GRIGORE T. POPA UNIVERSITY OF
MEDICINE AND PHARMACY IASI

MANAGING, TRAINING AND EXPLORING ANATOMY

- HABILITATION THESIS -

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CONTENTS

ABBREVIATIONS

CRC	Colorectal cancer
SMAS	Superficial musculo-aponeurotyc system
CT	Computed tomography
MRI	Magnetic resonance imaging
ICCs	Interstitial cells of Cajal
α-SMA	A -smooth muscle actin
ICC-MY	Interstitial cells of Cajal intermuscular
ICC-IM	Interstitial cells of Cajal intramuscular
ICC-SM	Interstitial cells of Cajal submucosal
ANO1	Anoctamin-1
NCCs	Neural crest cells
ETCs	Endothelial tip cells
PD	Peritoneal dialysis
FOA	Fronto-orbital artery
ACA	Anterior cerebral artery
ACoA	Anterior communicating artery
ARFOA	Aberrant right fronto-orbital artery
BCP	Biphasic tricalcium phosphate
ORIF	Open Reduction and Internal Fixation
CPC	Phosphocalcic cements
CaP	Calcium phosphates
HA	Hydroxyapatite
SEM	Scanning electron microscope
CS	Chitosan medium molecular weight
TPP	Sodium tripolyphosphate
DMSO	Dimethylsulfoxide
CS-M	Chitosan-metformin
CS-MG	Chitosan-metfomin-glybenclamide
CS-G	Chitosan-glybenclamide
FT-IR	Fourier transform infrared
SD	Swelling degree
SGF	Simulated gastric fluid
TEM	Transmission electron microscopy
STZ	Streptozotocin
GBM	Glomerular basement membrane
F	Females
M	Males
G	Group

PDD	Peritoneal dyalisis duration - years
MD	Mesothelial denudation
STF	Submesothelial stromal fibrosis
FD	Fibrin deposits
SVH	Subendothelial vascular hyalinosis
VP	Vascular permeability
PI	Perivascular inflammation
Ca	Calcification
ESRD	End stage renal disease
AKI	Acute kidney injury

ABSTRACT

A habilitation thesis brings autobiographical evidence of the background of the main results in postdoctoral scientific, didactic and professional activity. It also highlights the perspectives which this background open. Academic carreer is a complex profession, which lays its success on persuasion and self-improvement, creativity and a continous search for new ideas and concepts.

The gross part of my academic career have been running under the sign and perspective of scientific research opportunity. I undiluted my work on two major goals: the research of different anatomical structures and scholar and research theme which is largely addressed to the pedagogical management of students and young practitioners.

My work in the Anatomy Chair has also been focused on selection and motivation of the new coworkers and to stimulate and encourage future physicians to explore these themes.

The leading perspectives of my professional career are to pursue research in the two major directions already mentioned.

Entire efforts made accomplishing scholarship results are about to permanently update our syllabus and to synchronize it with the one of the European Union countries. This will improve the performance of our students and our international visibility, with a huge socio-economic impact.

The inducement in the research activity started with the graduation of my PhD, under the coordination of Mircea Chiriac MD PhD. The theme of my doctoral thesis was "The systemic and telomic study of the external carotid system".

My academic activity materialized in approaching the most social and medical demanding current dissorders.

Following this direction we have conceived and published a total of 28 scientific in extenso manuscripts which gave me the right to participate in a number of 3 grant research projects.

My habilitation thesis is organised in the three major SECTIONS required by the CNATDCU, as recommended and approved criteria. The manuscript presents my entire postdoctoral academic activity within the Chair of Anatomy at ‘Grigore T Popa’ University of Medicine and Pharmacy in Iaşi.

SECTION I includes the abstract of the thesis.

SECTION II is divided into two distinguished parts: overview of personal, professional, academic and scientific achievements and a second part formed by another four chapters.

The first part is a biographical record of the most significant features of my professional achievements in all the fields of academic activity. They are the consistence of the background and the skillfulness which enable me to achieve the top of the academic career.

The second part comes out of my studies performed after I graduated the PhD research, including all the projects I have conducted.

Thus, in its CHAPTER 1, entitled *FROM HYPPOCRATES TO HARVEY - FROM FASCIES TO ARTERIES* I discuss about the disseminated research of my work in morphofunctional approach of fascias, skeletal muscle and arterial anatomical variants.

By the CHAPTER 2, entitled *THE PHOENIX OF BONE RESTORATION - FROM PATHOLOGY TO ANATOMY* I approach the current issue of osteosynthesis which affects so many orthopedic patients. This study is conducted in order to assess the biocompatibility, quality and extent of osseous healing of modern days synthetic bone substitutes.

CHAPTER 3, entitled *MORPHOFUNCTIONAL AND APPLIED DEVELOPMENTAL RESEARCH ON THE ENDOCRINE SYSTEM* undergoes the theme of the morphofunctional study on pancreatic endocrine system. It contains also, the results of a research project I have managed in laboratory study of diabetes mellitus type 2.

The last one, CHAPTER 4, entitled THE PROPHECY OF SURVIVAL - ADVANCES IN TUMOUR TRACKING approaches the issue of early diagnosis, staging and management of colorectal tumours. This theme has also a huge social and economic impact because colorectal malignancies have an increased incidence and became one the most common cause of death by cancer.

The purpose of the study is to evaluate the efficiency in use of neoangiogenesis biochemical markers and different radiologic high-end techniques in order to assess early informations about tumour proliferation rate and the prognosis of the patients.

All my scientific achievements so far have been outlined in books and book chapters, ISI articles in journals with impact factor and communications at congress and conferences. These would not have been possible without teamwork within Anatomy Chair and would not have been possible without the help and signature of the medical school trainers.

The continuity in studying the arterial system, started with the topic of my doctoral study, opened the way for my research career and empowered me with compulsory skills of an academic carreer. The achievements of my academic activity enhance the international visibility and the reputation of the University I am part of.

SECTION III presents in a detailed manner all the perspectives that my former academic activity has opened. There are described all my plans for future researches in chronological order of the originating idea.

Following this thinking I started to present the subjects from the PhD studies and I finished with the most recent ones.

In the last part of the habilitation thesis I added the most significant and outstanding literature works that I used in my career as a fundamental starting point for my research.

REZUMAT

O teză de habilitare aduce dovada autobiografică a fundamentului principalelor rezultate în activitatea științifică, didactică și profesională postdoctorală. De asemenea, subliniază perspectivele pe care acest fundament le deschide. Cariera academică este o profesiune complexă, care își găsește succesul în determinare și autodepășire, creativitate și căutarea continuă a unor idei și concepte noi.

Majoritatea carierei mele academice s-a desfășurat sub semnul și perspectiva oportunității de a realiza o cercetare științifică. Mi-am direcționat munca pe două obiective majore: cercetarea diferitelor structuri anatomice și a temei de cercetare care se adresează în mare măsură managementului clasei de studenți și al tinerilor practicieni.

Activitatea mea în cadrul Catedrei de Anatomie a fost, de asemenea, axată pe selectarea și motivarea noilor colaboratori și pentru stimularea și încurajarea tinerilor medici pentru a explora aceste teme.

Perspectivele principale ale carierei mele profesionale sunt de a continua aprofundarea celor două direcții majore de cercetare deja menționate.

Toate eforturile depuse pentru obținerea rezultatelor la nivel înalt în cercetare au ca obiectiv actualizerea programei noastră de studiu și să o sincronizeze cu cea din țările Uniunii Europene. Acest lucru va îmbunătăți performanța studenților și vizibilitatea noastră internațională, cu un impact socio-economic major.

Stimularea în activitatea de cercetare a început odată cu absolvirea doctoratului, sub coordonarea prof. Dr. Mircea Chiriac. Tema tezei mele de doctorat a fost "Studiul sistemic și telomic al sistemului carotic extern".

Activitatea mea academică s-a concretizat în abordarea celor mai de interes probleme sociale și medicale, acestea fiind totdată și printre cele mai solicitante.

De pe urma acestei direcții de cercetare am conceput și publicat un număr total de 28 de manuscrise științifice în extenso care mi-au dat dreptul de a participa la un număr de 3 proiecte de cercetare de tip grant.

Teza mea de abilitare este organizată în cele trei secțiuni majore, solicitate de CNATDCU, conform criteriilor recomandate și aprobate. Manuscrisul prezintă întreaga mea activitate academică postdoctorală în cadrul Catedrei de Anatomie, din cadrul Universității de Medicină și Farmacie "Grigore T Popa" din Iași.

SECȚIUNEA I include rezumatul tezei.

SECȚIUNEA a II-a este împărțită în două părți distincte: trecerea în revistă a realizărilor personale, profesionale, academice și științifice și cea de a doua parte este formată din patru subcapitole.

Prima parte a acestei secțiuni este o prezentare biografică a celor mai importante aspecte ale realizărilor mele profesionale în toate domeniile activității academice. Acestea sunt consistența fundamentul formării mele profesionale și a abilităților care mă consacră să obțin un rol esențial în educația academică.

A doua parte vine din studiile efectuate de după terminarea cercetării doctorale, inclusiv toate proiectele pe care le-am desfășurat.

Astfel, în CAPITOLUL 1, intitulat "DE LA HIPPOCRATES LA HARVEY - DE LA FASCII LA ARTERE", am discutat despre aportul meu personal în abordarea morfofuncțională a fasciilor, a mușchilor scheletici și a diferitelor variante anatomice arteriale.

În CAPITOLUL 2, intitulat "PASĂREA PHOENIX ÎN REFACEREA OSOASĂ - DE LA PATOLOGIE LA ANATOMIE, abordez problematica de actualitate legată de osteosinteză, care afectează atât de mulți pacienti ortopedici. Acest studiu este realizat pentru a evalua biocompatibilitatea, calitatea și amploarea vindecării osoase prin utilizarea diverselor materiale pentru osteosinteză.

CAPITOLUL 3, intitulat STUDIU MORFUNCȚIONAL ȘI CLINIC ASUPRA SISTEMULUI ENDOCRIN, se axează pe tema cercetării sistemului endocrin pancreatic. Acesta conține, de asemenea, rezultatele obținute printr-un proiect de cercetare pe care l-am condus în studiul de laborator al diabetului mellitus de tip 2.

Ultimul, CAPITOLUL 4, intitulat PROFEȚIA SUPRAVIEȚUIRII - ACTUALITĂȚI ÎN DEPISTAREA PRECOCE A CANCERELOR, abordează problematica diagnosticării precoce, stadializării și managementului tumorilor colorectale. Această temă are, de asemenea, un impact social și economic imens, deoarece malignitățile colorectale au o incidență crescută și au devenit una dintre cele mai frecvente cauze de deces prin cancer.

Scopul studiului este evaluarea eficienței utilizării markerilor biochimici ai neoangiogenezei și a diferitelor tehnici radiologice de ultimă oră pentru a evalua criteriile de diagnostic precoce, rata de proliferare a tumorii și prognosticul pacienților.

Toate realizările mele științifice de până în prezent s-au materializat prin publicarea de cărți și capitole de carte, articole cotate ISI din reviste cu factor de impact și comunicări la congrese și conferințe de profil. Acestea nu ar fi fost posibile fără o muncă de echipă și în echipă, în cadrul disciplinei de anatomie cât și fără ajutorul și aportul unor formatori de școală medicală care și-au pus semnătura asupra evoluției mele academice.

Continuitatea în studiul sistemului arterial, începută cu subiectul cercetării mele doctorale, a deschis calea în cariera mea de cercetător și m-a împuternicit cu abilitățile obligatorii ale unei cariere academice. Realizările activității mele academice sporesc vizibilitatea internațională și reputația universității din care fac parte.

SECȚIUNEA a III-a prezintă, într-o manieră detaliată, toate perspectivele pe care activitatea mea academică a deschis-o. Sunt descrise aici toate planurile mele pentru cercetări viitoare în ordinea cronologică a ideii originare.

În urma acestei gândiri am început prezentarea cu subiectele derivate din studii de doctorat și am terminat cu cele mai recente.

În ultima parte a tezei de habilitare am adăugat cele mai semnificative și marcante lucrări de literatură pe care le-am folosit în cariera mea ca o bază fundamentală de plecare pentru cercetarea mea.

SECTION I. PROFESSIONAL, SCIENTIFIC AND ACADEMIC ACHIEVEMENTS

Brief overview of the academic carreer

The development and perspectives of my academic career has both a professional and, especially, a human side. These sides are the cumulative, objective and appreciative result of my life experiences. All the efforts made to achieve a high level of academic recognition and performance represent the successful conquest of the efforts made until that moment.

In the context of rapid social evolution and a competitive market for academic service offerings, universities need to adapt to the needs and requirements in order to achieve an educational management standard. This enables them to further development and recognition among the top-ranking higher education institutions. It could only be done with the help of highly qualified teachers, who have to form competitive and adaptable specialists in the field.

The academic activities oblige each individual to didactic work and scientific research. European Union policy and management of research causes a fallacy between the two directions of action by the fact that institutional and individual assessment is determined by the achievement of performance parameters/indicators in research. Thus, while didactic activity evolves on a linear plateau, the research activity exponentially expands.

Teaching activity

My teaching experience is the result of 22 years of practical works and courses in the field of Anatomy. Over time, the teaching methods I have used have diversified with the modernization of the infrastructure, but the key instrument in the anatomy teaching process remains anatomical dissection. It is the main teaching method for anatomy, through which the student first needs to understand the notions and then memorize them are fulfilled. However, there is also a high demand for personalized bibliographic resources, correlated with the syllabus.

I have achieved all my teaching levels by competition:

- ↗ Associate Professor from 2015 until now at the Chair of Anatomy and Embryology, Faculty of Medicine, University of Medicine and Pharmacy "Grigore T. Popa" Iași;
- ↗ Lecturer from 2007 to 2015, at the Chair of Anatomy and Embryology, Faculty of Medicine, University of Medicine and Pharmacy "Grigore T. Popa" Iași;
- ↗ Assistant professor between 01.03.1999-22.09.2002 and 1.10.2003-31.09.2007, at the Chair of Anatomy and Embryology, Faculty of Medicine, "Grigore T. Popa" University of Medicine and Pharmacy Iași;
- ↗ Associate instructor from 01.10.1996 to 1999, at the Chair of Anatomy and Embryology, Faculty of Medicine, University of Medicine and Pharmacy "Grigore T. Popa" Iași.

I have finished the PhD study in 2005.

From my first step in the Chair of Anatomy to the present day, I have had as basic principles the permanent improvement of anatomical knowledge, skills and didactic methods, the integration of anatomy into clinical practice.

During this time I was looking to accomplish my teaching competences by:

- permanent updating of course contents and presentation of well-documented lectures;
- guiding good, systematized and stimulating practical classes;
- elaboration of course and practical textbooks for Romanian study directions;
- development of course and practical printed guides for all study directions;
- developing evaluation tests for practical and course hours for all study directions;
- capitalizing the results of the students' assessment for the improvement of didactic activity.

Research activity

I began my scientific research projects simultaneously with my my enrollment in the PhD studies. I was admitted to PhD training at ”Grigore T. Popa” University of Medicine and Pharmacy Iaşi and in 2005 I obtained the title of Doctor in Medical Sciences, Medicine Doimain, (OMEC 3956 from 25.04.2005) – with the thesis entitled: "The systemic and telomic study of the external carotid system", scientifically coordinated by Prof. Mircea Chiriac, MD, PhD. During the preparation of the PhD thesis, I updated and improved a series of anatomical dissection techniques of cervico-cranial structures.

With my new condition of being an Anatomist, I gradually integrated into the research activity.

I continued my research work, constantly improving myself by attending two postgraduate courses entitled: "Adrenal gland - controversy and certainty. Cortico-medullar paracrine interactions and their involvement in the development of 20th century syndrome", and "The Impact of Cell and Molecular Biology Studies in Deciphering Mechanisms to Provide Hypothalamic-Hypophyso-Adrenal Axis Function and Their Implications in Pathology". They allowed me to acquire the necessary skills in performing macro- and microscopic morphological studies, *in vitro* experimental tests, statistical analyzes.

The results of the research activity have been disseminated by scientific papers presented at congresses, symposiums, national and international conferences, scientific papers published in ISI journals as well as participation in 3 research contracts as project manager or team member.

My scientific portfolio includes:

- 28 ISI papers;
- *H-index* 6;
- 20 scientific papers published in extenso in BDI journals;
- 45 abstracts published in the volumes of national and international scientific events;
- 1 CNCSIS award;
- project manager in an Internal Grant from UMF "Grigore T. Popa" Iași, entitled "Improving the pharmacological profile of oral antidiabetic drugs using polymer matrices" - funding period: 2015-2016;
- team member of two research projects in which I was involved:

1. Project Action COST BM 1402 entitled "Development of a European Network for Preclinical Testing of Intervention in Mouse Models of Age and Related Illnesses (MouseAGE)" - Running period: 2014-2018;
2. Project Action COST BM 1302 entitled "Joining Forces in Corneal Regeneration Research" - Running period: 2013-2017.

I was looking constantly for improving my personal research skills by:

- promoting a competitive scientific research by continuing research into ongoing projects and accessing new national or international research projects;
- identifying, promoting and diversifying new research directions;
- information and training courses on the scientific research projects;
- stimulating interdisciplinarity in research by supporting intra- and inter-departmental human resourses exchange.

Research activity plays an essential role in scientific and professional recognition, through the quality of the published articles and the research projects won, which offers a real dimension and scale of performance.

All of my professional achievements ensure my success and academic recognition, but also they are increassing my international visibility, as member of the Academic Corpus of the University of Medicine and Pharmacy "Grigore T. Popa" from Iași.

In order to increase the international visibility I attend to concentrate on:

- continue the dissemination of the results of my research activities by elaborating scientific manuscripts and articles published in journals with national and international recognition and appraisal;
- promoting research results through active participation in national and international scientific events;
- interdisciplinary collaboration with research structures from other universities and research centers;
- the exchange of experience/research internships with similar educational/research units;
- making usage of hosting services such as SlideShare to disseminate research results;
- working on ORCID platform that provides a persistent identity for humans.

CHAPTER 1. FROM HYPPOCRATES TO HARVEY - FROM FASCIES TO ARTERIES

1.1. State of the Art

The term embryology derives from the Greek words ἔμβρυον which means *embryon*, "the unborn, embryo" and λογία *-logia* and it is the branch of biology that studies the prenatal development of gametes (sex cells), fertilization, and development of embryos and fetuses. Additionally, embryology encompasses the study of congenital disorders that occur before birth, known as teratology (Carlson and Kantaputra, 2014).

Aristotle proposed the currently accepted theory of epigenesis that organisms develop from seed or egg in a sequence of steps. The alternative theory, preformationism, that organisms develop from pre-existing miniature versions of themselves, however, held sway until the 18th century. Modern embryology developed from the work of von Baer, though accurate observations had been made in Italy by anatomists such as Aldrovandi and Leonardo da Vinci in the Renaissance (De Felici and Siracus, 2000).

In 1931 embryologist and historian Joseph Needham published a well-received three-volume treatise titled Chemical Embryology. The first four chapters from this work were delivered as lectures on Speculation, Observation, and Experiment, as Illustrated by the History of Embryology at the University of London. The same lectures were later released as a book published in 1934 titled A History of Embryology. This monograph represents one of the first general accounts of the history of embryology and presents embryology as a history of intertwined ideas, a style of historical writing advanced by noted biology historian Jane Oppenheimer (Cave, 1935; Needham, 1959; Bodemer, 1961; Oppenheimer, 1975).

In 1416 BC in Garbha Upinandas, hindu text was found a paragraph which mentions that "from the conjugation of blood and semen the embryo comes into existence. During the period favorable to conception, after the sexual intercourse, (it) becomes a Kalada (one-day-old embryo). After remaining seven nights it becomes a vesicle. After a fortnight it becomes a sperical mass. After a month it becomes a firm mass" (Lopata, 2009).

