325	6304	5592
3	176.28	4787	5455	6428	4941.5
4	160.24	2159	2169	5920	4548
5	130.09	2230	222.3	5997	5072.5
6	160.71	2410.1	2032.4	5869.4	4555.8
7	158.43	2120.9	2110.9	4481.4	4061.8
8	176.25	2721.5	2754.4	6043.2	4442.6
9	205.93	3361.6	3951.9	5652.1	4224.4
10	121.56	5097.1	5060.7	5664.4	5079.5
11	148.36	2927.6	3256.3	4019.6	3334.5
12	196.03	6772.7	6732.7	5983.3	4343.1
13	116.95	2800.6	2922.4	5790.9	4376.1
14	191.53	4013.15	4239	5774.2	4000.2
15	157.51	4578.1	4569.5	6036.1	4686.1
16	119.06	2636.4	2416.3	4557.4	3420.5
17	169.78	3241.3	3092	4334.7	3610.5
18	190.66	3380	3506	3938.6	3143.2
19	213.05	2478.8	2616.2	4165	3213.3
20	166.35	3003.1	3146.9	5690.2	4256.2
Average doses (cGy)	162.82	3256.30	3226.33	5458.575	4260.64

Discussions

The normal stimulated and un-stimulated salivary flow rate averages 1.5–2.0 mL/min respectively 0.3–0.4 mL/min. A stimulated salivary flow rate ≤0.5–0.7 mL/min and the unstimulated salivary flow rate is ≤0.1 mL/min is considered hypo salivation and when saliva flow is less than the rate of fluid absorption and fluid evaporation xerostomia is diagnosed (Dawes, 2004).

Due to the anatomical proximity between the tumor and the radiosensitive structures in the head and neck, preserving the function of these organs is difficult in order to maintain the quality of life of the patient. Radiotherapy has an important role in the treatment of head and neck cancers and in recent years techniques that better covered the target volumes and protect radiosensitive organs contribute to reducing toxicity rates. However, the use of systemic

chemotherapy and molecular target therapy for locally advanced disease in concomitant or sequential with radiotherapy contributes to a superior loco regional control and survival benefit but also associated with a high rate of toxicity. The introduction of 3D conformational radiotherapy (3D-CRT) and Intensity Modulated Radiotherapy (IMRT) was a major improvement over conventional radiotherapy. These methods are based on treatment planning systems (TPS), using computer tomography (CT) simulation with a careful delineation of target volume and OARs. The spatial relationship between target volume and OARs can be evaluated by 3D volumes reconstruction. The use of IMRT and VMAT techniques offers the possibility of conformation and high-dose irradiation for "banana shape" target volumes without delivering high radiation doses to the OARs. A mean dose on the parotid glands below 26Gy leads to a decrease in xerostomia rate. Not just the mean dose is a predictor of xerostomia. Multiple studies have shown that sub-volumes in the gland that receive certain doses contribute to toxicity, taking into account the parallel architecture of the parotid from a radiobiological point of view (Fig 86). In the case of locally advanced cancers with bulky tumor volumes, obtaining these doses is difficult, both the parotid glands and the submandibular glands (Deasy, 2010, Pointreau, 2016).

70% of the total mucus secreted by the salivary glands is produced by the minor salivary glands which are dispersed in the oral cavity so a dose limitation of the oral cavity could contribute to reducing xerostomia but could also help prevent mucositis and loss of taste. The non-implicated tumor oral cavity should be delineated as OAR and dose constraints should be used for radiotherapy planning if possible, with a dose ≤ 30Gy being recommended. A low priority compared to other radiosensitive organs is recommended to be applied in order not to compromise the correct irradiation of the target volume (Sciubba, 2006).

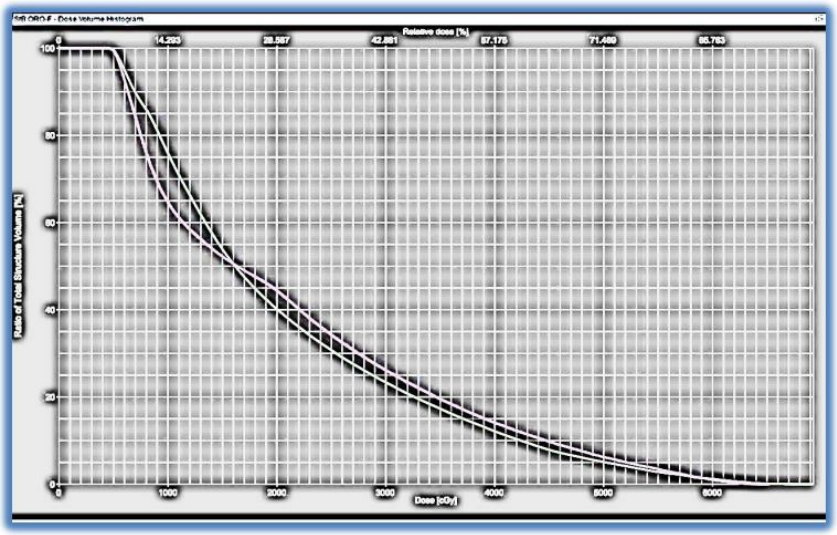

Figure II.8.7
Dose volume histograms for left (violet) and right (blue) parotid glands - image from Varian Eclipse ™ TPS

Teeth located in the irradiated field during head and neck cancers irradiation are at risk of developing radiation cavities that can progress rapidly to peri-apical disease and chemotherapy may worsen subclinical dental pathologies. Effects are potentiated by associated toxicity like xerostomia and rarely by oral lichen planus (Wang. 2011). Decreased salivary flow and oral microbial flora change are factors that aggravate the evolution of dental pathologies. Full dental assessment and dental treatment are recommended as a starting point for multimodal therapy. Teeth with advanced carious or peri-apical lesions and periodontal infections need to be extracted prior to the start of the treatment in order not to

affect the delivery of the treatment (Villa, 2018). The incidence of the ORN jaw is estimated between 2.6% and 44% with a lower ORN incidence to the maxilla because it is more vascular than the mandible. All patients who receive doses greater than 50Gy in the head and neck region are at risk of developing ORN, but dental and jaw trauma and dental surgery treatments or dental surgeries may increase the risk (Hong, 2010).

Conclusions

Oral health care assessment, including clinical and radiological evaluations prior to the start of multimodal therapy for head and neck cancers can reduce morbidity and improve the quality of life of these patients. Even if VMAT, a modern radiotherapy technic is used for the radiotherapy treatment delivery, for locally advanced cases, radiosensitive organs involved in oral cavity toxicity and teeth will receive increased radiation doses. In this context, ensuring good hygiene of the oral cavity and including dental control in the therapeutic protocol of multimodal maneuver of head and neck cancers is necessary to minimize toxicity and reduce the risk of severe late onset toxicity.

II.9. Metformin's role in response to induction chemotherapy for multimodal treated oral cancers in diabetic patients

The HNSCC (the sixth most common malignancy) genomic demonstrate the potential of targeting the mTOR pathway in the treatment of these malignancies. Metformin is one of the most widely prescribed oral anti-diabetic drugs but some preclinical evidence suggesting that metformin has anti-cancer properties leading to the inhibition of m-TOR pathway. Metformin plays also a role in modulation of the immune system by potentiating the action of T lymphocyte on the tumor cell and reduces the Warburg effect characteristic of the tumor cell where tumor cells generate ATP from glycolysis in conditions of poor nutrition and hypoxia. The association of Metformin with platinum-based chemotherapy and radiotherapy has shown in some studies potentially synergistic and radio-sensitization. Some trials demonstrate the benefit of sequential chemotherapy, (induction chemotherapy followed by radiotherapy or concurrent chemo-radiotherapy). The purpose of the study is to evaluate comparatively the imaging response (RECIST CRITERIA) to induction chemotherapy in patients diagnosed with diabetes and head and neck cancers treated with Metformin or not with the induction chemotherapy response of patients without metabolic comorbidities. The results can comparatively evaluate the platinum sensitivity for diabetic patients and the influence of Metformin in the modulation of this sensitivity. The study also aims to compare the benefit of induction chemotherapy to patients who associate diabetes mellitus with advanced local head and neck cancers proposed for multidisciplinary treatment.