Around 1400 BC Egyptians made reference to the placenta and its importance as the seat of the external soul. However, they did not consider an embryo alive until the baby was born. The early Egyptians also discovered that chick eggs could be removed from nests and artificially incubated in ovens. This important finding allowed for observation of chick embryos during different periods of development.

A Judeo-Christian text from 1000 BC, entitled Book of Job mentions that "Your hands formed me and made me - will you now absorb me? Remember that you formed me as if with clay - will you return me to dust? You poured me out like milk, and pulled me together like cheese. You clothed me with skin and flesh, and [inside me] did you interweave bones and sinews".

Another Judeo-Christian text from 500 BC says that "for you created my inmost being; you knit me together in my mother's womb. I praise you because I am fearfully and wonderfully made; your works are wonderful, I know that full well. My frame was not

hidden from you when I was made in the secret place. When I was woven together in the depths of the earth, your eyes saw my unformed body. All the days ordained for me were written in your book before one of them came to be" (Anagnostopoulos, 2013).

The first written record of embryological research is attributed to Hippocrates (460 BC–370 BC) who wrote about obstetrics and gynecology. Hippocrates should be recognized as the first true embryologist. He believed that the embryo began development by extracting moisture and breath from the mother and he identified a series of condensations and fires that were responsible for the development of bones, belly, and circulation in the embryo and fetus (Barnes, 1985).

He also supported the view that the human fetus gained nourishment by sucking blood from the placenta. Hippocrates could be one of the first to allude to the concept of preformationism with the Greek physician's belief that organisms were fully formed in miniature inside germ cells. This belief helped give rise to theological embryology or the idea that various souls entered the embryo as it grew (Nancy, 2006).

Aristotle studied embryos of different organisms by opening up bird eggs at different stages of development and dissecting mammalian and cold-blooded embryos. He also sustained that semen supplied the form or breath to embryos and mothers supplied some type of substance to aid in embryonic development. The role of menstrual blood was targeted by Aristotle as the most likely substance out of which the embryo was made. He observed that young embryos of different species all possessed universal characteristics and that as the embryos aged, differentiating characteristics arose (Annas, 2001).

Galen of Pergamos mainly wrote from 150 AD to 180 AD - through the Renaissance - and was known as a vitalist (life arises from or contains a nonmaterial vital principle) and teleologist (all life and actions are driven by an ultimate purpose) whose main contribution to embryology was his steadfast belief that the umbilical cord was necessary for respiration (Aird, 2011).

After Galen, embryology is studied among the Arabs but not as successful as studying optics and astronomy (de Lacy, 1992).

Albertus Magnus (also known as Albert of Cologne) is credited with the reawakening of scientific embryology. He resembled Aristotle in his observational techniques and attention to detail and he frequently discussed embryology in his books. Albert believed that women had seeds and that female seeds coagulated, much like cheese, after coming in contact with male seeds. When a coagulated seed made contact with menstrual blood, the seed now had the nutrition necessary for proper development. Albert also studied chick and fish embryos and wrote extensively about each organism's development, helping to bring embryology back into the observational and scientific realm (Emsley, 2001).

The embryological findings of Leonardo di ser Piero da Vinci dominated science in the late 1400s and early 1500s. Leonardo is noted for his dissection of the human fetus and his quantitative measurements of embryonic growth. He was the first to provide evidence that embryos can be measured chronologically and that they change in weight, size, and shape over time (de la Chiesa, 1967).

The sixteenth century also saw the recognition of the field of gynecology. Clinical textbooks were published and helped fuel an emerging new interest in human development.

The growth of midwifery during the late 1500s has a direct connection with the availability of illustrated obstetrical literature that became more mainstreamed during this time.

Meanwhile, seventeenth century introduces the embryology-related work of William Harvey (1578 AD–1667 AD). As early as 1652, Harvey dissected and examined deer and chicken embryos with the aid of low powered lenses. Harvey determined the position where the embryo arises in an egg, the so-called white spot, and described the blastoderm as the unique place of origin of the embryonic body. He also wrote of the importance of the amniotic fluid, believing that it was absorbed into the blood of the embryo and later, the fetus. Harvey also lent his voice to the refutation of spontaneous generation in describing how even the lowest organisms arise from eggs (Haddad and Khairallah, 1936).

Italian biologist Marcello Malpighi (1628 AD–1694 AD) is the person responsible for the rise of preformationist doctrine. Malpighi described embryo development as a simple unfolding of an already miniature adult organism. At about the same time, Jan Swammerdam, a noted frog embryologist also supported preformationism after seeing folded butterflies in chrysalises. To Swammerdam, adult butterflies were simply masked (preformed) inside of caterpillars (Needham, 1931).

Nicholas Stensen discovered the follicles of the mammalian ovary in dogfish and demonstrated that the human female ovary was homologous to the ovaries in previously studied oviparous animals. Stensen declared that the human ovary housed eggs, yet not all breakthroughs revolved around eggs. As rudimentary microscopes became more available, so too did the number of observations of spermatozoa, mainly from different species of fish. During the late 1600s embryos with severe congenital malformations, called embryonic monsters at the time, began to receive scientific descriptions. A detailed 1686 drawing of a teratoma with well-formed teeth and hair is presented in the text (Needham, 1931).

Epigeneticists asked how embryonic monsters and regeneration of starfish arms fit into the preformationplan of a God that had made sure that all normal adult structures were in the egg or the semen, waiting to unfold. The preformation-epigenesis debate grew and culminated in a series of arguments between epigeneticist Caspar Friedrich Wolff and preformationist Albrecht von Haller. Wolff published De Formatione Intestinorum in 1768 and demonstrated that the chick intestine forms by the folding of tissue that detaches from the embryo's ventral surface. The folds eventually transform into a closed tube. Wolff argued that this observation proved that the intestine was not preformed and that organs appeared gradually. Wolff also examined embryonic monsters, declaring that they were formed by nature and stood as examples of epigenesis rather than preformationism. Haller, however, was much better known to scientists than Wolff, and Haller's powerful influence did much to sustain preformationism through the late 1700s (Needham, 1931).

Hermann Boerhaave wrote for the first time a detailed account of chemical embryology in his book Elementa Chemiae, published in 1724. Boerhaave separated egg white from the yolk and added various acids and bases, heated them, shook them, and boiled them to see the chemical and physical effects that each procedure had on albumin (Wilmut et al., 1997).

Muscles, connective tissue and blood supply are another topics discussed over the centuries.

The connective tissues in our body is an almost continous superficial layer, from the base of the skull to under the feet to the toes, and anteriorly just under the mandible at the hyoids.

Of course, there are different layers and different thicknesses, but the front and back lines collectively are really a stirrup, that we "suspend" in. Fascia wraps our muscles and tendons, and also buffers and supports the internal organs. Recent studies show fascia is a supreme sensory organ, with many more sensory nerve receptors than muscle tissue (Adstrum et al., 2017).

These receptors, such as proprioreceptors and nociceptors, sense body position and pain respectively. Without them, we would not sense our bodies in space, nor feel temperature or pain.

Fascia is a connective superhighway which transmits forces that used to be held accountable to only muscle, so forces applied to the body are shared by fascia. "The fascial continuum is essential for transmitting the muscle force, for correct motor coordination, and for preserving the organs in their sites: The fascia is a vital instrument that enables the individual to communicate and live independently. The transmission of the force is ensured by the fascial integrity, which is expressed by the motor activity produced; the tension produced by the sarcomeres results in muscle activity, using the various layers of the contractile districts (epimysium, perimysium, endomysium), with different directions and speed" (Bordoni and Zanier, 2014).

The anatomical meaning associated with fascia has varied during the 400 years that this term has been incorporated in English language medical literature. Fascia has been diversely portrayed as a range of macroscopically discernable body parts, the tissues they are composed of, and a pervasive soft connective tissue network structure. Over the last four centuries, fascia has been described in many ways. Anatomical understanding of fascia has developed over the years and is likely to continue to change with evolving research technologies. Multidisciplinary advances in fascial knowledge could conceivably contribute to improving individual and societal health care (Adstrum and Nicholson, 2019).

The study of the muscles started probably when someone tried to understand how he can move from A to B and executes movements at will. During the Italian Renaissance (end of XIVth to beginning of XVIth century), muscles were first descriptive, based on dissection by artists like Leonardo da Vinci (1452-1519) and Michael Angelo (1475-1564).

The first concern was the influence of the volume of the superficial muscle on the surface modelling represented in their paintings and sculptures. About this aspect, Leonardo da Vinci multiplied the number of bundles of some muscles but most of his representations of tendon insertions are imprecise. He applied mechanical principles to rib, elbow kinematics and kinetics of the foot.

Andreas Vesalius (1514-1574) in his remarcable plates are remarkable described the morphology of the muslces (O'Malley, 1964).

After that, Galileo (1564-1642), Borelli (1608-1679) and Newton (1642-1727) thought that physics and mechanical laws governed motility of animal and human body alike. Incidental discovery of electro stimulation effect on muscle in Galvani's laboratory and electric current concomitant of muscles contraction by Matteucci and Du Bois Reymond

were major breakthroughs. Recording of this current was the starting point for ECG, EMG and EEG. ECG entered first in the clinic (Vinci, 1929).

Duchenne de Boulogne (1806-1875). Matteucci's (1811-1868) publications inspired and stimulated Du Bois Reymond.

Du Bois Reymond (1818-1889) repeated and completed his experiments on frogs. He designed a very sensitive galvanometer with which he recorded his own global EMG (Pearce, 2001).

"Throughout the body the animal arteries are mingled with veins and veins with arteries, and both veins and arteries are mingled with nerves and the nerves with these...And of course the usefulness of such a complete interweaving is very evident, if, that is to say, it is a useful thing for all parts of the animal to be nourished" wrote Galen, in 2nd century AD.

The veins and arteries of the human body have been objects of study as long as there has been interest in anatomy. Their significance, while not always well understood, has been an important question in the history of anatomy and physiology. Galen, for instance, described the aorta as "a trunk divided into many branches and twigs" that nourished the body. Ancient medical practitioners were not initially even sure that arteries and veins did different things for the body, though they quickly understood that they acted differently when cut veins being full of blood and arteries seemingly empty. Once this was established, understanding their relationship to each other became an especially thorny problem.

The idea of a single circulatory system remained unique that the great Flemish anatomist Andreas Vesalius continued to discuss the two systems separately in the 1530s and 1540s. Yet by the time he completed On the Fabric of the Human Body (1543), he had begun to wonder how exactly venous blood found its way into the arteries, questioning the idea of the porous septum in the heart. Many anatomists already knew that the walls of the arteries were thicker than those of the veins, but attributed this to the nature of the substance the passed in each of them. Further anatomical work led to the identification of puzzling features of the veins (1543), he had begun to wonder how exactly venous blood found its way into the arteries, questioning the idea of the porous septum in the heart. Many anatomists already knew that the walls of the arteries were thicker than those of the veins, but attributed this to the nature of the substance the passed in each of them. Further anatomical work led to the identification of puzzling features of the veins (Kemp, 1970).

William Harvey was writting in 1653 that "Wherefore there are many valves in the veins opposed to the heart: the arteries have none except at the exit from the heart. Hence the first veins are pulsating, the latter are non-pulsating". These "little doors," as their discoverer, the Paduan anatomist Hieronymus Fabricius ad Aquapendente called them in 1603, seemed to prevent blood from flowing in more than one direction. His famous pupil Harvey transformed these observations into a new physiology. He established experimentally that the arteries and veins belonged to a single circulatory system that connected the heart and lungs. In his On the Circulation of the Blood (1628), Harvey employed animal vivisection, human and animal dissections, and observations of living patients to establish this new view of the circulatory system (Sean et al., 2004).

The research in this field gain great results in the last decades, together with the progress in radiology and informatics.

This research direction has been reflected in publishing the following articles:

1. Hinganu D, **Stan CI**, Taranu T, Hinganu MV. The anatomical and functional characteristics of parotid fascia. Rom J Morphol Embryol 2017; 58(4): 1327-1331.
2. Hînganu D, **Stan CI**, Ciupilan C, Hînganu MV. Anatomical considerations on the masseteric fascia and superficial muscular aponeurotic system. Rom J Morphol Embryol 2018; 59(2):513–516.
3. Hînganu MV, **Stan CI,** Ţaranu T, Hînganu D. Morphological changes in support mechanism of superficial face layers in Moebius syndrom. Rom J Morphol Embryol 2017; 58(3): 851-855.
4. Nedelcu AH, Tepordei RT, Sava A, **Stan CI**, Aignatoaei AM, Taranu T, Ursaru M. Supernumerary fronto-orbital arteries arising from contralateral anterior cerebral artery associated with partially duplicated anterior communicating artery - case study and literature review. Rom J Morphol Embryol 2016; 57(3): 1159-1163.
5. Ţăranu T, Florea L, Păduraru D, Georgescu ȘO, Frâncu LL, **Stan CI**. Morphological changes of the peritoneal membrane in patients with long-term dialysis. Rom J Morphol Embryol 2014; 55(3): 927-932.
6. Rusu MC, Poalelungi CV, Vrapciu AD, Păduraru L, Didilescu AD, **Stan CI**. Anoctamin 1 positive esophageal interstitial cajal cells in late stage human embryos. The Anatomical Record 2014; 297:301–307.

1.2. Sustentaculum facies - everlasting facial youth

1.2.1. Introduction

The superficial musculoaponeurotic system of the face (SMAS) was first described as a surgical entity by Tessier and colleagues at the French Society of Plastic Surgery meeting in Paris in 1974. It was first described in the literature with some accuracy but also inconsistency by Mitz and Peyronie (1976) and called the *superficial muscular and aponeurotic system.* Since 1976, many diverse descriptions of the SMAS and its anatomical relationships have been presented in the literature (Evans, 2017).

The facial SMAS is a heterogeneous structure of fascia, fat tissue, blood vessels, nerves and muscle. It thickens with age into an aponeurosis (broad, thin sheet of connective tissue attached and serving as the origin or insertion of a flat muscle) inferior to the zygomatic arch, anterior to the ear, posterior to the zygomaticus major muscle and inferior lateral part of the orbicularis oculi muscle, and variably superior to a line from the lobule of the ear to the oral commissure, which coincides with the risorius muscle or the superior aspect of the platysma muscle.

The fascia invests the risorius and facial platysma muscles, and this complex in the face is part of the facial SMAS.

The fascial segment of the facial SMAS continues anterior medial to invest or at least cover most of the facial mimetic muscles (muscles of facial expression) and continues into the nose and the upper and lower lips, where it covers the superficial portion of the orbicularis oris muscle. In the area of the anterior cheek and melonasolabial fold, it is histologically indistinct and occasionally interrupted.

Many authors have continued to call this typical anterior superficial fascia extension the SMAS. We refer to the superficial facial complex of the middle and lower thirds of the face as the *facial SMAS,* that of the neck as the *neck SMAS,* that of the temple as *temple SMAS,* and that of the forehead as *forehead SMAS.*

The facial SMAS is connected to the dermis by fibrous septae in the superficial fat layer. The muscles of facial expression do not work independently and are coordinated during facial expressions by the "SMAS system". Movement occurs between the facial SMAS and deep facial fascia anterior to the parotid gland (Cormac et al., 2017).

Two SMAS types exist. Type I SMAS occurs in the forehead, parotid, zygomatic, and infraorbital areas and comprises fibrous septae. Type II SMAS is a dense mesh of collagen, elastin, and muscle fibers and is found in the lip area (Ghassemi et al., 2003). SMAS thicknesses vary throughout the face from 2 to 3 mm (Har-Shai et al., 1996).

The face is arranged in five layers as follows: layer 1: skin; layer 2: subcutaneous fat tissue, including the retinacula cutis (composed of fibrous connective tissue); layer 3: superficial musculo-aponeurotic system (SMAS); layer 4: deep fat tissue; and layer 5: periosteum and deep fascia. This arrangement varies between facial regions, especially when the line of ligaments is incorporated into the model. The facial fat compartments are located in layers 2 and 4; each layer has unique characteristics and spatial relationships with the surrounding tissues.

The concept of the layered arrangement is a new way to understand the spatial relationship and functional interplay of the soft tissues of the face. Understanding the layers, the precise location of the superficial and deep facial fat compartments and their boundaries is crucial for the conduct of safe and effective minimally invasive facial procedures.

Structurally, the soft tissue of the face is disposed into a series of concentric layers: skin, subcutaneous fat tissue, superficial fascia, muscles of facial expressions, deep fascia (parotidomaseteric), the plan of the facial nerve, the plan of the parotid duct and buccal fat (Stuzin et al., 1992).

Superficial fascia covers the muscles of the facial expressions (platysma, orbicularis oculi, and zygomaticus major and minor) and deep facial fascia, the subsistence of the facializate cervical fascia, easily identifiable, covers and protects the terminal branches of the facial nerve located subjacent to fascia at the cheek level.

There are two types of spatial relations between superficial and deep fascia: in some regions facial plans are separated by an areolar plan, in other regions the two fasciae are intimately adherent to each other through a series of dense fibrous attachments. In the last decades, the easily improving and increasing accessibility to the facial rejuvenation techniques, requested intensified studies regarding the superficial facial structure.

Thereby, Tessier (Tessier, 1989) proposed the term of musculoaponeurotic superficial system (SMAS) to describe an anatomical and surgical structure, which is distinguished from the platysma muscle. Its Princeps description was made by Mitz V and Peyronie M (1976) in

the parotid and cheek regions (buccal after NA), which led to some controversy between the surgeons and anatomists, this structure being initially recognized only by the surgeons who use it in various facelift techniques (Stuzin et al., 1992; Tessier, 1989; Mitz and Peyronie, 1976; Moss et al., 2000; Mendelson, 2001).

The superficial layers of the face are under the permanent action of gravitational force in a unique manner, on the one hand due to the bipedal resort, on the other hand as an expression of the human specific functions (mimics, socialization).

The microscopic anatomy and the biomechanics of the superficial facial surface structure include a set of shapes which interpose to the gravitational force and to the aging and "falling" of the face, which consist of the continuity with the epicranial fascia, adhesions and fixing perizygomatic fixation ligaments, the adhesion of the superficial structures to the peri- and inter-orbital muscles, and the presence of fibro-adipose tissue regionally organized, with a role in facilitating/stop dragging during the contractions (Mendelson, 1997; Gola, 2005).

In the periorbital region, the adipose supraSMAS and the infraSMAS layers are crossed by conjunctive fibers fascicles (microseptum) oblique arranged, more or less, which come from the superficial and profound surfaces of the SMAS. The infraSMAS fibrous connecting tissue precisely compartmentalizes the face fat and acts like a barrier, which restrains the progressive movements of the superficial plans.

There has been recently demonstrated that these compartments gain and loose in weight in different proportions (Rohrich and Pessa, 2007). In time, the gravity tends to weaken the ligament support and to initiate the progressive tissue migration (Yousif, 1995; Marten, 1997), with the changing of the face tissues spatial relations.

The rate of face aging partially depends on the faces fat modifications in time (Dallara, 2009). The SMAS exists in the periorbital region as a very well defined entity and it is acknowledged by the researchers (Ghassemi et al., 2003; Coleman and Grover, 2006).

The superficial fascia of the periorbital region and implicitly the SMAS, have some characteristics which apart them from the other regions because there is a craniofacial junction area, to passage from one compartment of the head to another (from face to forehead), reflected in profoundness by a pneumatized craniofacial junction area, specifically human.

There is one similar compartment in another junction area, the cervicofacial one, in the submandibular region, a limit that is profoundly continued by the existence of the buccal floor. It was signaled that there fixing and tense areas of the SMAS, such as the medial side of the mandibular branch or of the orbit assessing their importance is limited (Ghavami et al., 2008).

The stabilizing effect that occurs in these ligamentary connections ensures the movement that results from the muscular contraction of each zone, so that, at least in youth, the movement is not transmitted to the superficial fascia of the adjacent areas. The ligament fixation has a shock-absorbing effect, which modulates the degree of sliding during the muscle contraction (Mendelson, 1997).

At this level, the SMAS has very powerful fixation means embodied by the supraorbital ligament adhesion and by superior and inferior temporal septum nearby. In some topographic regions can exist "weak points", which yield first during aging. Periorbital, such

areas are only in the inferior and the lateral parts of the region, where the adhesions and the cutaneous muscular attachments are weaker and less dense and where progressive decreasing of fibroadipose infraSMAS layer and the tissue around the eyes is moving inferiorly.

Therefore the morphological substrate of lower eyelids fall, the orbital fat herniation ("palpebral bags") and the aspect of descended face are built (Gola, 2005; Coleman, 2004). In the superior part, the dragging of the frontal tissues is orientated to the resistance point of the supraciliary arch, involving the movement of the eyebrows, which becomes more protruding.

Following the weakening of the periorbital tissue and the change of spatial distribution of the fibroadipose layer (Gosain, 2005), the arches and the convexities disappear and give a rounded aspect of young faces (Hamra, 1996). The structural related differences give particular abilities in the emotional face expressions, but also have different capacities of maintaining the anatomical substrate of the face and ensure flaps nutrition in the facial lifting.

We have also identified and researched the morphology and function of SMAS in the Moebius syndrome. Congenital atresia of the facial nerve leads to important functional deficiencies of the perioral muscles which, over time, affect adjacent regions by means of elongation of their fixation mechanisms and by the appearance of superficial soft tissue prolapse.

Moebius syndrome, also called congenital facial paralysis, congenital ocular-facial paralysis or nuclear aplasia is a rare neurological disorder, whose cause has not yet been elucidated. It affects especially facial and oculomotor cranial nerves and its clinical feature is peripheral facial paralysis (Kumar, 1990).

Clinically, it is highlighted from birth, most often by:

- lack of facial expressions, failure of making facial mimics and presence of various types of speech disorders;
- paralysis of the oculomotor nerve, eyelid ptosis, strabismus;
- upon affection of the glossopharyngeal nerve, it may give disturbance in swallowing and chewing and tongue muscle atrophy.

These symptoms may occur by themselves or in various combinations, according to the nerves that are affected, the age and the associated pathology (Carta et al., 2011; Magnifico et al., 2017; Kuhn et al., 1990). The treatment is symptomatic and it aims to improve the patient's quality of life. Thus, logopedic treatment, physiotherapy and home care services can be considered. Surgical cure has specific indications and can provide part of face mobility, on different levels (Terzis and Noah 2002; Terzis and Anesti, 2011; Di Blasio et al., 2014).

Facial paralysis is a major health problem. It is devastating to the patients as it prevents accurate communication and expression of feelings and emotions, thus adversely affecting the quality of life (Mendelson, 1997).

Knowing and understanding subcutaneous layers in related regions of the face, such as the masseteric layer is important in various surgical specialties. The superficial muscular aponeurotic system (SMAS) of the face is a guiding structure for the plastic and oro maxillo facial surgeon, especially in the masseteric region. In view of these considerations, a number of clarifications are needed regarding the notion of SMAS of the face, its origin and embryogenesis. Also, a systematisation of some of the anatomical conclusions adapted to the new techniques of plastic and repair surgery would equally be of interest, but these would

have to take in accont the great variability in the histological aspect of SMAS in different facial regions in the same individual, but also between the same region in different individuals SMAS presents as a distinct fibromuscular layer, consisting of the platysma muscle, the parotid fascia and the fibromuscular layer covering the cheek (Thaller et al., 1990). A continuous, organized, fibrous mesh, specific to the nasolabial fold and upper lip, frontal, parotid, zygomatic and infraorbital regions, is achieved (Ghassemi et al., 2003).

The parotid fascia is forming a capsule around the gland, together with the masseteric fascia. It sheaths the parotid gland, its excretory duct and the branches of the facial nerve as well. Commonly is considered that parotid fascia proceeds of the superficial layer of the deep cervical fascia, which splits to cover the gland (Tamplen et al., 2016). It is named parotid fascia because it is related to the lateral side of capsule. The fascia itself is made of two layers: *lamina superficialis* that runs upwards to be continued by the temporal fascia and lateral by the masseteric fascia, *lamina profunda* that covers the stylohyoid, the styloglossus and stylopharyngeus muscles. The superficial layer attaches to the zygomatic arch and to the mandibular body.

Plastic surgeons consider that the superficial fascias of the mentonian, parotidian and cervical regions are interconnected (Berry et al., 2010). The entire connective tissue that makes this connection together with the skeletal muscles attached to it is nowadays considered to form a muscular aponeurotic cervico-facial unit (Khan et al., 2014).

Basically, by preserving a flap of SMAS in parotid interventions, it can be reduced the risk of parotid Frey syndrome (gustatory sweating), and also minimizes the risk of postoperative infection and allows reconstruction of the parotid lodge (Lee et al., 2017; Quer et al., 2017). This is possible by maintaining characteristics of the facial SMAS at this level: it forms fascial tunnels for arteries and nerves and continues in neighboring regions. SMAS transmit, distribute and amplify the activity of all facial muscles (Owsley et al., 1983).

The existence of SMAS at this level is not fully recognized by researchers (Gola et al., 2005). Indirect has long been used in plastic surgery techniques, but maxillofacial surgery partially adopts this concept and not from long time ago (Meningaud et al., 2006; Stathopoulos et al., 2018). Our study brings quantitative and qualitative evidence of the existence of SMAS at the parotid level.

The main purpose of this study is to objectify the origin of the superficial parotid fascia, which is considered to be a duplication of the deep fascia, by analyzing its quantitative and qualitative anatomy. The importance of our study derives from the practical utility of the superficial muscular aponeurotic system (SMAS) concept, which is continues also at parotidian level. This finding allows surgical techniques to minimize postoperative incidents. The main surgical approach to parotid tumor formations based on the SMAS concept is extracapsular lumpectomy with SMAS flap (Bonanno and Casson 1992; Giannone et al., 2008; Dell'Aversana Orabona et al., 2015). ***We have also identified the particular morphofunctional features of the SMAS in masseteric region and to show their practical significance.***

Considering the social and personal impact of facial nerve paralysis, our research goal was also to highlight the anatomical and functional changes in the Moebius syndrome, as well as the clinical changes that occur in the face statics and dynamics. We also tried to establish criteria that should be the basis for reparative surgery in this disease.

We centralized all the data to develop a map encompassing anatomic and functional changes occurring after long-term lesion evolution.

Personal contribution – published papers:

1. Hinganu D, **Stan CI**, Taranu T, Hinganu MV. The anatomical and functional characteristics of parotid fascia. Rom J Morphol Embryol 2017; 58(4): 1327-1331.
2. Hînganu D, **Stan CI**, Ciupilan C, Hînganu MV. Anatomical considerations on the masseteric fascia and superficial muscular aponeurotic system. Rom J Morphol Embryol 2018; 59(2):513–516.
3. Hînganu MV, **Stan CI,** Țaranu T, Hînganu D. Morphological changes in support mechanism of superficial face layers in Moebius syndrom. Rom J Morphol Embryol 2017; 58(3): 851-855.

1.2.2. Material and methods

1.2.2.1. Anatomical study

Our study was conducted on 2 cephalic extremities, formalized and dissected at "Ion Iancu" Institute of Anatomy within University of Medicine and Pharmacy "Grigore T. Popa" Iași. The macroscopic study on the dissection specimens was performed using the SOM 62 Kaps operator microscope owned by "Ion Iancu" Institute of Anatomy, U.M.F. "Grigore T. Popa" Iași. Dissections were performed layer by layer and, on each stage of dissection mesoscopic images were captured with Kaps SOM 62 operating microscope. The conclusive aspects were acquired, examined and further processed to remark the regional stratigraphic differences. On the dissection specimen, the following superimposed layers were identified, in different topographic locations (frontal lateral, inferior temporal, preparotid, premasseteric, infraorbital): dermoepidermic, subcutaneous fat tissue, superficial fascia, superficial muscular layer, deep fascia, and periosteum and deep musculoglandular items. The conclusive aspects were acquired, examined and further processed to highlight the regional stratigraphic differences.

1.2.2.2. Clinical study

The study was conducted on a total of six patients with Moebius syndrome lesions that presented or were admitted in "St. Mary" Emergency Hospital for Children, Iaşi, Romania.

The information was obtained from observation charts of each patient and intraoperatively (for patients who have had such treatment recommendation), where tissue specimens were taken. Based on these, we analyzed changes that occur in these cases, from the clinical, anatomical and histological point of view. We pursued, on the studied cases, changes in functional anatomy, of superficial layers of the face, which we grouped in terms of clinical manifestations, correlated with individual pathophysiological substrate.

The clinical observation and palpation was done through specific maneuvers, which highlighted contraction and action of each individual muscle groups and by blocking others.

In the cases where surgical intervention was performed, we collected tissue fragments that have been assessed qualitatively by common staining techniques.

Surgical study conducted a study on a group of 10 patients admitted to the Clinic of Maxillofacial Surgery, "St. Spiridon" Emergency Clinical Hospital, Iaşi. Patients who were clinically and imagistically diagnosed [computed tomography (CT), magnetic resonance imaging (MRI)] with parotid tumors and underwent surgical interventions for total or partial parotidectomy.

1.2.2.3. Histological study

For the qualitative microanatomical study of SMAS in the stratigraphic layers of the face, we sampled all the soft parts of the facieses of the anatomical specimens, from the skin to the bone, in the form of small blocks, with the following topography: premasseteric and masseteric. The dissection was performed perpendicular to the surface of the epidermis in order to be able to follow the correct sequence of the planes.

We also used operatory fragments removed from superficial layers to deep fascia, during facial lifting and other interventions on this region and compared these with CT and MRI aspects.

The collected specimens were processed by paraffin technique and stained with special techniques for muscular and connective tissue (Verhoeff and Szekely).

Stereology was used with the standard Weibel parallel grid to quantify the percentage volumes of the main structural parietal components in the studied vessels.

1.2.3. Results

1.2.3.1. Results of the parotid SMAS study

In parotid region, superficial fascia presents numerous fat lobules and is part of the SMAS of head and neck, together with the mimic muscles, blood vessels and nerves that run across (**Figure 1.1**). They are separated by vertical supraSMAS and horizontal subSMAS fibers. The presence of infraSMAS horizontal tract has allowed us to obtain a layer of cleavage and surgical approach between the superficial and deep fascia. Highlighting of vertical tracts allowed us to affirm that it is a common feature with neighboring facial regions. These vertical tracts permit the removal and reattachment of a SMAS flap and obviously represents the anatomical substrate of a major cosmetic result in parotidectomy techniques based on the existence of SMAS.

In the posterior part of the parotid gland, the superficial fascia exhibits a condensation that forms a true hill for the gland. Here, the facial nerve, the facial artery and the external jugular vein enter the parotid gland. After dissecting the superficial and deep parotid fascia, we identified the branches of the facial nerve. They follow the fibrous expansions of the superficial fascia.

Thus, we can assert that the superficial fascia forms tunnels for the terminal branches of the facial nerve, which allows us to map and preserve them.

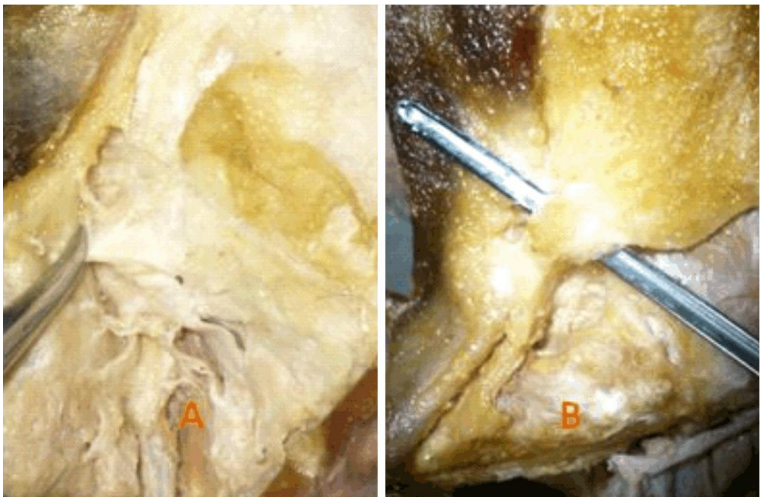

Figure 1.1. Parotideo-masseteric fascia retaining ligament: the superior region (A) and the inferior region (B). Dissection specimen.

Thus, we can assert that the superficial fascia forms tunnels for the terminal branches of the facial nerve, which allows us to map and preserve them. Continuing in depth dissection, we identified the formation of the external jugular vein and the terminal segment of the external carotid artery. Vascularization and innervation of face skin is done directly through the superficial fascia.

Essential constituent of the SMAS is superficial fascia (**Figure 1.2**), which continues in all regions of the face, only muscles being different. Blood vessels and terminal branches of the facial nerve, crossing each several regions of the face are closely related to the superficial fascia, which mechanically protects them.

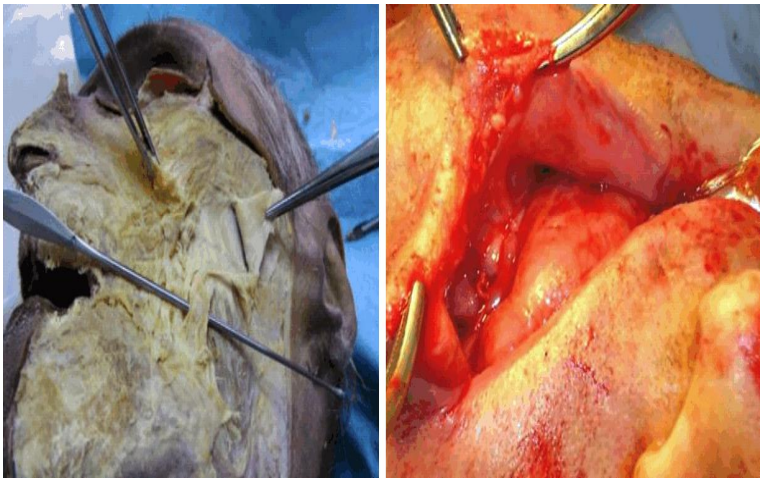

Figure 1.2. Removing the superficial muscular apo- neurotic system and parotid fascia: dissection specimen (left) vs. intraoperative image (right).

The classical theory regarding origin of parotid fascia as derived from deep facial fascia is invalidated by its anterior continuity with platysma muscle fascia. In upper of the cheek, it makes the transition to the zygomatic region. Morphological, macroscopic and

mesoscopic differ- ences between the two regions stand out as a net line, “as a border strip” (**Figure 1.3**).

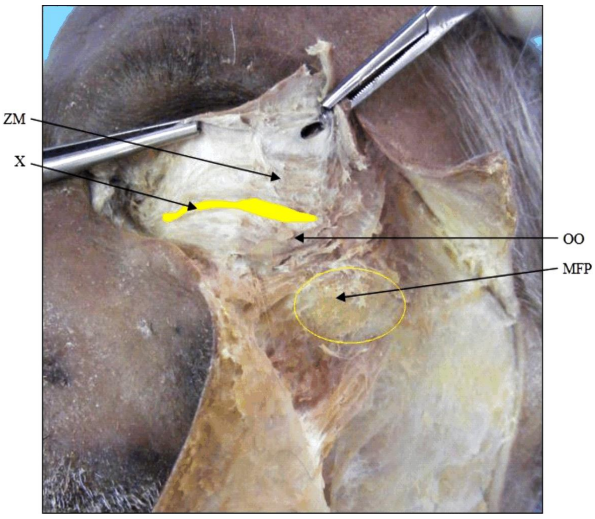

Figure 1.3. The boundary between the upper and lower face (X) given by the great zygomatic muscle; strati- graphy of infraorbital region – great zygomatic muscle (ZM), inferior fascicle of orbicularis occuli (OO) muscle; molar fat pad (MFP).

Although SMAS is intimately applied to the surface of the parotid gland, a thin, well-defined parotid fascia is identified between the gland and the SMAS. Superficial fascia facilitates adherence of mimic muscles to the dermis and deep fascia (**Figures 1.4 and 1.5**). This suggests that oral superficial fascia will follow chewing movements of the masseter muscle.

SMAS is well represented, with thick collagen fibers condensations, mostly disposed longitudinally, with dimensionally reduced interstitial spaces (**Figures 1.6**), in continuity with the neighboring regional fascias. The blood vessels are found only on the periphery of the lamina and are very small.

Figure 1.4. SMAS adhesions to deep fascia that secures the side of the parotid region. Dissection specimen.

Distance to the deep, parotideo-masseteric fascia, is reduced, the fat infraSMAS layer being also reduced and represented by a very thin lamina of adipose cells, crossed by thin collagen fibers obliquely oriented. Elastic fibers are completely absent.

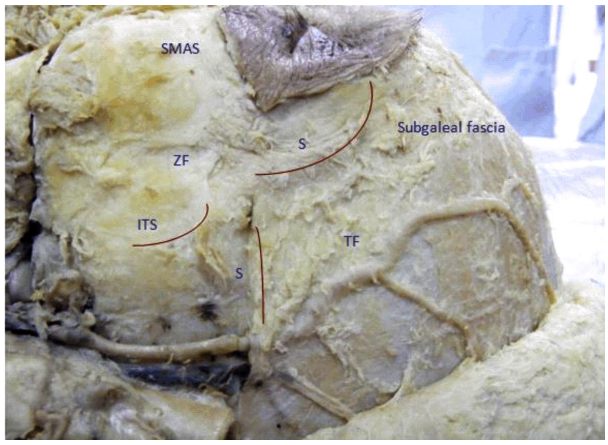

Figure 1.5. Ligamentary supraorbital (S) adhesion, superior temporal septa (STS) and the inferior one (ITS). SMAS: Superficial muscular aponeurotic system; TF: Temporal fascia; ZF: Zygomatic fascia.

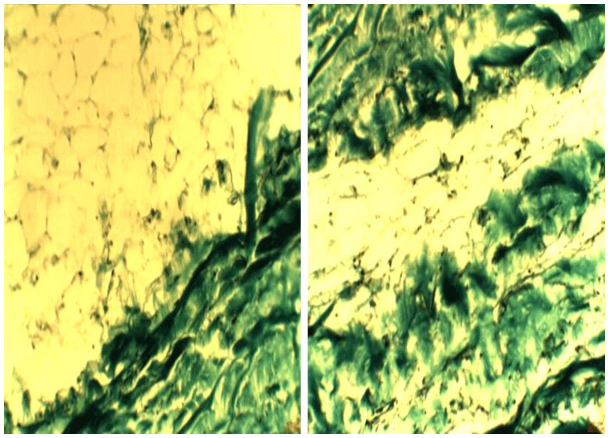

Figure 1.6. Fibrous-adipose supraSMAS (left image) and infraSMAS (right image) tissue is well represented in the parotid region. Szekely staining, ×200.

Fibro-adipose superficial layer is well represented with connective fascicles, observing numerous fat lobules sepa- rated by connective tracts and almost vertical or slightly oblique in one direction or another. There are predomi- nantly medium-sized collagen fibers and rare elastic fibers.

Our observations support the quantitative data obtained on CT images from the group of patients that underwent surgery. In parotid region, fibroadipose superficial layer has an average thickness by the 4.31±3 mm, and deep fat layer is very thin, 0.34±0.47 mm. SMAS appears as a hyper- dense line in the intimate relation to gland, with a thickness of 0.44±0.74 mm. In the parotid region, the superficial layers have different thicknesses: the supraSMAS fibrous tissue is well represented (**Figure 1.7**), the SMAS is a structured, dense organized connective tissue, the infra SMAS fat tissue forms a very thin lamina under which lies deep parotid fascia.