Metformin acts by inhibition of the mitochondrial complex I and oxidative phosphorylation. Several studies have shown correlations between improved outcomes in oral squamous cell carcinoma and the administration of Metformin. The mechanism of action involves disruption of tumor metabolism and alteration by immune mechanisms of the tumor microenvironment. The antineoplastic activity of metformin varies depending on the metabolic status of the patients and the molecular particularities of the tumors. Adenosine monophosphate-activated protein kinase (AMPK) has been shown to be primarily responsible for the antineoplastic effects of metformin (Curry, 2018). The American Diabetes Association and the American Cancer Society formulated some consensus on the link between risk factors, common biological and prognostic features in diabetes and cancer (Giovannucci, 2010).

Materials and Methods

The study included 30 patients diagnosed with non-metastatic squamous oral cell carcinoma. The analyzed cases included squamous cell carcinomas of oropharynx (12 cases), hypopharynx (2 cases), larynx (9 cases) and floor of mouth (7 cases). Patients who were staged using CT imaging pretreatment followed induction chemotherapy of 2-4 cycles and who were evaluated for CT imagines were included. Induction chemotherapy included platinum-based mono-therapy, platinum-taxanes doublet (TP) or platinum-5- fluorouracil (PF) or triple association platinum-taxane-5- fluorouracil (TPF). 6 patients received Carboplatin or Cisplatin mono-therapy, 16 patients received TP or PF platinum doublet 8 patients received triple association TPF protocol. Subsequently, all patients received intensity modulated radiotherapy (IMRT) or volumetric arc therapy (VMAT) with curative intent. Radiotherapy was delivered using the sequential boost technique up to a total dose of 70Gy in 35 fractions on primary tumor volume, 66Gy in 33 fractions on the high-risk cervical lymph nodes target volume and 50Gy in 25 fractions for the target the low-risk supraclavicular node volume.

Patients receiving concomitant chemo-radiotherapy and patients who did not benefit from the response to chemotherapy induction were not included in the study. 3 patients in the study group were diagnosed with diabetes and received treatment with Metformin at the time of diagnosis of diagnosed diabetes with diabetes. Patients were aged between 46 and 67 years, diabetic patients aged 58.6, respectively 67 years. Diabetic patents associated comorbidities like hypertension and dyslipidemia corresponding to the profile of the metabolic syndrome. Hemoleucogram and biochemistry were analyzed weekly and established. Treatment related toxicities were evaluated using the CTCAE version 4.02. Acute toxicity was evaluated during treatment and supportive treatment was given for all patients who developed oral mucositis, body weight and fluid balance were monitored. Mouth washes with local analgesics, coating agents or anti-inflammatory corticosteroids were administered in all cases with caution on blood glucose levels and the risk of hypertension.

Results and discussion

No patient had fully responded to induction chemotherapy; 12 patients had partial response (PR), 8 patients had stable disease (SD) and 5 patients had disease progression (PD). For all diabetic patients, PF or SD was obtained after induction chemotherapy. A study by Baur et al highlighted an increased risk of mortality among diabetic patients and for those treated with insulin the relative risk was 2-4 higher compared to the non-diabetic cancer patients. The authors conclude that both diabetes and associated treatments increase the risk of mortality among patients diagnosed with cancers except for patients treated with Metformin (Baur, 2011). Alcusky et al. evaluated the effect of Metformin exposure after the diagnosis of head and neck cancers on the risk of all-cause mortality in a population-based Italian cohort. The authors analyzed 7,872 patients, 708 (8.99%) exposed at some point after the diagnosis of cancer to metformin. 3,626 (46.1%) died during a 2.98 years median follow-up. The authors failed to identify a significant association between exposure to metformin and reduced risk of all-cause mortality in case of head and neck cameras without being able to prove validated theories in preclinical studies (Alcusky, 2015).

Although systematic review and meta-analysis demonstrate the improvement in the overall survival of the majority of patients diagnosed with cancer and treated with metformin, especially colorectal cancer, broncho-pulmonary and hepatocellular carcinoma; on a cohort of Ontario diagnosed with squamous cancer of the larynx, hypo-pharynx, and naso-pharynx no survival benefit was observed (Alexandra, 2018, Noto, 2012). In a study conducted in patients diagnosed with oropharynx cancer treated by chemotherapy with modern techniques, Spratt et al. assesses the effect of metformin exposure of diabetic patients on local failure-free

survival (LFFS), regional failure-free survival (RFFS), distant metastasis-free survival (DMFS), and overall survival (OS). The LFFS have demonstrated improved DMFS for non-diabetic patients and improved DMFS for metformin users. LFFS and RFFS were significantly influenced by diabetes status and metformin use (Spratt, 2016). For patients receiving radiotherapy, there are both in vitro and in vivo studies that show the benefit of metformin that can improve the efficacy of radiotherapy for cancer patients who have diabetes, assessing short-term response and long-term survival with 2-year and 5-yearoverall survival endpoints. Patients who received metformin had survival benefits and short-term response compared to patients who were not treated with metformin (Rao, 2018).

Patients receiving metformin treatment also have a risk of losing weight caused by reduced food intake, loss of appetite and gastrointestinal side effects. Weight loss is associated by many authors with an increased risk of toxicity. In patients with head and neck cancers, weight loss is also enhanced by swallowing impairments caused by the tumor itself or by the side effects resulting from multimodality treatment. It is demonstrated that weight loss during treatment is associated with poor prognosis and increased toxicity. Chang et al. demonstrates that multimodal treated patients for head and neck cancers require careful multidisciplinary management and support in order to complete treatment without severe associated toxicity, but may also benefit from metformin from a survival point of view (Chang, 2017). Mutations in tumor protein 53 (p53) tumor suppressor genes are common in head and neck cancers and metabolic changes from mitochondrial respiration to glycolysis.

There is a possibility that glycolysis inhibitors may have a radio sensitizing effect in this subtype of patients. p53 mutant tumors are radio resistance compared to HNSCC cells that have wild type p53. Inhibition of respiration using metformin increased glycolysis in wild type TP53 tumors with a possible radio sensitivity effect (Sandulache, 2012). Another hypothesis is based on the concept that metformin acts distinctly on stem cells from head and neck cancers and on tumor cells. Cancer stem cells are involved in resistance to conventional chemotherapy. Kuo et al demonstrates the effect of metformin on malignant stem cells exposed to Cisplatin chemotherapy, but metformin has reduced the proliferation of non-stem cancer cells. The authors conclude that metformin targets complex III and has the effect of reducing reactive oxygen species, leading to differential effects on stem and non-stem malignant cells (Kuo, 2019).

Associating metformin with chemotherapy can benefit but in order to customize the treatment, it is necessary to understand the molecular mechanisms that can lead to potentiation or decrease effects to the cytotoxic effect of chemotherapeutic agents. Induction of apoptotic mitochondria and nucleus could be the explanation for the synergistic effect of metformin combination with cisplatin and down-regulation of lipoprotein or cholesterol synthesis may be molecular base theory forth effect of metformin in association with taxanes (Peng, 2017). Other authors associate metformin in the treatment of cancer with tumor-targeting microenvironment and suppression of inflammatory signals. The potential for metformin to target Axl and Tyro3 receptor tyrosine kinase inhibit cell proliferation and action on Cisplatin in ovarian cancer (Kim, 2015).

Preclinical research studies proved a synergy between immune-oncology drugs and metformin, demonstrating increase in response rate in the murine B16 melanoma model and MC38 colon adenocarcinoma model using an association between inhibitors of PD-1 combination with metformin, effect not observed for patients receiving metformin alone. The authors also demonstrated that metformin differentially impacts subtypes of oral cancers, a higher rate of apoptosis being identified in HPV-compared to HPV + carcinomas (Curry, 2018).