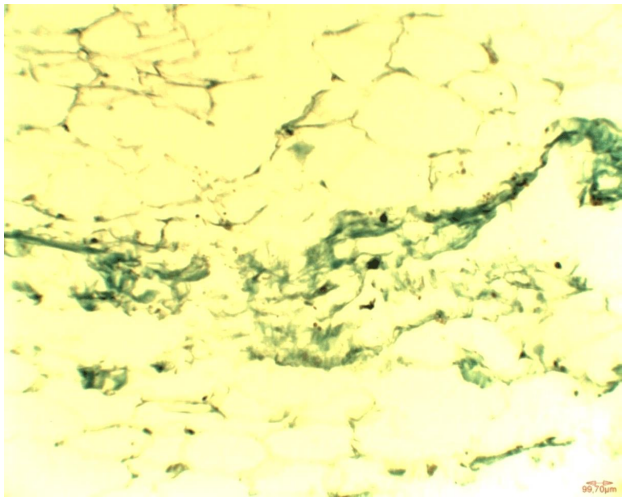

Figure 1.7. SMAS fibrous layer, well represented in the parotid region. Szekely staining, ×200. SMAS: Superficial muscular aponeurotic system.

CT investigation shows that the boundary between the two lobes of the gland is considered to be an area of continuity between the superficial and deep fascia (which forms the posterior capsule). Posterolaterally, parotid fascia is continued by a conjunctive part, which interconnects with superficial fascia and sternocleidomastoideus muscle fascia. Anteriomedially, fibrous tissue forms tunnels through which branches of the facial nerve are running (**Figure 1.8**).

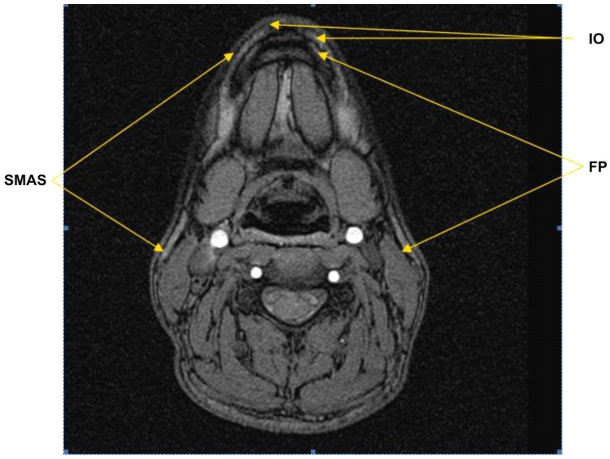

Figure 1.8. Horizontal section through the lower edge of the mandible; SMAS and deep fascia (FP) between the parotid and mental region; transSMAS insert of the orbicular mouth, inferior fascicle (IO). SMAS: Superficial muscular aponeurotic system.

1.2.3.2. Results of the masseteric SMAS study

SMAS can be seen macroscopically and microscopically in the masseteric region. Laterally, it forms the anterior sheet of the parotid fascia. Although SMAS is intimately applied to the superficial surface of the parotid, a distinct parotid fascia is identified between the gland and SMAS, which extends to the masseteric region (**Figure 1.9**).

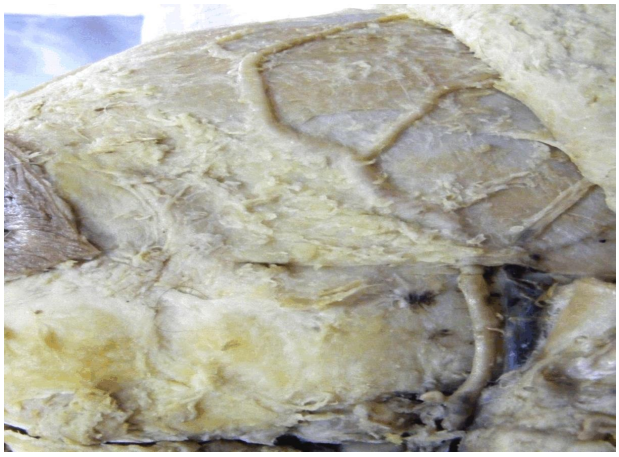

Figure 1.9. Parotid cervicofacial SMAS continues to be superior with parietotemporal fascia. Vessels are observed superficial and their branches. Dissection specimen. SMAS: Superficial muscular aponeurotic system.

The cervicofacial cutaneous muscular aponeurotic unit is separated from underlying, musculo-aponeurotic or periostic planes by deep adipose tissue that functions as a sliding plane. If deep subcutaneous adipose tissue is reduced, SMAS adheres to the underlying planes and the skin loses part of its mobility.

The neurovascular elements in its immediate neighborhood are numerous and complex. One must emphasize the complexity of the parotid region where the facial nerve and the branches of the external carotid artery are found. The lesser (superficial) masseteric nerve, together with superficial masseteric artery and vein are running between SMAS and the masseteric fascia. The branches of the facial nerve are also in the vicinity of the SMAS of massteric region, as well as branches of the trigeminal nerve.

The facial nerve branches are considered the most variable anatomical elements, but the use of landmarks allows us to locate them accurately (**Figure 1.10**). These nerve threads are accompanied by branches of the external carotid artery. When removing skin flaps or performing pretragal incision for facial lifting, surgeons must consider these relations of the superficial masseteric fascia (**Figure 1.11**).

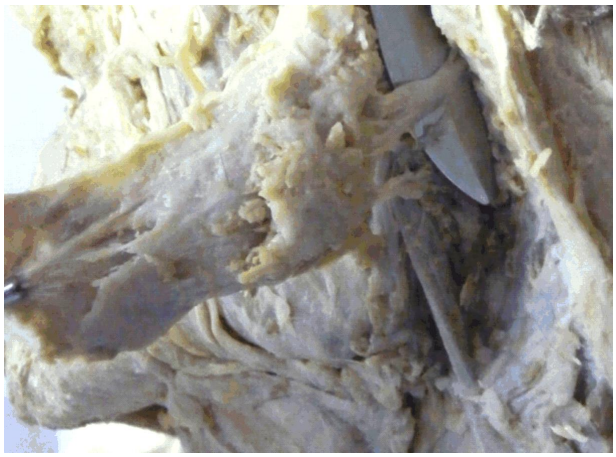

Figure 1.10. Branches of facial nerve related to SMAS. Dissection specimen. SMAS: Superficial muscular aponeurotic system.

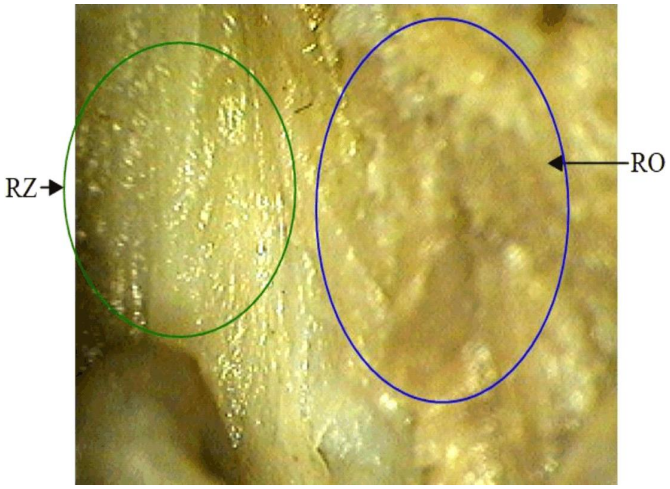

Figure 1.11. The limit zone between the zygomatic region (RZ) and oral region (RO). Mesoscopic images.

The masseteric region makes the transition to the zygomatic and temporal regions. The morphological, macro and microscopic differences between these regions are very concise. Superficial thick masseteric fascia continues anterior and superior with the zygomatic fascia, and posterior and superior with the temporal one. Inferior and posterior, at the level of the parotid gland, the superficial fascia gives rise to the anterior capsule of the gland.

Anterior and medially, it is continued by the superficial fascia of the cheek and it forms fibrous tunnels through which branches of the facial nerve are passing (**Figure 1.12**).

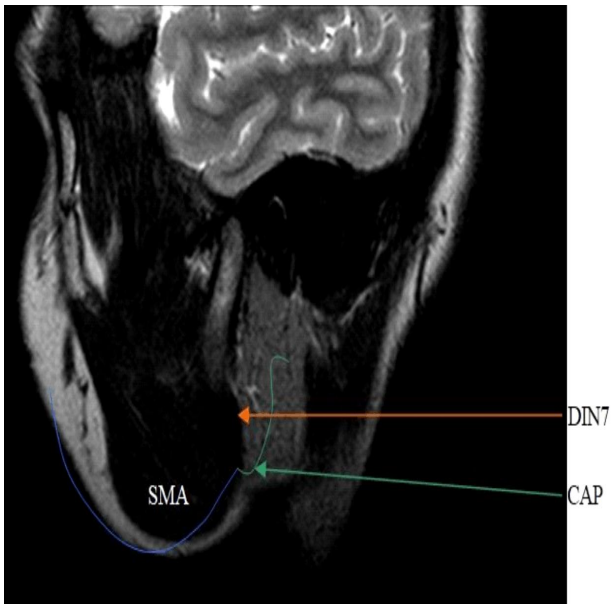

Figure 1.12. Anterior parotid capsule (CAP); intraparotid division of the facial nerve (DIN7).

Superficial masseteric fascia is continued downwards by superficial parotid and cervical fasciae, where it originates from (**Figure 1.13**).

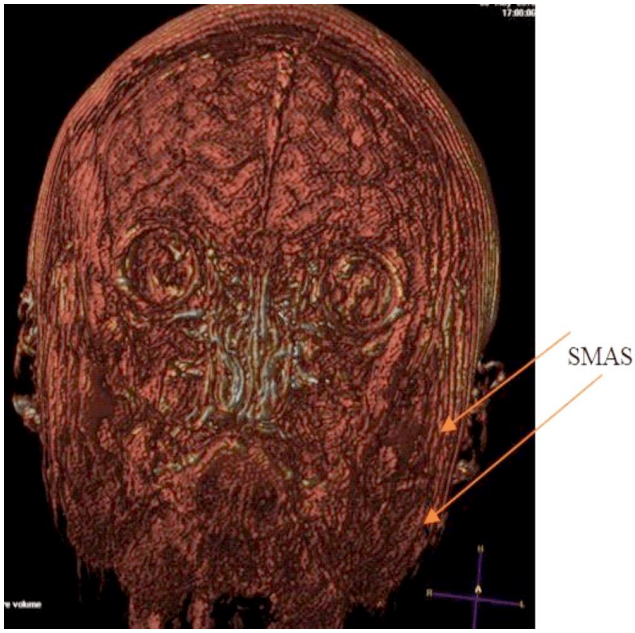

Figure 1.13. Three-dimensional (3D) coronal reconstruction that highlights the topographical plan of SMAS from tragus to gonion. SMAS: Superficial muscular aponeurotic system.

The most important features of soft tissues stratigraphy at this level are:

- the superficial fascia (SMAS) is best represented at this level, consisting of dense connective tissue;
- the superficial fascia is connected to surrounding fasciae and works as one great muscular aponeurotic complex;
- subcutaneous adipose tissue is predominantly prefascial but its consistency and form is different from zygomatic, temporal and parotid regions;
- the superficial fascia gives rise to the zygomatic and inferior temporal ligament;
- the superficial fascia is crossed by vascularnervous elements;
- the superficial layers have very low mobility in the center of the region.

In conclusion, we can systematize that the lateral region of the face has as layers: cutaneous, dermoepidermic;

- subcutaneous fat;
- superficial fascia;
- the branches of the facial nerve;
- deep, masseteric fascia;
- the muscular layer,
- represented by the masseter muscle.

The distance to the deep fascia is increased, the infraSMAS fat layer being well represented with oblique orientation; the elastic fibers are completely missing. The superficial connective layer is well represented, with many fat lobes separated by connective tracts with almost vertical or slightly oblique direction. There are predominantly medium-sized collagen fibers, rare elastic fibers, almost similar to the arrangement in the parotid region.

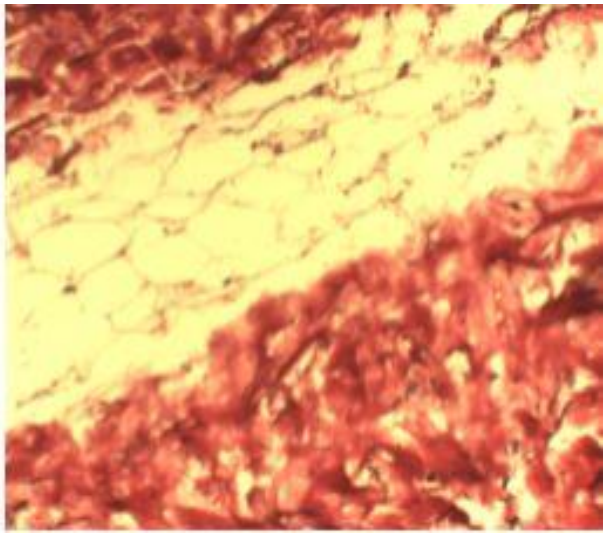

Figure 1.14. Succession of superficial layers in the parotid region, top-down: SMAS, adipose tissue infra-SMAS and parotid fascia. Verhoeff's staining, ×400. SMAS: Superficial muscular aponeurotic system.

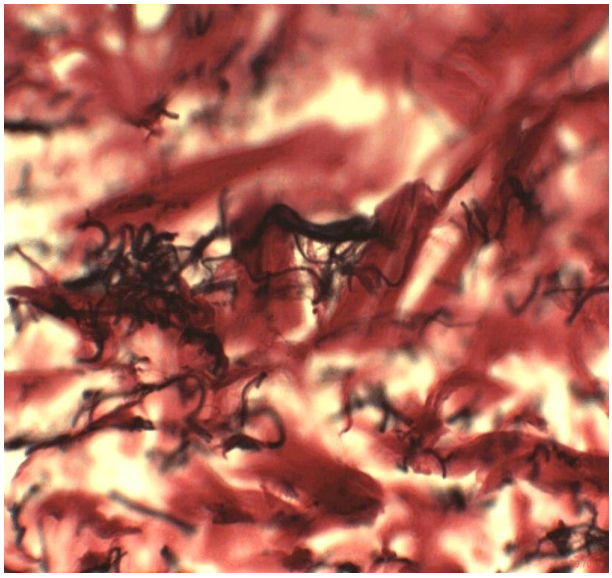

Figure 1.15. Parotid dense structure, with many collagen fibers thick and thin, fragmented elastic fibers. Verhoeff's staining, ×600.

Our observations support the quantitative data obtained on CT images. In the masseteric region, the superficial fibroadipose layer has an average thickness of 4.32± 2.9 mm, and the deep fat layer is very thin, 0.33±0.48 mm. SMAS appears as a hyperdense line intimate to the gland, with a thickness of 0.76±0.43 mm (**Figures 1.14, 1.15, 1.16).**

Microscopically, SMAS is well represented, with condensed colagene thick fibers, orderly disposed, mostly longitudinal, with diminished interstitial spaces. Blood vessels meet only at the periphery of the lamina and have very small dimensions.

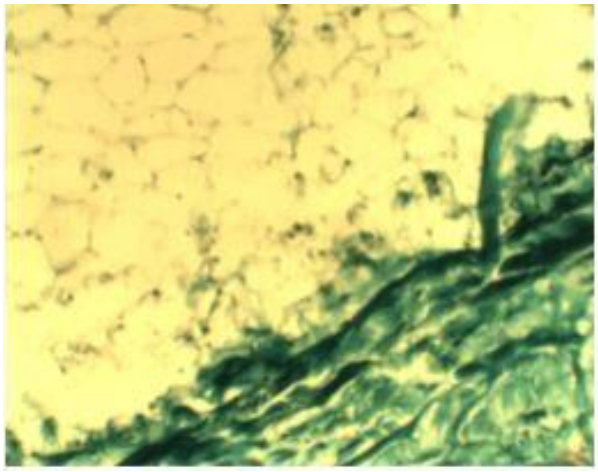

Figure 1.16. SupraSMAS fibro-adipose tissue, well-represented, in the parotid region. Szekely staining, ×200. SMAS: Superficial muscular aponeurotic system.

1.2.3.3. Results of the clinical study – Moebius syndrome

The clinical examination and functional exploration of the first patient showed the following aspects related to atresia of the facial nerve: asymmetric facies, lagophthalmos, impossible closure of the eyes and mouth, decreased lacrimation, loss of taste.

Besides these aspects, the patient experienced symptoms related to damage of other cranial nerves, such as: abducens nerve – impossible lateral gaze, convergent strabismus, photophobia, myopic astigmatism; hypoglossal nerve – difficulty in chewing, preferring soft food, tongue protrusion, difficulty in swallowing. Cranial nerve injuries are associated with other congenital malformations: microcephaly, tear duct changes, hypoplasia of the wing nose, hooked nose, short filter.

In another patient, we observed clinical signs given by facial nerve damage, with craniofacial dysmorphia, eyelids opening upward and laterally. Lesion of the oculomotor nerve determined convergent strabismus, disorders in eyeball motility, with the absence of abduction and convergence of the eye, obliquity of palpebral fissureand divergent deviation.

All of these were correlated with congenital atrophy of the optic nerve (blindness), impossible closure of mouth, micrognatism, posterior rotated ears, triangular facies, psychomotor retardation.

The third patient also presented symptoms associated with damage of the facial nerve, facial dysmorphia with myopathic facies and ptosis of the eyelid more precisely, but also by with injuries associated with lesions of other cranial nerves – paresis of oculomotor nerve, bilateral paralysis of the abducens nerve with strabismus and bilateral palpebral ptosis; hypoglossal nerve injuries – swallowing disorders.

All these were associated with circular facies, hypertelorism, mongoloid eyelid slits, facial hypoplasia, broad bridge of the nose, low-set ears, pectus excavatum, prominent nose base, ears dysplasia, weak voice, intellectual disability, cognitive and psychomotor delay.

Another patient had clinical signs of facial nerve injury at the face level: facial dysmorphia, inexpressive facies, narrow slits eyelid, associated with small nose, small mouth, lowered commissures, micrognatism, ears dysplasia both being small and round, discrete right

hemiparesis and moderate delay in psychomotor development.

The fifth patient had the following changes of the face: inexpressive facies, short eyelid slots – given by bilateral facial nerve injury. He had specific clinical signs given by oculomotor nerve damage: limited movements of the eyeballs associated with prominent upper lip, evident micrognatism.

The last patient had the following symptoms: bilateral facial paresis (inexpressive facies), cranial nerve paresis (ocular mobility disturbance) associated with small, asymmetrical tongue, significant phonation disorders, dental malposition. Neurologically, this patient had medium/severe delay in mental and language development (intelligence quotient – IQ = 35–40).

After studying these patients, we found that clinical symptoms were directly correlated with the level of cranial nerve injuries. Unilateral lesion of the facial nerve causes facial dysmorphism by ptosis of the superficial tissues on the same side and by flattening wrinkles expression. In cases where the damage occurs bilaterally, the patient's face is like an inert mask. In both cases, the evolution in time of symptoms leads to dysfunction of the facial muscles, especially the muscles of the jaw, controlled by the trigeminal nerve.

We found that the most exposed areas of the face to present dysmorphism are the central ones: oral, nasal, orbital. On the lateral regions of the face, where superficial fascial has insertions on the bone, there are two forces that act on these attachments: those caused by facial muscle contraction (engaged in a whole as SMAS) and those caused by masticatory muscles.

Clinical signs of the damage in this lateral region appear in time, after a mean duration of five years from the onset of central facial dysmorphism.