To elucidate the mechanisms of clinical benefit to some cancers of the head and neck by metformin administration, we must take into account the new Warburg effect theory.

Initial theory ruled that cancer cells metabolize glucose through aerobic glycolysis and normal cells become cancerous since glucose metabolism is altered from oxidative phosphorylation to aerobic glycolysis. Current theory states that Warburg effect corresponds to the initial stage of carcinogenesis and is mediated by one of the known carcinogens (excess carbohydrate in food). Metformin has been shown to be beneficial in preclinical and clinical studies by inhibiting the m-TOR pathway in prostate, pancreatic and breast cancers, with the implication that m-TOR pathway inhibition is not the only mechanism by which metformin has antineoplastic action (Devic, 2016, Ben Sahra, 2011, Sadeghi, 2012, Jiralerspong, 2009).

In other preclinical study, Verma et al. treated severe combined immune deficient mice bearing orthotopic head and neck squamous cell xenografts treated with metformin for 5 days evaluated tumor oxygen saturation and hemoglobin concentration and observed increases in these values in the treated group compared to the control group and also a significant decrease in Ki-67 staining and MR-based tumor volume in the group of mice exposed to metformin. The authors conclude that the administration of metformin induces changes in tumor microenvironment (Verma, 2018). In a cohort of patients taking metformin compared to patients not taking metformin incidence of head and neck cancer was 0.64 times lower and a lower incidence for patients> 40 years old taking metformin. However, evidence of clinical efficacy in therapeutic associations in head and neck cancers is limited to several studies. The most extensive systematic review and meta-analysis performed by Decensi et al. (2010) of metformin and cancer risk in diabetic patients, did not analyze head and neck cancer patients (Rêgo, 2015, Decens, 2010).

Conclusions

The presence of diabetes did not negatively influence the response to induction chemotherapy, but it is likely to increase the rate of complications and prolong the time until the beginning of radiation therapy. Other prognostic and predictive factors for toxicities (smoker status, presence of HPV infection, Charlson index of comorbidities, presence of diabetes complications) should be analyzed in relation to the response of diabetic patients diagnosed with head and neck cancers to induction chemotherapy on a larger lot of patients.

Instead of final judgments,

Radiomics in oral cancers – a new beginning?

Radiomics, a subdomain of artificial intelligence, consists in extracting a large volume of data from all medical imaging techniques and correlating them with clinical data in order to build predictive and prognostic models. Radiomics is related to radio-genomics that correlates genetic mutations and molecular and biological characteristics of tissues with information extracted from medical imaging. Both are state-of-the-art fields of translational biomedical research. The ability to predict early patient survival and response to treatment, but also the capacity to identify tumor subtypes non-invasively, could make radiomics a key player with an essential role in personalized oncology. In head and neck cancer radiotherapy, radiomic algorithms can predict not only the response to radio-chemotherapy or induction chemotherapy but also the need for planning through adaptive radiotherapy (ART). Radiomics can also predict the risk of severe toxicities, especially that of xerostomia.

Given the benefit that a de-escalation of treatment can bring in selected cases to improve the quality of life, radiomics is expected to be part of the therapeutic decision for head and neck cancers in the near future, and may help identify cases where de-escalation of multimodal therapy will not jeopardize the therapeutic benefit.

Section II

FUTURE DIRECTIONS IN PROFESSIONAL, ACADEMIC AND SCIENTIFIC ACTIVITY

The proposals for career development do not start from a fixed moment in life, but cover the past, the present, assuming that in the sequence of occupied positions it determined not only quantitative, but also qualitative increases, in terms of accumulated experience, based on professional training and skills acquired and demonstrated in the didactic activity. Teaching Physiopathology in a medical university requires a permanent training as well as the continuous acquisition of new knowledge.

I consider my professional mission is

• to be a teacher respected both by students and by colleagues from the academic world and the extra-academic environment. I will continuously evaluate, correct and optimize my teaching tools and performance standards used.