Affection of the infraorbital, zygomatic and jugal regions is a consequence of the appearance of laxity in the fixation structures of SMAS, combined with changing vector forces acting in these regions, due to gradual installation of central facial dysmorphism and "fatigue of masticatory muscle". The main defect that occurs in this medial region is quantitative loss of adipose tissue (the reduction of Bichat's fat pad).

Moebius syndrome affects both the sequentiality and the clinical manifestation of these three regions. Initially, malfunctions occur in the medial regions, especially in those with occlusive functional characters. Impairment of the medial transition regions installs secondly, substantially due to secondary functional lesions in the medial regions. Finally, clinical changes in the lateral regions occur latest, due to ongoing straining manifested for long time.

The pathophysiological mechanism of these clinical manifestations is quite similar to the phenomenon of "aging face" and consists in loss of the quality of various mechanisms of anti-gravity fixation of superficial cervical and facial structures. These phenomena have resulted in facial soft tissue ptosis that further affects the functionality of other facial regions.

Emergence of these clinical manifestations represents criteria for surgical treatment, which is the only treatment option capable of yelding mechanical and anti-gravitational support of the structures of the SMAS, achieved though cutting of the original, incompetent means of fixing (rejuvenation) and transfer of the temporal muscle function to the mimic muscles (orbicularis oris).

Common stainings revealed the atrophied muscle fibers correlating with the appearance

of excess fibrous connective tissue, especially in the medial, relational region of the face (**Figure 1.17**).

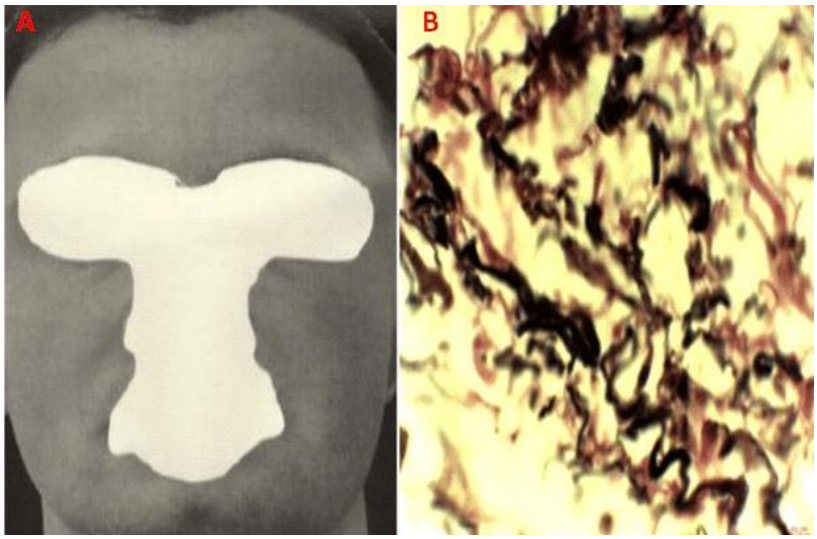

Figure 1.17. A=median region of the face; B=numerous elastic and collagen fibers, medium size and muscular fibers underrepresented in the structure of SMAS from the jugal region. Verhoeff staining, ×600.

Laxity of fixation elements can be identified at the level of the lateral regions of the face (**Figure 1.18**), through the proximity of the periosteum, by collagen fiber elongation.

In the medial passage region (**Figure 1.19**), the changes appear most probably at a later stage. It is the last region of the face that suffered degenerative changes following the loss of tone and contractility of the mimic muscles. The nasolabial groove, in particular, remains last redoubt. At its level, we have not detected any significant changes in advanced stage morphology of the disease in the studied patients.

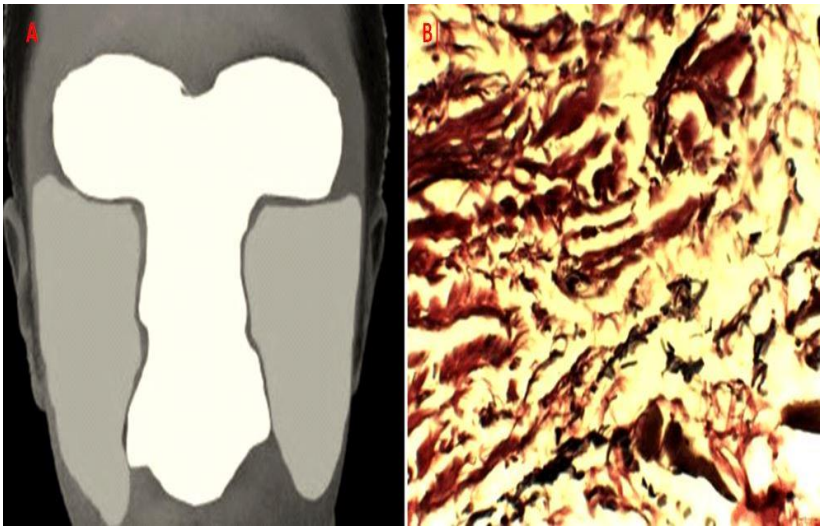

Figure 1.18. A=medial regions of the face; B=collagen and elastic fibers from SMAS in lateral part of nasolabial groove.Verhoeff staining, ×400.

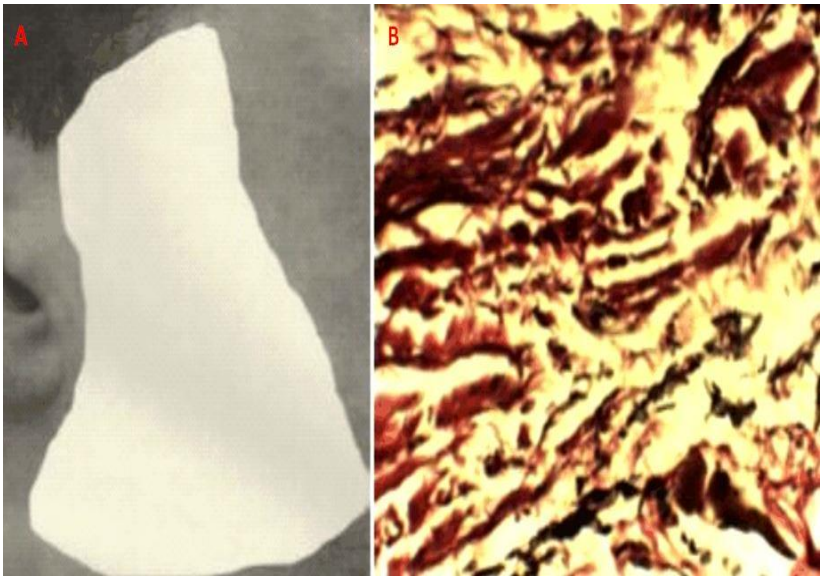

Figure 1.19. A=lateral regions of the face; B=periorbital SMAS, with dense collagen fibers, medium and abnormally long, arranged longitudinally. Van Gieson staining, ×600.

1.2.4. Discussions

1.2.4.1. Parotid SMAS

In the parotid region, the most important neighboring relation of the parotid fascia is that with the branches of the facial nerve and the maxilla. These nerves fibers are accompanied by branches of the external carotid artery. Preserving them is the greatest care of a surgeon who intervenes at this level (Siemionow et al., 2006). Nowadays, by preserving these neurovascular bundles, it is possible to operate a complete face transplant. Data obtained by imagistic records are consistent with those in the literature – quantitative measurements performed on CT (Macchi et al., 2007) showed regional differences in thickness of the superficial layers of the face.

At the parotid level, the SMAS has individual features and also common morphofunctional characteristics with other facial regions (Zigiotti et al., 1991). The common features are that superficial fascia facilitates the adhesion of the mimic muscles from the region to the dermis, forms portvas and portnerve blades and presents an adipose supra and infraSMAS layer. Particular aspects of the parotid region, which we highlighted in this study, refer to the fact that the superficial layers have different thicknesses, the supra SMAS fibrous tissue is well represented, the SMAS is an ordered, dense organized connective tissue and the infraSMAS adipose tissue forms a very thin blade under which there is a deep parotid fascia.

The latter has very dense structure, its collagen fibers constituting a dense structure with homogeneous masses in which the separated fibers are not individualized. We can affirm they have a lamellar disposal (Gola et al., 1994, Jost and Levet 1984).

This reveals that the oral superficial fascia will follow the masticatory movements of the buccinator and masseter muscles, but also those of the great and small zygomatic muscles. These muscles are the infraSMAS layer in parotid region and thus take part in the formation of a unitary complex together with the superficial fascia. The parotid SMAS

continues with that of the adjacent regions, being fixed superiorly by the lower part of periorbital septum and zygomatic arch (Wassef, 1997).

Medially, it adheres to the maxilla through the fibrous condensation of the nasolabial fold. Inferiorly it is inserted at the lower edge of the mandible, from where it continues with the surface of the platysma. Laterally, it forms the anterior sheet of the parotid fascia. The great zygomatic muscle adheres to its front, facing the superficial fascia. The latter has a much dense structure, its collagen fibers constituting a dense structure with homogeneous masses in which fibers (Macchi et al., 2010) are not individualized. *We can say, they have a lamellar disposal and elastic fibers are thin and fragmented.*

Therefore, elevation of the SMAS is concomitant with the muscle (Mendelson, 1995). Although it is intimately applied to superficial surface of the parotid gland, a weak but distinct parotid fascia is identified between the gland and the SMAS.

The CT examination of patients supports the previous results, and shows that while reaching the parotid gland, the superficial fascia gives rise to the anterior capsule of the gland. This data is also acknowledged in the literature (Baek et al., 2007).

The existence of a SMAS layer on the parotid capsule allows the application of techniques that have been used in facial lifting procedures and parotid tumors ressection. This aspect is however a novelty in maxillofacial surgery techniques and improves the results of the interventions at this level. The identification of the facial nerve in its quadrilateral intraparotid dissection, and especially the fascial tunneling of its terminal branches allows a better preservation of the nerve, yielding improved results for the patient (Ikoma et al., 2014). The decrease in the incidence of postoperative infections to less than 1% and the higher aesthetic results (Wong and Ahetty, 2017) prompt the use of the concept of the single surface layer demonstrated by us in parotid surgery.

In the parotid region, the superficial fascia presents numerous lobules. They are separated above SMAS by vertical fibrous tractus and subSMAS these tracts have a horizontal disposition. In the posterior part of the parotid gland, the superficial fascia exhibits a condensation that forms a true hill for the gland. Here the facial nerve, the facial artery enters to parotid gland and the external jugular vein exits the parotide.

In the genian region, SMAS's most important relationship is that of the maxilla and facial nerve branches. These nerve threads are accompanied by branches of the external carotid artery. Their preservation is the greatest concern of a surgeon who intervenes at this level (Siemionow et al., 2006). Nowadays, by preserving these neurovascular bundles, complete facial transplants are possible. *The classical theory of the origin of parotid fascia being derived from the deep facial fascia is invalidated by the continuity of its anterior layer with the platysma muscle fascia.*

Parotid masseteric SMAS is well personalized and may contain some muscular fibers and nerves fibrous tunnels (Gola, 2005). It covers the parotid gland and masseter muscle (Pidgeon et al., 2017). Riolan, in "Le Double", signaled "portio musculi cutanei supra parotidem ad aurem ascendentis" (Riolan, 1897), a part of the platysma muscles that ascends to the ear, passing over the parotid. It is fixed to the auricular cartilage and establishes deep adhesions with the parotid capsule. In the area located inferiorly to the mandibular angle and in the mastoid region, SMAS adheres to the superficial cervical aponeurosis that covers the sternocleidomastoideus muscle.

1.2.4.2. Masseteric SMAS

In the masseteric region, the superficial fascia features numerous lobules. They are superimposed by vertical fibrous tracts. Infra-SMAS these tracts have a horizontal disposition. In the posterior part of the parotid gland, the superficial fascia exhibits a condensation that forms a true hill for the gland. *At this level, the facial nerve, the facial artery and the external jugular vein enter the parotid gland. Jugal SMAS is thin, discontinuous and difficult to dissect; it becomes gradually thinner from the posterior to the nasolabial above and does not go beyond the groove. It contains the risorius muscle that moves paracomisurally and pulls it back in the smile. This muscle develops in the SMAS thickness, before the masseteric aponeurosis, but without inserting it*.

SMAS forms together with the skin a functional, tegumentary, adipose and neurovascular unit, physiologically inseparable, the cervicofacial cutaneous muscular aponeurotic unit (Zhang et al., 2013). On axial CT, SMAS appears as a relatively hypertensive, tortuous line between superficial fibrous tissue and deep hypodense adipose tissue. Quantitative measurements performed on CT by researchers (Macchi et al., 2007, Macchi et al., 2010) showed regional differences in thickness of the superficial layers of the face. *We have also used MRI and CT images to determine morphological aspects of other muscle. In the parotid region, the superficial fibroadipose layer has an average thickness of 4.32±2.9 mm and the deep fat layer is very thin, 0.33±0.48 mm.*

SMAS appears as a hyperdense line intimate to the gland, with a thickness of 0.76±0.43 mm. At the cheek level, the superficial fibroadipose layer is very well represented (5.57±1.17 mm), the thicker layer is thinner, 2.94±0.62 mm, and SMAS recognizable slightly (2.94±0.62 mm). At the level of the nasolabial groove, the superficial fibroadipose layer is poorly represented (0.37±0.06 mm), the deep fat layer has an average thickness of 2.15±0.63 mm, while SMAS continues with muscles of facial expression, also with average thickness (2.41±0.05 mm).

On MRI images, SMAS appears as a continuous hypotensive line in T1 and T2, from the parietal region to the nasolabial sulcus. It includes the muscles of facial expressions in the cheek regions and in the nasolabial sulcus.

Our anatomoradiological study confirms the architectural composition of the face from multiple layers of tissue that connects the facial muscles to the dermis. Aspects encountered on MRI images at various incidences support microanatomical observations, obtained on sections at various levels, according to which, the arrangement of SMAS suggests a gradual centrifugal thinning towards adjacent regions.

Also, masticatory movements define important force vectors in this area. In facial nerve damage, these are greatly damaged and require specific reconstruction techniques (Pippi, 2014).

1.2.4.3. Moebius syndrome

The facial dermis is fixed to the bones through a fibrous support, which contains ligaments and superficial fascia, and at the same time allowing the movements and facial gestures. This attachment must also resist external forces such as gravity and traction.

The ligaments, which fix the superficial fascia to the skeleton, can divide the face into several distinct regions. Three of them are in the mental and infrazygomatic regions: chin, lateral side of the chin (Jost and Levet 1984).

Besides these regions, we can include those of lower eyelids, upper eyelids, infratemporal and frontal.

In these regions, the ligaments between the posterior aspect of superficial fascia and facial skeleton "put in quarantine movements caused by muscle contraction, at least in young adults, so that movements of superficial fascia will not be transmitted to neighboring regions".

Outside the classical division of the face in three zones – superior, middle and inferior – one can be added based on aesthetic, anatomical and functional considerations. Anatomical pattern of ligamentous attachment of the superficial fascia to the facial skeleton defines the limits that divide face in several regions.

Three of these are parts of what is seen on the outside such as: the cheek, lateral cheek, pre- and infrazygomatic sides medial cheek, in addition to the other regions: lower eyelid, forehead and upper eyelid. Congenital atresia of the facial nerve has a different impact on these parts of the face, both in terms of morphology and in of appearance of clinical signs over time. In severe cases of bilateral congenital atresia, its clinical manifestation is more severe and presents earlier.

The weakening of connecting tissue through muscular shortening and stretching determines the displacement on different directions depending on the configuration of each muscle, separately. There are multiple movement vectors in facial aging because each muscle of facial expression has its own connection direction of the lax tissue. The correction of each vector displacement requires a proper fixing vector.

The presence of Moebius syndrome associated pathologies speeds up appearance of connection tissue degeneration, leading to earlier clinical manifestations. Fortunately, SMAS concept usage provides increased opportunities for controlling the direction of lifting. We can hence say that the face is made up of three distinct functional morphological regions: median, medial, lateral and posterior (Berkovitz and Holland 2002).

The median region *of the face includes superficial and deep layers around the face openings, having the shape of a butterfly with raised wings. The main feature of this region is the fixityof superficial structures to the deep onemore precisely powerful, flared muscular insertions on the deep part of the dermis.*

It can be said that it is the visceral segment of the face. Most muscles play the role of sphincter of the openings they surround, while the others represent their functional extensions. The medial part of the face is predominant in individualizing the facies and hence, the relational life of each individual.

Therefore, we call it the "relational" part of the face, and we can divide it into four subregions: orbital, nasal and oral. Although muscles are different in each of the regions, there are some common considerations, in terms of stratigraphy, quantity and quality:

- *the skin gradually thins as we move towards the mediosagittal area;*
- *the same behaviorhighlights in the subcutaneous adipose tissue,the difference being that the layer beneath the superficial fascia is almost nonexistent;*

- *superficial and deep fascia lose their elasticity, being made up of dense connective tissue.*

In some situations, both fascia are reflected with one another and can adhere to the periosteum region. This happens periorbitally (ligamental adhesion where it is impossible to perform a deep dissection) at the lower edge of nasal cartilage and at the midline union of the two nasal wings (Bosse and Papillon 1987).

The medial region (intermediate) of the face is a paired region, mainly characterized by a high degree of mobility of the superficial tissue compared to deep layers. It is bounded superiorly by the zygomatic arch (Delmar, 1994) and inferiorly by mandible arch. Laterally, it spreads to the parotid gland capsule and medially to a linewhich joins the angle of the mouth with the lateral edge of the nasogenian groove.

The exacerbated mobility of these regions, compared to the other two, is due to the fact that it contains two fat peds (malar and jugal)of considerable size.Connective fibers (Gardetto et al., 2003) of the superficial fascia of these fat pads adhere strongly to the deep layer of the dermis. In depth, they are "anchored" only by the cutaneous insertions of zygomatic muscles.

This region is also one of "passage", SMAS (Gola, 2005) realizing connective tunnels through which branches of the facial nerve, Stenon's duct and branches from facial vessels cross from the lateral part to the medial part or vice versa. Duplication of SMAS with the formation of tunnels for nerves and vessels is done through a trans-versely oriented connective tissue (Gosain et al., 1993), while the superficial layer, whose origin is orientated from superior to inferior, isin the direction of the traction vectors of zygomatic and infraorbital insertions.

Another important role of these regions is to form an interconnection between the medial and the lateral region of the face, providing an additional support to actions that occur at this level. Thus, it actively intervenes in far-reaching facial expressions, such as making a wide smile. This stands out when the region is developing an inflammatory or tumoral process, which disrupts its functionality. In this case, it can cause active facial dysmorphism (in an attempt to express facial gestures) or passive dysmorphism through retraction of tissue and loss of anti gravity support (Hughes and Salinas 1999).

The role of the most powerful masticatory muscles (temporal, masseter, buccinator) is amplified, through a leverage mechanism, by the connections of their insertion regions with the perioral one (Piccioloni et al., 2016).

Mimics in this area can occur only on its boundary where the muscles of facial expression take insertion.

The p*osterior lateral region of the face is a paired region, fixing the face to the exoskeleton. Here lie strong ligaments and ligamentary adhesions that, on one hand anchor soft tissues of the face and, on other hand, delimit it by the neighboring regions: frontal, temporal, lateral-cervical and cervical.*

Being particularly visible from profile, this region intervenes indirectly in creating facial expressions. At this level, in deep plan masticatory muscle inserts on mandibular ramus. The region extends superiorly to the zygomatic arch, practically covering the infratemporal fossa. Laterally and posteriorly, one can make up the parotid region, while the deep fascial layer is made up by parotidian masseteric fascia.

1.2.5. Final remarks

Musculofascial superficial aponeurotic segment in masseteric region is distinguished in the subcutaneous fat layer only in the posterolateral areas of the face, where there is a deep fascial support, functionnaly tensed. Collagen fibers are dispositioned orderly on successive longitudinal and transverse planes, or have individual structure, forming layers of varied shapes and sizes, most obviously at the level of the modiolus. Muscle fibers belonging to the superficial layer of the skin that form SMAS in some areas, leave or cross to the deep surface or the osteoperostic plane. These anatomical findings could be useful for the understanding of the SMAS concept and for various types of facial surgery.

TIn Moebius syndrome, the mimic muscles of the central regions of the face have a significantly decreased role. Through fibrous extentions in the medial region, SMAS determines the robust adherence of the fat pad at this level. This provides the contour of the cheek, and SMAS has an active role in raising the soft tissues of the cheek. The anterior capsule of the parotid gland is formed by SMAS. From this level it is continued posteriorly and laterally with the fascia of sternocleido-mastoid muscle and anteriorly and medially with fibrous tunnels for facial nerve branches. At the supraorbital level, there are strong adhesions between the ligaments of super-ciliary arches and the dermal insertions of the superior fascicle of orbicularis oculi muscle, which are becoming increasingly lax. Continuities of this fascia with the surrounding fascia – frontal, cervical, temporal – are due to pathological changes associated with Moebius syndrome.

1.3. Anatomic variations in arterries

1.3.1. Introduction

Detailed knowledge of the cerebral arterial blood supply is crucial for neurosurgeons, neurologists and interventional radiologists. Due to particularities of the cerebral arterial vascularization, each major artery plays an important role in the brain's blood supply. Amongst them, the anterior cerebral artery particularly stands out due to its vast distribution territory. Moreover, anatomical variations of the anterior cerebral artery are quite common and are often described in the medical journals (Makowicz et al., 2013). These variants refer to hypoplasia or aplasia, accessory anterior cerebral artery (Maruyama et al., 2005), anomalous origin of the callosomarginal artery (Krishnamoorthy et al., 2006) or uncommon origin of anterior communicating artery (Lee et al., 2000). Despite that, only a small number of patterns regarding the fronto-orbital artery have been described, some authors mentioning its anomalous origins (Lee et al., 2000; Maruyama et al., 2005).

The fronto-orbital artery (FOA) is the first cortical branch of the anterior cerebral artery (ACA), normally arising from the A2 segment (Bhat et al., 2014; Avci et al., 2004). Some authors mention its origin from the A1 segment of ACA (Makowicz et al., 2013), the pericallosal artery (Lee et al., 2000), the callosal marginal artery or sometimes from the fronto-polar artery (Aso et al., 2015). The trajectory of the fronto-orbital artery usually follows an anterior course, along the medial surface of the fronto-orbital gyrus. This artery

typically supplies the *gyrus rectus*, the olfactory bulb and tract, the medial and inferior surface of the fronto-orbital gyrus and the anterior and medial surface of the superior frontal gyrus (Rhoton 2002).

In the current case report, we describe a unique vascular pattern represented by two anomalous right fronto-orbital arteries arising from the A2 segment of the contralateral anterior cerebral artery, associated with a partially duplicated anterior communicating artery (ACoA). These aberrant arteries are associated with a hypoplasic right fronto-orbital artery.

The aim of this study is to document the origin of the fronto-orbital artery in relation to its embryological development and to warn the clinicians about the anatomical variations of this artery. Furthermore, the duplication and fenestration of ACoA and their major complications, respectively arterial cerebral aneurism and subarachnoid hemorrhage are being discussed. . In this paper, we are intending to identify the anatomical formations, which can be defined as sustentaculum facies, with an important function in supporting the superficial layers and the possibilities of various techniques in rhytidectomy.

At the junction level of the craniofacial and cervicofacial areas, there are muscular transfascial attachments, fibrous adhesions and ligaments, which fix SMAS to the profound layers. Due to the stabilizing role, we have described all these compact structures as the sustentaculum facies.

The periorbital adhesions represent a support point, which converges toward all the adjacent ligaments orientated to those three axes of the space, and together with the zygomatic ligament form the upper portion of sustentaculum facies. They are the main anti-gravity suspension mechanism of the face.

The infraSMAS plan is connected to the profound layer by the adhesions and a connective conjunctive adipose tissue crossed by the neurovascular branches, a port-vessel tissue and a portnerve similar to one from other regions. The decreasing of the percentage volume of the collagen fibers from their structure or of the sliding fibroadipose tissue during aging, explains the decreasing of the functional capacity followed by face fall.

Personal contribution – published paper:

Nedelcu AH, Tepordei RT, Sava A, **Stan CI**, Aignatoaei AM, Taranu T, Ursaru M. Supernumerary fronto-orbital arteries arising from contralateral anterior cerebral artery associated with partially duplicated anterior communicating artery - case study and literature review. Rom J Morphol Embryol 2016; 57(3): 1159-1163.

1.3.2. Material and methods

The anatomical material consisted of a 64-year-old female brain fixed in a 10% formalin solution for 15 weeks. We dissected the arterial circle of Willis with the aid of an operator microscope (OPTRON – Zeiss OPMI 6), focusing on the anterior cerebral artery and the anterior communicating artery. In order to highlight the blood vessels and their distribution territory, we injected red ink into the left ACA, followed by injection of blue ink into the right ACA. The images were acquired with a Nikon D7000 digital camera equipped

with a 60 mm AF Micro-Nikkor f/2.8D lens and were processed with Adobe Photoshop CS5 and Capture NX2 software.

1.3.3. Results

During the dissection, we discovered a unique con- genital abnormality regarding the arterial supply of the right fronto-orbital gyrus. Normally, this gyrus is vascu- larized by the fronto-orbital artery, which is the first cortical branch of the ipsilateral anterior cerebral artery. In our case, the arterial supply of the right fronto-orbital gyrus had three sources: one ipsilateral fronto-orbital artery, one contralateral fronto-orbital artery and another accessory branch originating from the contralateral ACA (**Figure 1.20**).

The first source is represented by the right fronto- orbital artery that originates from the A2 segment of the ipsilateral ACA. This artery has a short trajectory on the surface of the brain, situated into the *inferior rostral sulcus*, and then it splits in three branches: superior, inferior and anterior (Figure 2). The superior branch perforates the corresponding slope of the *sulcus* supplying the deep cerebral tissue. The inferior branch is directed towards the inferior border of the fronto-orbital gyrus. The anterior division is the terminal branch and continues the right fronto-orbital artery into the *inferior rostral sulcus*. At the distal extremity of the sulcus, this branch disappears into the cerebral tissue (**Figure 1.21**).

The second source, that we named aberrant right fronto-orbital artery (ARFOA), has the origin in the A2 segment of the left ACA, close to anterior communicating artery. This artery, that seems to be the main arterial source of the right fronto-orbital gyrus, is directed anteriorly and to the right, then passes under *falx cerebri* and before it reaches the medial surface of the right fronto-orbital gyrus, it gives off thin collateral branches for the posterior part of the gyrus situated under *superior rostral sulcus* (**Figure 1.21**). The artery has a postero-anterior direction along with the medial surface of the fronto-orbital gyrus and splits in superficial branches, generating a “medusa head” aspect. The superior branches supply the inferior part of fronto-polar gyrus corresponding to area 10 Brodmann. The anterior branches exceed the superior border of the right hemisphere supplying the cortex of frontal pole, while the inferior branches direct to *gyrus rectus* crossing the hemispheric border. The main distri- bution territory is represented by the medial aspect of fronto-orbital gyrus (area 11 Brodmann) (**Figure 1.21**).

The third source is a small branch that we named accessory right fronto-orbital artery (AccRFOA). It origi- nates in the A2 segment of the left anterior cerebral artery, distal to the previous branch. It is directed anteriorly and to the right, goes under the *falx cerebri*, crosses the previous branch on its dorsal aspect and reaches the medial surface of the right fronto-orbital gyrus. It supplies the segment situated above the *inferior rostral sulcus* (**Figures 1.20 and 1.21**).

In this case, the variations in the number and position of the FOA are also associated with a partially duplicated anterior communicating artery. The duplication occurs in the right half of ACoA. No aneurysm formation has been detected (**Figure 1.22**).

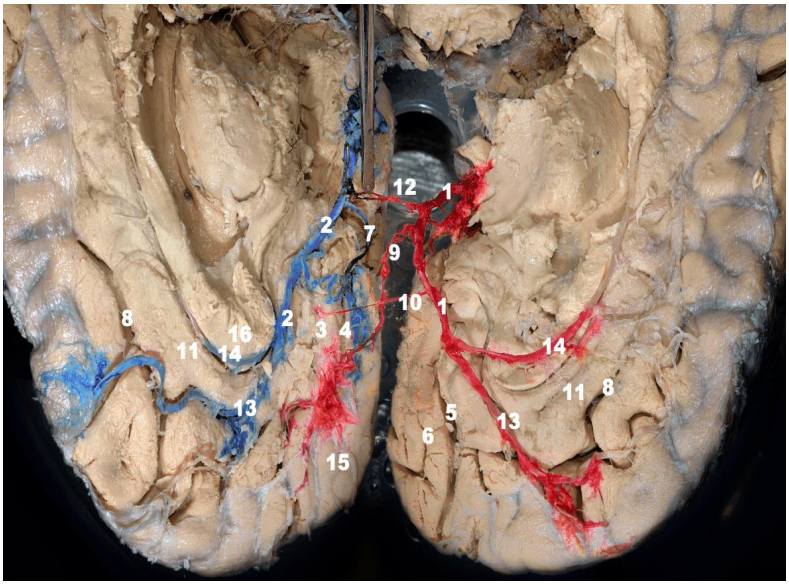

Figure 1.20. Ipsilateral and contralateral fronto- orbital arteries. Superior view of the medial aspect of both cerebral hemispheres. 1: Left anterior cerebral artery; 2: Right anterior cerebral artery; 3: Right superior rostral sulcus (Eberstaller); 4: Right (accessory) inferior rostral sulcus; 5: Left superior rostral sulcus; 6: Inferior rostral sulcus; 7: Right fronto-orbital artery (first source); 8: Cingulate sulcus; 9: Aberrant right fronto-orbital artery (ARFOA); 10: Accessory right fronto-orbital artery (AccRFOA); 11: Cingulate sulcus;12: Partially duplicated anterior communicant artery; 13: Frontopolar artery; 14: Pericallosal artery; 15: Right fronto-orbital gyrus (gyrus rectus); 16: Genu of corpus callosum.

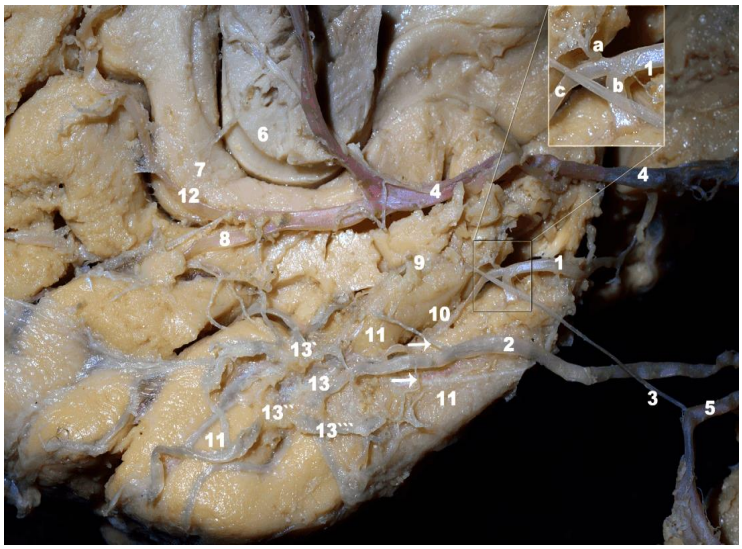

Figure 1.21. Territory of distribution for the aberrant left (contralateral) fronto-orbital artery – second arterial source. Medial aspect of the right frontal lobe. 1: Right fronto-orbital artery; 2: Aberrant right fronto-orbital artery (ARFOA); 3: Accessory right fronto-orbital artery (AccRFOA); 4: Right anterior cerebral artery; 5: Left anterior cerebral artery; 6: Corpus callosum; 7: Cingulate gyrus; 8: Right fronto-polar artery; 9: Superior rostral sulcus (Eberstaller); 10: Inferior rostral sulcus; 11: Right fronto- orbital gyrus (gyrus rectus); 12: Right pericallosal artery; 13: "Medusa head" aspect of the aberrant contralateral fronto-orbital artery: 13' – superior branches, 13'' – anterior branches, 13''' – inferior branches. Inset: Branches of the right fronto-orbital artery: a – superior, b – inferior, and c – anterior branch. Arrows: collateral branches of the aberrant contralateral fronto-orbital artery.

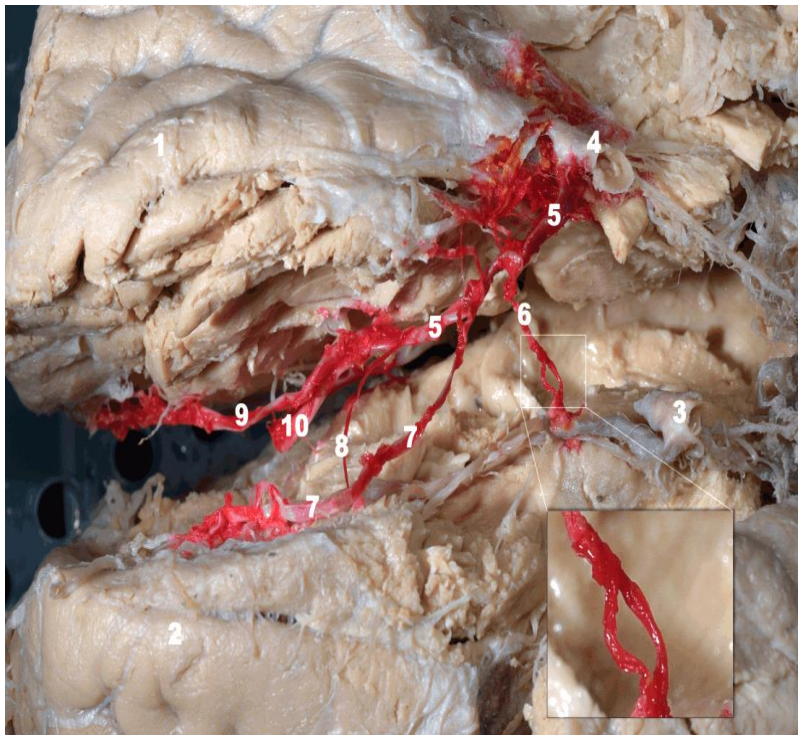

Figure 1.22. Fenestration of the anterior communicating artery. Basal view. 1: Left frontal (orbital) lobe; 2: Right frontal (orbital) lobe; 3: Right internal carotid artery; 4: Left internal carotid artery; 5: Left anterior cerebral artery; 6: Partially duplicated anterior communicating artery; 7: Aberrant right fronto-orbital artery (ARFOA); 8: Accessory right fronto-orbital artery (AccRFOA); 9: Left frontopolar artery; 10: Left pericallosal artery.

1.3.4. Discussion

The vascular anatomy related to the anterior cerebral artery is quite complex due to its embryological development. During the formation process of the anterior communicating artery, a large plexiform anastomosis appears between the two anterior cerebral arteries. Normally, the vessels of this plexus will fuse together to form the anterior communicating artery. A fenestration process may appear instead, explaining the high incidence of anatomical variations (de Gast et al., 2008). During this process, the mesenchymal tissue that normally forms the left fronto- orbital artery remains connected to the mesenchyme of the right hemisphere and supplies the contralateral fronto-orbital gyrus. Incomplete fusion of the plexiform anastomosis also explains the anatomical variants of ACoA, including duplication and fenestration (Menshawi et al., 2015).

We reported the unique case of an anomalous origin of the right fronto-orbital artery in the left ACA that is associated with an accessory branch emerging from the same source. To our knowledge, this is the first report of this pattern in the literature. Lee and Maruyama reported two cases of anomalous origin of FOA observed during angiography (Lee et al., 2000; Maruyama et al., 2005). *The current study stands out by presenting a unique association of vascular malformations and by showing for the first time, an anomalous origin of FOA identified during anatomical dissection.*

The abnormal position of the fronto-orbital artery can determine clinical symptoms such as tonic and clonic seizures related to an epilepsy focus in the right fronto-orbital gyrus. These anatomical variants are also a predisposing factor for arterial aneurysms and intracranial hemorrhage (Hong 1997), bringing the new techniques of angiography, especially angiographic projections, roadmapping and endovascular access to ACA to a useful area of diagnosis and treatment (Graves 1998).

The duplication of ACoA is a rare finding. In a large cadaveric study, Kapoor et al. (Kapoor et al., 2008) identified duplicated ACoA in 10% of cases, triplicated in 1.2% and plexiform in 0.4%. Gunnal mentioned in their anatomical study that the duplication was seen in 16 (10.66%) formalized brains (Gunnal et al., 2014). The imagistic studies realized by Krzyżewski and Kovač report duplication of ACoA in 0.49% and respectively 0.4% of the investigated subjects (Krzyżewski et al., 2015; Kovač et al., 2014). *The discrepancy between the results of cadaveric and imagistic studies might be attributable to various factors: low diameters of the vessels, insufficient blood flow or imaging artifacts. To our knowledge, the partially duplicated ACoA has not been previously reported.*

The difference between duplication and fenestration is a subject of debate for scientists. Makowicz considered that segmental duplications are, in fact, fenes- trations as long they represent a division of a vessel (Makowicz et al., 2013). To sustain his findings, he proposes a morphological classi- fication: type 1 – small, slit-like fenestration; type 2 – large, lenticular fenestration. Other authors such as Menshawi and Kovač concluded that the arterial fenestrations are completely different from arterial duplications (Menshawi et al., 2015; Kovač et al., 2014). They claim that a fenestration is characterized by an artery that splits into two branches that rejoin. Furthermore, these authors affirm that the common origin is reserved to arterial fenestrations, while duplications should have distinct origins (Menshawi et al., 2015).

In contrast to duplications, cerebral arterial fenestrations are quite common. 2.1% of all the patients undergoing digital subtraction angiography were reported (Cooke et al., 2014). The fenestration is most often located in the posterior circulation (73.2%), especially in the basilar artery (52.6%). Fenestrations of the anterior circulation vessels are reported in 24.6% cases, mainly on ACoA. The rest of 2.2% are found on the internal carotid artery and the middle cerebral artery. The prevalence of aneurysms within the anterior and posterior fenestrations is almost similar: 60.7% *vs.* 61.1% (Cooke et al., 2014).

De Gast concluded that there is a direct relation between fenestration of ACoA and aneurisms of the anterior brain circulation: 83% of the patients with fenestrated ACoA where diagnosed with ACoA aneurysm and 5.3% of the total number of patients with anterior circulation aneurysm associate ACoA fenestration (de Gast et al., 2008). Moreover, in 4.4% of those cases, the aneurysms were located in ACoA and the rest of 0.9% in other arteries. The reported fenestration of ACoA in dissected bodies is between 7.5% and 40% (Dimmick and Faulde 2008).

Subarachnoid hemorrhage is often associated with arterial fenestration and especially with arterial aneurysms. The prevalence of hemorrhage in patients with aneurysms arising directly from fenestration is slightly higher (66.7%) compared to those patients with aneurisms situated at distance from the fenestration (58.6%) (Cooke et al., 2014). In a study

which included 174 subjects, Hudák concluded that 95.2% of the patients with "unexplained sub- arachnoid hemorrhage" where diagnosed with arterial fenestration (Hudák et al., 2013).