• to guide students to learn, how to learn, to be able to know where and what to look for, to form their own thinking in the medical field, to develop their personality by making the most of their potential and creativity;

• to treat and evaluate students in a fair way, to capitalize and periodically adapt my own skills and competences to the requirements of the quality standards regarding the teaching-learning-evaluation activity

• to support students, colleagues from the university, with the best quality plans, with reports and effective solutions based on the knowledge assimilated and the experience accumulated over the years.

The university environment requires continuous improvement in all fields of activity (teaching activity and research activity), keeping the connection with their final applicability: the optimization of medical activity. That is why I propose that the university career should be carried out in two main directions: didactic and research, without excluding their interdependence with medical activity.

The correlation of the medical activity with the didactic and research activity is feasible for the following reasons:

- obtaining skills in the use of modern laboratory techniques and learning the rigors of work, the basis for a quality research activity and allow improving performance and assuming a more active role within the research teams;

- exercising the profession of a doctor, through access to a varied and extremely interesting case study, provides the framework for supporting the practical activity of students who choose this field for the completion of their diploma work;

- the experience gained in the Laboratory of the County Emergency Clinical Hospital "St. Spiridon" Iași creates the possibility of involvement in the guidance of resident doctors and, at the same time, opens the perspective of identifying new topics of interest in research regarding the use of molecular biology techniques to optimize diagnosis.

My professional goals are related to the three points on which the development of my career is supported: teaching, research and medical activity.

Objectives in the Teaching Practice

The improvement of the didactic activity will require :

a. Making the educational offer more flexible by the introduction of new methods of diagnosis of diseases from the subject of the course and practical works of the discipline, the preparation of didactic material, the purchase of equipment, diagnostic kits, reagents in

accordance with the new diagnostic possibilities, studying to the same extent the classical methods, as a diagnostic principle and training and awareness of the future dentist with the current extended possibilities but also the limits of paraclinical diagnosis.

b. The improvement of didactic practices by developing tests for the year and license exams in the discipline.

c. Capitalizing on all the opportunities to connect with students, encouraging individual study and dialogue with each individual student, the development of the formative dimension (in addition to the informative one), of the educational process, in the spirit of training students for integration on the labor market and the development of successful careers and the development of online training, with its limits.

Objectives in the Perspective of Scientific Research

The research activity I will carry out will be materialized around the competence fields of Physiopathology and Microbiology, especially Clinical Microbiology, due to my position as primary physician in laboratory medicine, with 20 years of practice in the field of microbiology.

In summary, my general objectives related to the development of scientific research activity are:

- Increasing involvement and expanding collaborations in research, increasing the number of publications in reference journals and in the volumes of high-level international conferences, the presentation of scientific works in oral communications, round tables, posters at national and international scientific events, involvement in research projects with topics in the field of the position for which I am applying, participation in national and international research programs and identifying the application methods for a research grant with an interdisciplinary research team in order to develop joint projects with a high degree of impact and practical applicability
- Continuing the development of positive relations with students by stimulating real scientific partnership, training students in solving scientific research problems, the results of which should be valued in the annual student scientific communications or within the diploma projects
- Close communication with other teaching staff, from related disciplines, for the realization of interdisciplinary projects and the creation of research teams adapted to the needs of the project.
- Increasingly intense involvement in the activity of the professional societies in which I work, Increasing visibility on a national or international level and continuous valorization of scientific experiences.

Given the scientific concerns so far, my specific goals in the research areas that I want to expand in the future include Considering the scientific concerns so far, the research areas that I want to expand in the future include:

i. **Microbiota and Inflammation in The Oral Cavity**
ii. **Microbiota and Pathogenetic Mechanisms Underlying Oral Squamous Cell Carcinoma (OSCC)**
iii. **Microbiome and Oral Cancer Prognosis**
iv. **Radiomics in Oral Cancers.**

Bacterial concentrations in the saliva and dental plaque are high. Comprehensive information about the bacterial species present in the oral cavity is publicly available (Expanded Human Oral Microbiome Database (EHOMD). Bacteria are not uniformly distributed in the oral cavity on all surfaces, but their proliferation is different depending on

their metabolism and the health of oral cavity. For example *Streptococcus mitis* and *Streptococcus oralis* have been associated with bacterial endocarditis in patients with prosthetic valves. In addition there were bacteria associated with periodontal disease, like *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Knowledge of the alterations in the oral microbial flora can help in the development of antimicrobial therapies useful for the prevention of OSCC.