The treatment of the ACoA aneurysms hemorrhage is surgical or interventional. Most of the cases benefit of balloon assisted coil embolization, a minimally invasive technique with a low rate of procedure related complications (Fang et al., 2014). The endovascular treatment ensures the complete or near-complete occlusion of the aneurisms for long-term and is a good alternative to traditional clipping procedure. The classical surgical treatment is reserved for specific patients because of the potentially dangerous dissection of adherent vessels or vascular anatomical variations (Mugikura et al., 2014; Zada et al., 2009). A minimally surgical procedure was developed recently to expose the fronto-basal area of the brain. This approach, called supraorbital trans-eyebrow craniotomy, permits clipping or trapping aneurism techniques as well as the resection of different tumors such as gliomas and meningiomas, avoiding the classical pterional and fronto-temporo-orbito-zygomatic paths (Prat-Acín et al., 2013; Ladziński et al., 2010; Ormond et al., 2013; Ormond et al., 2014).

Noninvasive imaging studies often fail to visualize the anterior communicating artery, but it does not mean that this artery is absent. Based on examination during surgical procedures reported incidence of this variant is estimated at 5% (Dimmick and Faulder 2009).

In large post-mortem studies fenestrations of anterior communicating artery have been reported with a frequency of 40%. As these vessels are very small, it is rarely possible to visualize fenestrations with the same frequency in imaging studies. Their incidence in CT angiography is about 10% (Bozek et al., 2012**)**. Anterior communicating artery aneurysm is the most common form of intracranial aneurysm, accounting for 25-38% of total cerebral aneurysm cases (Koo et al., 2005).

Variations of anterior cerebral circulation may be asymptomatic and may not produce complications, although some of them increase the risk of aneurysm formation and acute intracranial hemorrhages, play an important role in planning of neurosurgical procedures or may be mistaken for serious pathologies. Rapid technological progress in the imaging area and availability of non-invasive angiographic studies will certainly lead to increased number of diagnosed anatomical variants among examined patients.

1.3.5. Final remarks

Detailed knowledge of congenital anomalies related to cerebral vascular system is important for neurosurgeons and clinicians due to numerous arterial variations. The relevance of this case in medical practice lies in the fact that it brings to attention a rare anomaly of the anterior cerebral artery vascular system, where most of the cerebral aneurysms associated with congenital abnormalities occur. Nevertheless, recognizing and reporting the anatomic variants of the fronto-orbital arteries could be helpful in planning surgical procedures involving the ACA and ACoA, especially since these congenital abnormalities modify the regional landmarks.

1.4. Anatomical substrate of peritoneal dialysis

1.4.1. Introduction

The peritoneal serosa behaves as a composite anatomical and functional membrane (Williams et al., 2003). In normal subjects, it consists of a monolayer of mesothelial cells on a basement membrane and a layer of connective tissue embedding cells, blood vessels, and lymphatics (Fang et al., 2004). The peritoneal mesothelium is composed by a single polygonal cell layer with apical microvili, which are widening the transperitoneal exchange surface (Dobbie 2000). In inflammation, the fibrinolytic activity of the peritoneal mesothelium is reduced while procoagulating activity is enhanced; thus, a fibrin pellicle is formed on the mesothelial surface. The mesothelial basement membrane includes collagen fibers and acts as a selective barrier for cells; it is also involved in mesothelial cells regeneration (Dobbie 1993).

Peritoneum functions as a semipermeable imperfect and selective dialysis membrane for water and solutes between endoperitoneal and blood compartments and its usage as support for long-term PD are progressively impaired by the association of three elements: (1) chronic placement of the dialysis catheter; (2) usage of hypertonic incompatible dialyzing solutions; (3) severe acute or recurrent episodes of infectious peritonitis.

Peritoneal dialysis is used for the treatment of chronic renal failure. Morphological changes of peritoneum due to the process of long-term dialysis involve the mesothelium, the intercellular junctions, the interstitial tissue and blood vessels. Morphological changes of peritoneum are also found in complications of chronic PD (Grzybowski et al., 1997). In such situations, the permeability of peritoneum is modified and the lymphatic drainage is enhanced (Grzybowski et al., 1997). PD can modify peritoneal morphology and structure and the progressive alterations may lead to peritoneal failure (Fang et al., 2004). On other hand, the morphological alterations of peritoneum impair the accuracy of PD (Lambie et al., 2013; Chung et al., 2003; Jovanović et al., 2013; Devuyst et al., 2002; Grzegorzewska 2006).

The aim of this retrospective study was to evaluate morphological changes of peritoneum in chronic peritoneal dialysis (PD) at 4, 8, 12, and 14 years.

Personal contribution – published paper:

Țăranu T, Florea L, Păduraru D, Georgescu ȘO, Frâncu LL, **Stan CI**. Morphological changes of the peritoneal membrane in patients with long-term dialysis. Rom J Morphol Embryol 2014; 55(3): 927-932.

1.4.2. Material and methods

The present retrospective study assessed the morphological changes of peritoneum in 110 patients (58 females/ 52.72% and 52 males/47.27%) with terminal renal chronic failure enrolled for PD procedures (lasting from one to 168 months) in the Department of Nephrology of "Dr. C. I. Parhon" Hospital, Iași, Romania. Patients were hospitalized in the

II[nd] Surgical Clinic of "Sf. Spiridon" Hospital, Iași, between 1.01.2003–31.12.2010 for catheter removal due to various causes: (1) abdominal parietal sepsis on the output catheter site (recurrent episode, with/ without infectious peritonitis) in eight (7.27%) patients, five females and three males; (2) severe iterative microbial peritonitis, non-responsive to conservative treatment, in 46 (41.81%) patients, 25 females and 21 males; (3) dialysis inefficiency, in 26 (23.6%) patients, 14 females and 12 males; (4) association of the two later-discussed pathologies, in 10 (9%) patients, six females and four males; (5) patient request, in six (5.45%) cases, two females and four males; (6) prior to renal transplantation, in eight (7.27%) patients, two females and six males; (7) bowel occlusion/subocclusion by adherential pathology, in six (5.45%) patients, four females and two males.

Surgical exploration of the peritoneal cavity performed a macroscopic evaluation of the mesothelial serosa and of peritoneal filling, if present.

Intraoperative biopsies were sampled usually from the antero-lateral parietal peritoneum (8–12×8–12 mm sized samples); there were also samples harvested from the visceral peritoneum (segmentary enterectomy samples), fibrous enterolysis samples and samples from the resected viscero-parietal bridles. Samples were divided, according to PD duration, in four study groups and assessed (**Table 1.1**).

From the 290 episodes of infectious peritonitis in the patients from Department of Nephrology involved in the PD program (for the considered duration), 46 required surgery. Maximal number of peritoneal infectious episodes, correlated with a functional PD catheter ranged between 5–11/patient in Group A, 9/patient in Group B, 6/patient in Group C and none in the last group (ultrafiltration impairment – UF).

Table 1.1. Peritoneal biopsies distribution in patients with terminal stage renal failure according to peritoneal dialysis duration

Group	PD duration [months]	No. of patients			%		
		Total	F	M	Total	F	M
A	1-48	92	48	44	83.63	43.63	40.00
B	49-96	12	7	5	10.90	6.36	4.55
C	97-144	5	2	3	4.54	1.81	2.72
D	145-168	1	1	-	0.90	0.90	-

PD - Peritoneal dialysis; F – Females; M – Males

Peritoneal and fibrous samples were paraffin embedded, cut and stained for histological examination (Hematoxylin– Eosin, trichromic Szekely, Gordon–Sweet and Van Gieson stains). Biopsy samples were histologically stereologically assessed to emphasize mesothelial cells integrity, the presence of denudation areas, submesothelial interstitial changes, the presence of acute or chronic inflammation, vascular abnormalities generated by subendothelial hyaline sclerosis in post-capillary venulae and arteriolae.

Stereological assessment

Stereological interpretation for quantitative measurements was performed on representative microscopic samples from biopsies in the four groups, using a 40× objective. Images were recorded by an image acquisition system and interpreted by Prodit 5.2 image

analysis software. Thus, we were able to perform specific measurements by selecting an automated method.

Stereology option was used to evaluate percentage volumes (%) for structural elements in the peritoneal samples (mesothelium, connective tissue, blood vessel lumen, inflammatory lesion) in patients with different PD duration (at four years/48 months interval) by comparison with normal peritoneum. This method combines test-dots and test-lines in a standard surface, allowing the calculation of the selected percentage volumes.

Digital superposition of a standard grid over the acquired histological image is performed. The grid was adapted by the observer to the specific tissue parameters, in order to produce optimal quantification conditions.

A 40× objective together with the Weibel parallels test grid was used; the distance between two points was of 19.39 μm. For each sample, the test grid was displaced by the observer starting from mesothelium toward lower limit, and then reversely in the near area until reaching a test surface of 528 points.

Statistical evaluation reported percentage volumes for reference elements in each sample, then on study groups, while statistically important data were depicted in specific diagrams.

1.4.3. Results

The capillary endothelium can include pores among neighbor endothelial cells (**Figure 1.23**). As referred to peritoneal dialysis (PD), the vascular endothelium corresponds to the blood compartment, in the dialyser and to the lymphatic compartment. Capillary and postcapillary permeability is controlled by the endothelium.

At surgical inspection of the peritoneal cavity in patients with infectious peritonitis, peritoneal fluid was purulent in 50% cases. Parietal and visceral peritoneum in patients with PD over five years showed a tanned aspect with hyperpigmented areas (of 1–2 cm) and hard-bound on palpation (**Figure 1.24**).

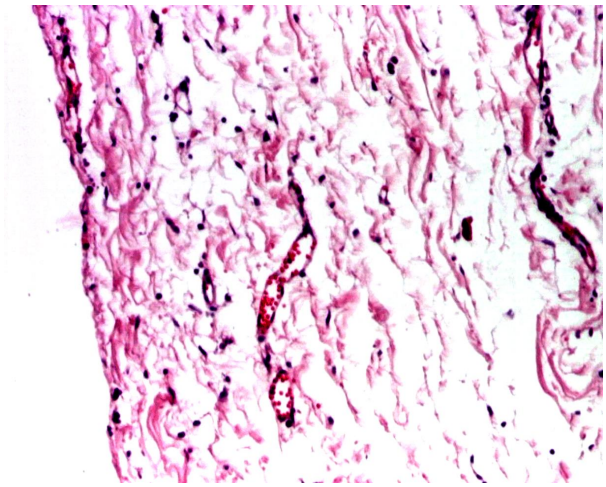

Figure 1.23. Normal peritoneum (with a single layer
of mesothelial cells, stromal and permissive vascular compartments). HE staining, ×100.

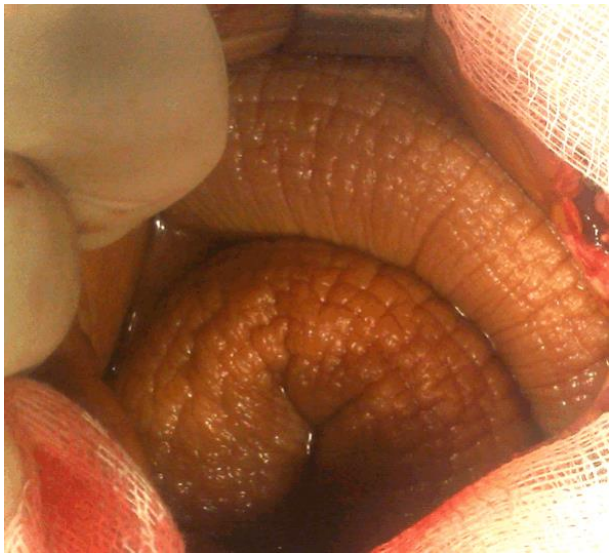

Figure 1.24. Tanned and hardbound peritoneum. Macroscopic aspect.

Proximal to distal investigation of the peritoneal barrier (from the interface of peritoneal areas artificially induced by PD toward endo- capillary space) showed the following changes, according to histological and stereological results: (1) mesothelial denudation was present in a percentage volume of 5.49% for patients in Group A, 16.10% in Group B, 16.68% in Group C, and 19.88% in Group D (**Figures 1.25–1.27**); (2) reduplication

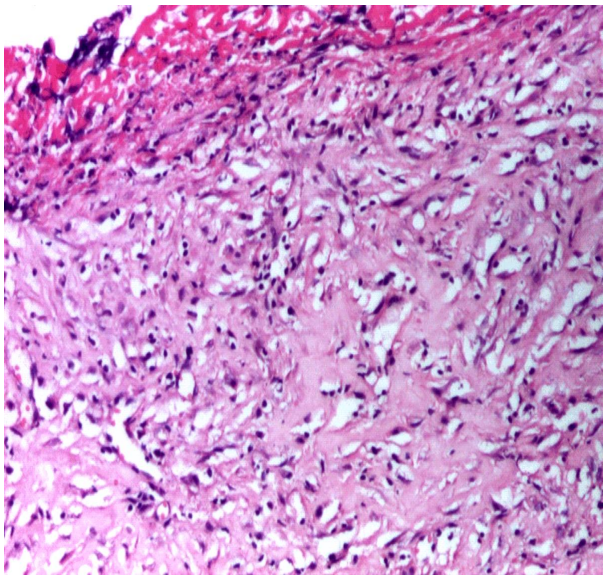

Figure 1.25. Mesothelial denudation, fibrin deposits and stromal inflammatory infiltrate (five years of PD). HE staining, ×100.

of the basement membranes (evaluated histologically) was recorded on the 11 patient samples, which undergone over five years (60 months) of PD (61.11%); (3) submesothelial interstitial stromal fibrosis was quantitatively evaluated in progressive percentage volumes, from 25.49% in Group A to 26.10% in Group B, 35.85% in Group C, and 56.63% in the patient with 14 years of PD (**Figures 1.28–1.30**);

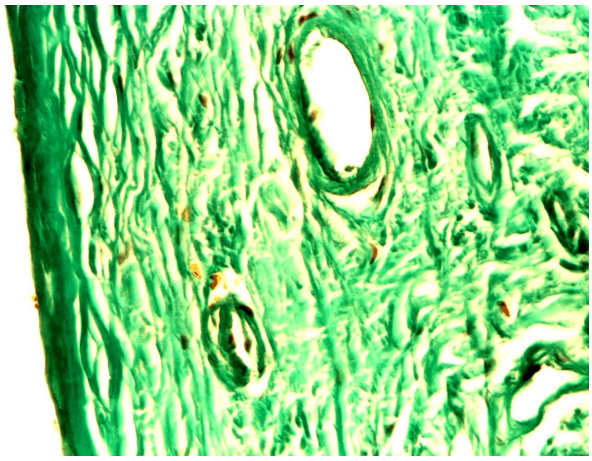

Figure 1.26. Fibrous mesothelial exfoliation, starting sub- endothelial hyalinization (four years of PD). Trichromic Szekely staining, ×200.

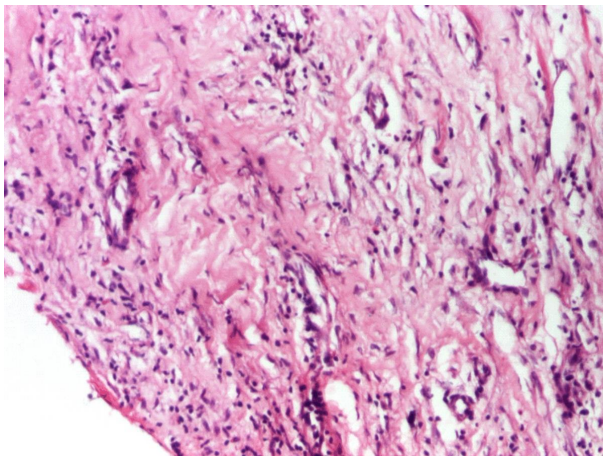

Figure 1.27. Infectious peritonitis (discontinuous meso- thelial layer and diffuse polymorphous inflammatory infiltrate, at three years of PD). HE staining, ×100.

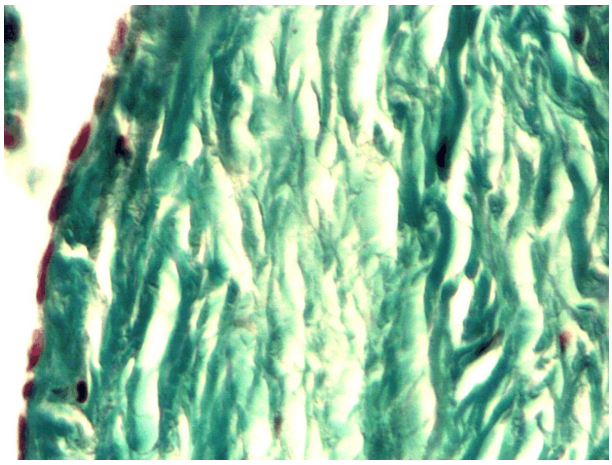

Figure 1.28. Peritoneum with a continuous layer of mesothelial cells and subsequent “band” fibrosis (at four years of PD). Trichromic Szekely staining, ×400.

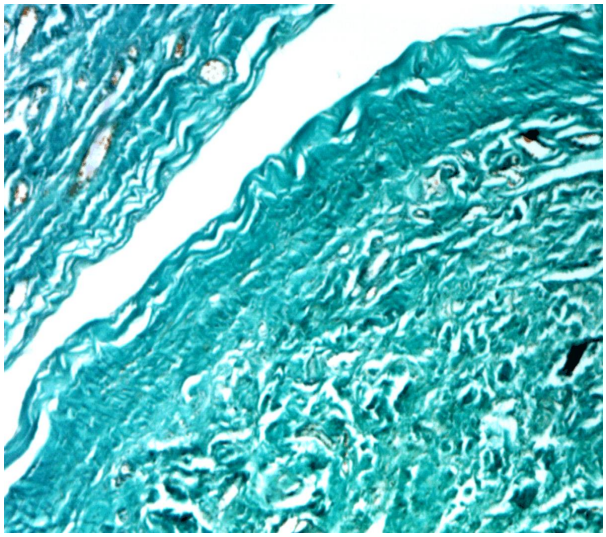

Figure 1.29. Stromal fibrosis, after eight years of PD. Trichromic Szekely staining, ×40.

(4) fibrin presence was noticed in percentage volumes of 3.03% (Group A), 26.22% (Group B), 29.77% (Group C), and 30.06% (Group D) (**Figure 1.31);** (5) hyalinizing vasculopathy by subendothelial hyaline deposits was observed in increasing percentage volumes from 2.22% to 6.63%, 9.16%, and 9.20% (**Figure 1.32**); (6) together with progressive subendothelial vascular hyalinosclerosis, it was noticed a vascular lumen distortion with different stenosis degrees up to complete vascular obliteration. Thus, vascular permeability was progressively reduced in percentage volumes from 22.59% in Group A to 12.81% in Group B, 7.77% in Group C, and 7.37% regarding the peritoneum with 14 years of PD exposure (**Figure 1.33**); (7) perivascular inflammatory process was more important in patients in the Group A (4.55%), with progressively decreasing percentage volumes, consistent with PD duration, of 2.98%, 1.87% and 1.33% in the remaining patient groups; (8) calcification occur rence (mainly in the hyalinized vessel walls) was observed 1.63% up to eight years, 3.74% up to 12 years and 4.03% following five years of PD (with percentage volumes of at 14 years of PD) (**Table 1.2**, **Figure 1.34**).

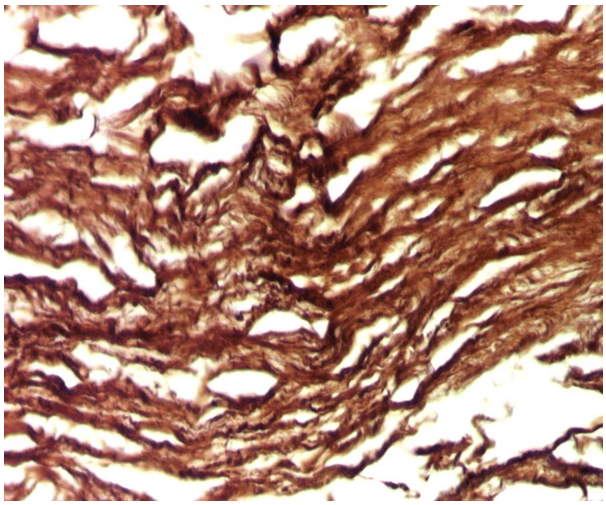

Figure 1.30. Fibrous peritoneum with submesothelial elastic layer. Gordon–Sweet staining, ×200.