Oral health depends on a particular balance between microbial populations (oral microbiota). When this balance is lost the dysbiosis appear. Dysbiosis can lead to the generation of numerous oral diseases, including carious pathology but also periodontal disease and gingivitis (characterized by inflammation process). Correct inflammatory responses assure a correct resolution but when the inflammatory reactions persist over time there are serious locally and systemic damages.

Bacteria may induce carcinogenesis by the following mechanisms (Al-Hebshi, 2019):

1. Stimulation of chronic inflammation
2. Cell proliferation
3. Inhibition of cellular apoptosis
4. Promotion of cellular invasion
5. Production of carcinogenic substances.

In the immune responses there are complex processes regulate by cytokines which have the function of regulating, suppressing, or amplifying the mechanisms of inflammation. Inevitably, periodontitis leads to an increase of proinflammatory cytokines such as interleukin- (IL-) 1α, IL-1 β, tumor necrosis factor-alpha (TNFα), and IL-6. IL-8 as we also show in our studies. The proinflammatory cytokines facilitate induction of the inducible isoform of nitric oxide (NO) synthase (NOS), growth the production of NO who increased the vascular permeability, which accelerates nutritional supply to the tumor and promotes the tumor growth.

The relationship between inflammation and cancer is not a recent subject; many malignancies are initiated by infections involving a series of the following physiopathological mechanisms: Antiapoptotic pathways—such as the JAK/STAT and phosphatidylinositol 3-kinase (PI3K)/Akt—can be activated by infected gingival epithelial cells, the JAK/STAT pathway activates NF-κB which increases the transcription of antiapoptotic genes and stimulates TNF-α production. P53 mutations are also seen in tumors. The PI3K/Akt pathway, on the other hand, is involved in the increase of Toll-Like receptor-4 (TLR4) mRNA, in response to bacterial lipopolysaccharide (LPS). Coinfection studies using *Fusobacterium nucleatum* and *Porphyromonas gingivalis* show that they induce a synergic virulence response with a stronger inflammatory response triggered by elevated levels of TNF-α, NF-κB, and interleukin IL-1β, as well as higher levels of attachment and invasion into host cells (Pignatelli, 2022). A large number of studies show how the treatment of periodontitis substantially decreases markers of inflammation, but more investigation is also needed to assess how improved periodontal disease prevention and management strategies may impact cancer risk (Nwizu, 2020, Pignatelli, 2022).

Studies have shown that compared to healthy subjects, periodontitis-correlated taxa were significantly increased in the microbiota of the OSCC, including Porphyromonas gingivalis, *Fusobacterium nucleatum*, *Pseudomonas aeruginosa*, *Fusobacterium periodonticum* their pathogenicity being determined not so much by their simple presence in the oral cavity as by complex interactions between them.

Bacterial culturing has been the reference diagnostic technique for many years. Most of our current knowledge on microbiology derives from cultural data. The emergence of new techniques of microbial diagnostics, mostly based on immune and molecular technologies, has not only highlighted some of the shortcomings of cultural techniques but has also allowed

their introduction as easy and available adjunct diagnostic tools to be used in clinical research and practice. These techniques, especially the polymerase chain reaction (PCR), represent an opportunity for continued development. However, the ideal diagnosis for studying the oral microflora and its involvement in the progression to oral cancer is far away. Qualitative PCR provides limited information, while quantitative PCR is still in development. Good results were obtained using the real-time PCR technique for the pathogens targeted in inflammatory diseases and cancer of oral cavity. This method seems to be indicated for its simplicity, rapidity and reproducibility but it cannot analyze data for an antibiotic susceptibility test.

The reported results are more than promising, but their achievement is hampered by the high costs of the processing equipment. The multiplex real-time PCR strategy could be used to detect the bacterial species in oral cavity in closely related to clinical processes such as chronic inflammation and progression of lesions to neoplastic transformation. This assay may also serve as a quick tool for profiling and quantifying bacteria relevant to oral cancer development and likely be a valuable tool for clinical translational research.

Radiomics, a subdomain of artificial intelligence, consists in extracting large amounts of quantitative features from digital medical images and correlating them with clinical data in order to build predictive and prognostic models. Radiomics is related to radiogenomics that correlates genetic mutations and molecular and biological characteristics of tissues with information extracted from medical imaging. Both are state-of-the-art fields of translational biomedical research. The ability to predict early patient survival and response to treatment, but also the capacity to identify tumor subtypes non-invasively, could make radiomics a key player with an essential role in personalized oncology. In oral cancer radiotherapy, radiomic algorithms can predict not only the response to radiochemotherapy or induction chemotherapy but also the need for planning through adaptive radiotherapy (ART). Radiomics can also predict the risk of severe toxicities, especially that of xerostomia. Given the benefit that a de-escalation of treatment can bring in selected cases to improve the quality of life, radiomics is expected to be part of the therapeutic decision for head and neck cancers in the near future, and may help identify cases where de-escalation of multimodal therapy will not jeopardize the therapeutic benefit. Radiomics analysis can be performed using high-resolution structural imaging such as computer tomography (CT), magnetic resonance imaging (MRI), but also from digital radiography (RD) or ultrasonography (US). Hybrid imaging, and especially positron emission tomography – computer tomography (PET-CT), is also used to extract radiomic features. Subsequently, these features are correlated with clinical and biological data to build predictive and prognostic models with clinical utility.

In oncology, the correlation of mining data from malignant tumor images in order to "decode" them is a constantly expanding research direction. The growing need for biomarkers in the context of stratification of oncological disease turns radiomics into a key player in precision oncology. Radomics as „medical imaging biomarker" could be useful to predict the risk of recurrence after surgery and the response and duration of response either post-radiotherapy treatment or after systemic oncological treatments (chemotherapy, target molecular therapy, immunotherapy). The possibility of predicting non-invasively and with minimal data costs obtained by genomics and proteomics techniques is the object of the rule called „radiogenomics".

Radiomics prediction of the HPV etiology involvement in oral cancer is one of the most studied applications. The p16 immuno-histochemical evaluation, considered a surrogate biomarker, was frequently correlated with radiomics features extracted from CT images and less frequently from PET-CT and MRI. Zhu et al's radiomic study was not limited only to the identification of HPV status, but the analysis was also associating the TP53 mutation detection. The authors identified two biomarkers with potential of prognostic for oral cancer by using a random forest classification algorithm.

Radiomics is one of the most challenging areas of AI (artificial intelligence) application in medicine. Through the ability to predict survival and treatment response but also to identify non-invasive tumor subtypes, radiomics could play an essential role in personalized oncology with the ultimate goal of improving their quality of life by limiting toxicity.

Objectives In Medical Activity

The improvement of the medical activity aims to increase the efficiency and the performances that relate directly to the didactic and research fields within the discipline.

I strongly believe that the university's environment can't be separated from the medical activity, as the teaching activity finds its main purpose in the training of future generations of highly trained doctors and the research activity must find applications in the medical practice.

This is one of the reasons why I want to pay special attention to professional training as a doctor in the specialty of Laboratory Medicine by attending training courses and by acquiring new skills or learning new diagnostic methods and molecular biology techniques; they have proven to be very useful both in diagnosis and research activity and in improving the quality of management in medical laboratories.

I don't think that I am able to complete my university career on my own.

I hope that I will be able to attract the collaboration and support of my colleagues in as many teaching and research projects as possible. Achieving the proposed objectives, both in the didactic, research activity and in the medical activity, is possible only within a united team, in which the members complement each other, with the support of "Grigore T. Popa" Iasi University of Medicine and Pharmacy and, finally, of my family.

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