Table 1.2. *Evaluation of the percentage volumes regarding morphological peritoneal changes*

G	PDD	Lesion type (percentage volume)						
		MD	***STF***	***FD***	***SVH***	***VP***	***PI***	***Ca***
A	0-4	5.49	25.49	3.03	2.22	22.59	4.55	-
B	5-8	16.10	26.10	26.22	6.63	12.81	2.98	1.63
C	9-12	16.68	35.85	29.77	9.16	7.77	1.87	3.74
D	13-14	19.89	56.63	30.06	9.20	7.37	1.33	4.03

G - Group; PDD – peritoneal dyalisis duration - years; MD – Mesothelial denudation; STF – Submesothelial stromal fibrosis; FD – Fibrin deposits; SVH – Subendothelial vascular hyalinosis; VP – Vascular permeability; PI - Perivascular inflammation; Ca – Calcification.

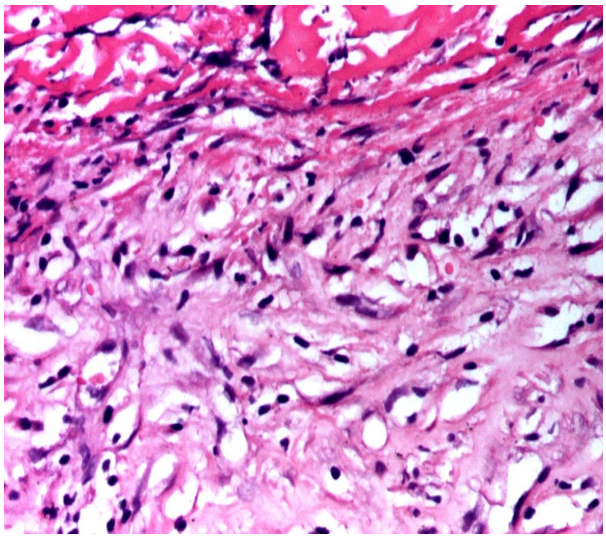

Figure 1.31. Fibrous peritonitis, with apical location. HE staining, ×200.

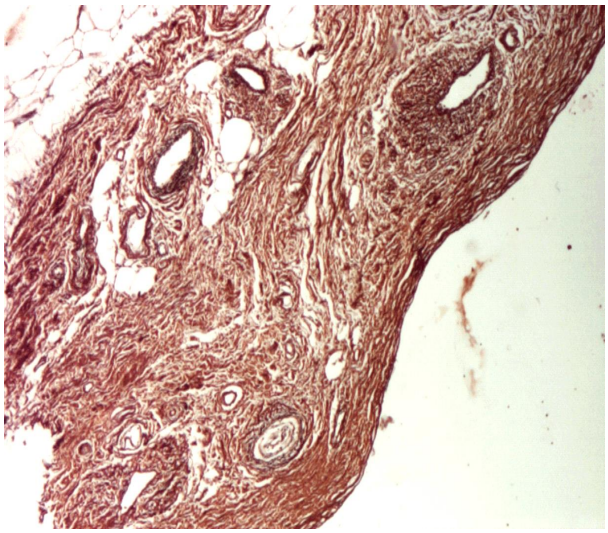

Figure 1.32. Vascular wall thickening after five years of PD. Gordon–Sweet staining, ×40.

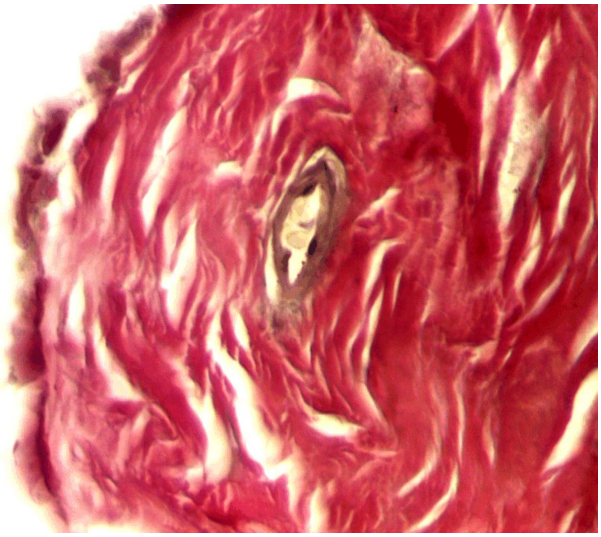

Figure 1.33. Hyalinization of a capillary with total obstruction (14 years of PD). Van Gieson staining, ×400.

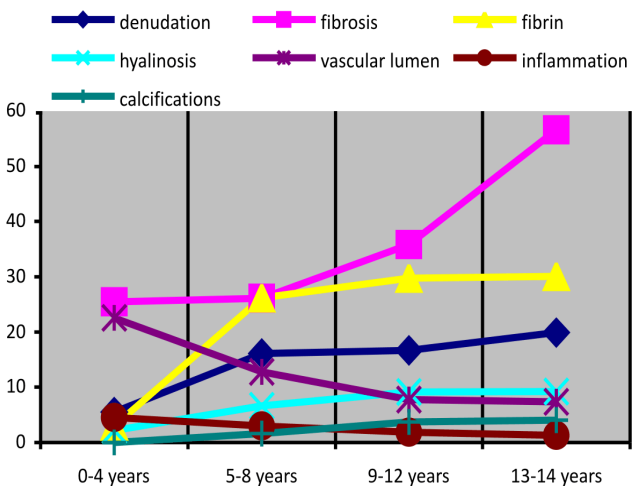

Figure 1.34. Graphic expression of peritoneal morphological changes.

1.4.4. Discussion

Technical innovations in peritoneal dialysis, now used widely for the long-term treatment of end stage renal disease (ESRD), have significantly reduced therapy-related complications, allowing patients to be maintained on PD for longer periods.

The first attempt to use the human peritoneum to dialyze uremic retention solutes was made almost 100 years ago. Over the next five decades, the therapy gradually evolved with an expansion in our understanding of solute and water kinetics that allowed for successful application of this mode of dialysis to AKI and ESRD (Mehrotra et al., 2016; Wear et al., 1938; Boen et al. 1962).

Tanned and hardbound peritoneal aspect was observed in patients with PD over five years (in which progressive fibrosis evaluated by stereology showed percentage volumes of

26.1%, 35.85% and 56.63%. Brown plates (of 1–2 cm) were found mainly in patients with peritoneal adhesions disease or with recurrent infectious peritonitis, which became surgical situations. The tanned aspect of the peritoneal surface and the presence of dispersed brown hardbound plates were previously observed in patients with PD, with starting fibrosis, confluence trend, hyperpigmentation all associated to extensive fibrosis in encapsulated peritoneal sclerosis. PD is deteriorating the functional morphology of the peritoneal serosa.

Stereological analysis performed in the present study emphasized progressive mesothelial exfoliation by percentage volumes from 5.49% (Group A) to 16.1% (Group B), 16.68% (Group C), and 19.88% in the patients with a 14 years PD (with no episode of microbial inflam- matory peritonitis). During histological evaluation, reduplication of the mesothelial basement membrane was assessed in 11 patients with PD over 60 months (a total of 10% and 61.11% in patients with PD from 5–14 years).

In chronic PD patients was found that besides mesothelial denudation, mesothelial aquaporines' activity is lost, with decreased CA125 levels as marker of active mesothelial cells in these patients; in chronic PD the ultrafiltration ability of the peritoneal membrane is arrested (Schoenicke et al., 2004).

Peritoneal inflammation in chronic PD induces a scar-forming healing process, which involves the submesothelial fibroblasts. Activation and proliferation of the peritoneal fibroblasts generates interstitial stromal fibrosis. The key mediator for the peritoneal fibrosis is TGFβ, which is produced by the inflammatory cells and inhibits the apoptosis of fibroblasts (Jiménez-Heffernan et al., 2004; Aroeira et al., 2005).

An intermediate, myoid/fibroblastic, phenotype of the stromal cells is absent in normal and non-PD patients but it shows a maximal density (myofibroblastic conversion) beneath the mesothelial layer in chronic PD patients with peritoneal impairment (Kawakita et al., 2005; Kang et al., 1999; De Vriese et al., 2001; Krediet et al., 2000; Kim 2009). In the presence of the inflammatory infiltrate, the stromal cells of the peritoneum are involved in progressive fibrosis, in modulating the inflammatory response, in tissue contraction, and in angiogenesis and vasculopathy repeated episodes of peritonitis or hemoperitoneum may accelerate these processes, which, ultimately, lead to ultrafiltration failure.

Submesothelial myofibroblasts originating from mesothelial cells through epithelial–mesenchymal transition (EMT) and from resident fibroblasts are involved in inflammatory responses, extracellular matrix accumulation, and angiogenesis. The mesenchymal phenotype of trans- differentiated mesothelial cells may retain a permanent mesenchymal phenotype as long as initiating stimuli persist, and contribute to PD-induced fibrosis and angiogenesis. Emerging evidence point to the peritoneal microvasculature as the main factor responsible for increased solute transport and ultrafiltration failure although the pathophysiology of peritoneal fibrosis and angiogenesis remains elusive. Experiments should find solutions to block, or reverse the EMT of mesothelial cells in order to prevent the fibrotic and angiogenetic mechanisms of ultrafiltration failure (López-Cabrera et al., 2006).

An autopsy study identified the correlation between the subendothelial hyalinization, the submesothelial fibrosis and the ultrafiltration decrease or failure, by severe impairment of the peritoneal membrane function after 3–4 years of PD (Jiménez-Heffernan et al., 2008).

Vascular subendothelial progressive hyaline sclerosis and stenosis up to occlusive lumen obliteration were identified in progressive percentage values from 2.22% to 6.63%,

9.16%, and 9.29% in our patient groups. At the same time, we noticed a reduction in permeability for the vascular lumen, updated to a complete obliteration and expressed in percentage volumes from 22.59% to 12.81%, 7.77%, and 7.37%. Our data are consistent with the observations of Devuyst and De Vriese who observed an increased vasculopathy incidence with PD duration and the degree of peritoneal fibrosis in patients with classic PD (Devuyst et al., 2002; De Vriese et al., 2001).

Janda and Nakazato demonstrated that osteopontin, which is also expressed in PD patients actively participates to the dystrophic calcification of the peritoneum (Janda et al., 2013; Nakazato et al., 2002). In this study, calcifications mostly occurred in the walls of hyalinized vessels and were observed at 5–8 years of PD, with percentage volumes of 1.63%, 3.74%, and 4.03%. Perivascular inflammation recorded decreasing values at five years of PD from 4.55% to 2.98%, 1.87%, and 1.33%.

In order to limit or to delay the pathological changes of peritoneum and to extend the functionality of the peritoneal membrane, several approaches could be considered:

- *glucose replacement with other biocompatible osmotic agents;*
- *protocol changes of the sterilization process and lactate buffer replacement with bicarbonate;*
- *limitation of microbial inflammatory episodes in the peritoneum;*
- *reduction of hypertonic dialyzing solution usage during infectious peritonitis episodes (to reduce peritoneal stress, to quicken mesothelial reepithelialisation, and to limit progressive fibrosis, encapsulated peritoneal sclerosis, angiogenesis and ultrafiltration failure);*
- recombined human erythropoietin treatment to prevent (Vorobiov et al., 2008) the dialysis fluid-induced apoptosis of mesothelial cells.

Peritonitis continues to be a major cause of morbidity and mortality in PD patients globally. Depending on the underlying causative organism, PD-related peritonitis is complicated by relapse in 3%–20% (14% overall), catheter removal in 10%–88% (22% overall), permanent HD transfer in 9%–74% (18% overall), and death in 0.9%–8.6% (2%–6% overall) of cases. After a single episode of peritonitis, the risks of death due to infection, cardiovascular disease, and dialysis withdrawal are markedly increased in the first month and continue to remain significantly elevated for up to 6 months afterward. peritonitis rates are generally improving globally over time, there have been marked and unacceptable variations in peritonitis rates and outcomes between centers in many countries (Mehrotra et al., 2016).

1.4.5. Final remarks

Histological and stereological data quantification illustrates structural and functional alteration of the dialysis membrane in long-term PD. These should be further evaluated with specific biomarkers in order to adequately assess the role of EMT and angiogenesis in chronic PD. Peritoneal dialysis is the therapeutic proce- dure in terminal stage chronic renal failure and may induce by itself or in association with peritoneal inflammatory infectious episodes, at 3–4 years of treatment, a cascade of self-maintained peritoneal structural changes, supported by the chronically stressed mesothelial cells. The recorded PD efficiency depends upon a reduced number of infectious peritonitis episodes.

1.5. Perspectives in clinical applied embryology

1.5.1. Introduction

Interstitial cells of Cajal (ICCs) are resident cells of smooth muscle organs, in which they perform various functions (Rumessen and Thuneberg, 1982; Rumessen et al., 1982; Faussone-Pellegrini and Cortesini, 1985; Thuneberg and Peters, 2001; Timmermans, 2001; Ward and Sanders, 2001; Sanders, 2006), such as pacemaker, mediator of neurotransmission and mechanosensory roles (Faussone-Pellegrini et al., 1990; Faussone- Pellegrini and Thuneberg, 1999; Timmermans, 2001; Huizinga and Faussone-Pellegrini, 2005a).

CD117/c-kit is a transmembrane receptor tyrosine pro- tein kinase (Young, 1999). CD117/c-kit antibodies reli-ably label ICCs (Young, 1999; Wu et al., 2000; Poole et al., 2004; Ciontea et al., 2005; Huizinga and Faussone-Pellegrini, 2005b; Popescu et al., 2005; Huang et al., 2009; Suciu et al., 2010; Rusu et al., 2011a,b). Networks built-up by ICCs are widely distributed within the intermuscular (ICC-MY), intramuscular (ICC-IM, ICC-DMP), and submucosal (ICC-SM) layers from the esophagus to the internal anal sphincter (Takaki, 2003). Recently, a novel cell type was described: the telocyte (Popescu and Faussone-Pellegrini, 2010).

It was discussed, on a rat experimental model, that in transmis- sion electron microscopy the standards for differentiating ICCs from telocytes are quite similar, and the peculiar morphologies of telocytes, the telopodes (long, slender, and moniliform cell processes), should make in transmission electron microscopy the difference; moreover, the structural association with smooth muscle cells should direct the diagnosis toward ICCs (Rusu et al., 2012). Anoctamin 1 has also been described as a specific marker of the ICCs (Hwang et al., 2009; Takaki et al., 2010; Chen et al., 2011; Sanders et al., 2012). In mice embryos, c-kit positive ICCs were also anoctamin 1 posi- tive (He et al., 2012).

The anoctamin-1 (ANO1, commonly known as DOG1) gene expression was found to be differentially expressed in gastrointestinal tumors, as compared with other mesenchymal tumors. It has also been demonstrated that ANO1 may be expressed in esophageal and head and neck squamous cell carcinoma (West et al., 2004; Carles et al., 2006; Miettinen et al., 2009; Lee et al., 2010).

It was shown that neural crest cells (NCCs) colonize the embryonic gut from week 4 to week 7, and further coalesce to form the myenteric and submucosal plexuses. Smooth muscle differentiation follows the NCCs colonization. ICCs emerge from the gut mesenchyme (Wallace and Burns, 2005).

With regards to the esophageal ICCs morphogenesis in human, to our knowledge, only one study specifically deals with this topic (Radenkovic et al., 2010). In that study, antibodies against c-kit, neuron specific enolase, smooth muscle actin, and desmin were used. The results revealed a continuous layer of esophageal c-kit positive cells surrounding by week 7 the elements of the myen- teric plexus (Radenkovic et al., 2010).

It was thus hypothesized that in late stage human embryos anoctamin 1 may also be a key marker of the esophageal ICCs, thus contributing to the pacemaker machinery. Therefore, an immunohistochemical study was performed to test that hypothesis.

Additional markers were designed to evaluate the esophageal structure in late stage embryos.

Personal contribution – published paper:

Rusu MC, Poalelungi CV, Vrapciu AD, Păduraru L, Didilescu AD, **Stan CI**. Anoctamin 1 positive esophageal interstitial cajal cells in late stage human embryos. THE ANATOMICAL RECORD 2014; 297:301–307.

1.5.2. Material and method

Five human embryos resulted from legal abortions were collected immediately postabortion. The lengths of these embryos varied between 23 and 29 mm, thus corresponding to late embryo stages (54–56 days) (Sadler and Langman, 2009). Approval for the present study was granted by the Bioethics Committee of the "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania.

Samples were fixed for 24 h in buffered formalin (8%) and were processed with an automatic histoprocessor (Diapath, Martinengo, BG, Italy) with paraffin embed- ding. Sections were cut manually at 3 lm, and were mounted on SuperFrostVR electrostatic slides for immunohistochemistry (Thermo Scientific, Menzel-Gla€ser, Braunschweig, Germany). Histological evaluations used 3-lm thick sections stained with hematoxylin and eosin.

Primary antibodies for CD117/c-kit (clone T595, Novocastra-Leica, Leica Biosystems Newcastle, Newcas- tle Upon Tyne, UK, 1:20), DOG1 (clone K9, Novocastra-Leica, Leica Biosystems Newcastle, Newcastle Upon Tyne, UK, 1:100), CD34 (clone QBEnd 10, Dako, Glostrup Denmark, 1:50), nestin (clone 10c2, Santa Cruz Biotechnology, Santa Cruz, CA, 1:500), a-smooth muscle actin (a-SMA) (clone 1A4, Dako, Glostrup, Denmark, 1:50), desmin (clone D33, Biocare Medical PM 036 AA, Biocare Medical, Concord, CA, 1:100) and CD31 (clone JC70A, Dako, Glostrup Denmark, 1:50) were used.

Sections were deparaffinized, rehydrated, and rinsed in PBS buffer solution at pH 7.4 (for the nestin, CD34, CD31, a-SMA and desmin antibodies) and in TBS buffer solution at pH 7.6 (for the CD117/c-kit and DOG1 antibodies). Retrieval by incubation in specific buffer was completed as follows: (a) for CD34: EDTA, pH 9; (b) for the other antibodies: 0.01 M citrate retrieval solution, pH 6. The standard ABC technique used a DAB protocol. Appropriate blocking of endogenous peroxidase was completed before immunolabeling (Peroxidazed 1, Bio-care Medical, Concord, CA). Sections incubated with nonimmune serum served as negative controls. Sections were counterstained with Hematoxylin.

The microscopic slides were analyzed and micrographs were taken and scaled using a Zeiss working station, as previously described (Rusu, 2013).

1.5.3. Results

In the late embryo stage, the lower esophagus presented a mucosa comprising a pluristratified epithelium and lamina propria, submucosa, and muscularis consisting, in turn,

of an inner circular layer and an outer longitudinal layer. The longitudinal and circular muscle layers were positively labeled by a-SMA and desmin antibodies (**Figure 1.35**) and were separated by a distinctive layer corresponding to the myenteric plexus. Moreover, a-SMA consistent positive labeling was associated with the lamina propria and the adjacent inner part of the submucosa; a discrete desmin-positive phenotype was corresponding to the strong a-SMA phenotype in this location (**Figure 1.35**). In submucosa, a-SMA seemingly labeled also microvessels; however doubts were kept on this diagnosis.

With CD31 antibodies (**Figure 1.36**) endothelia were positively labeled: a distinctive microvascular layer was demonstrated in the inner part of the submucosa.

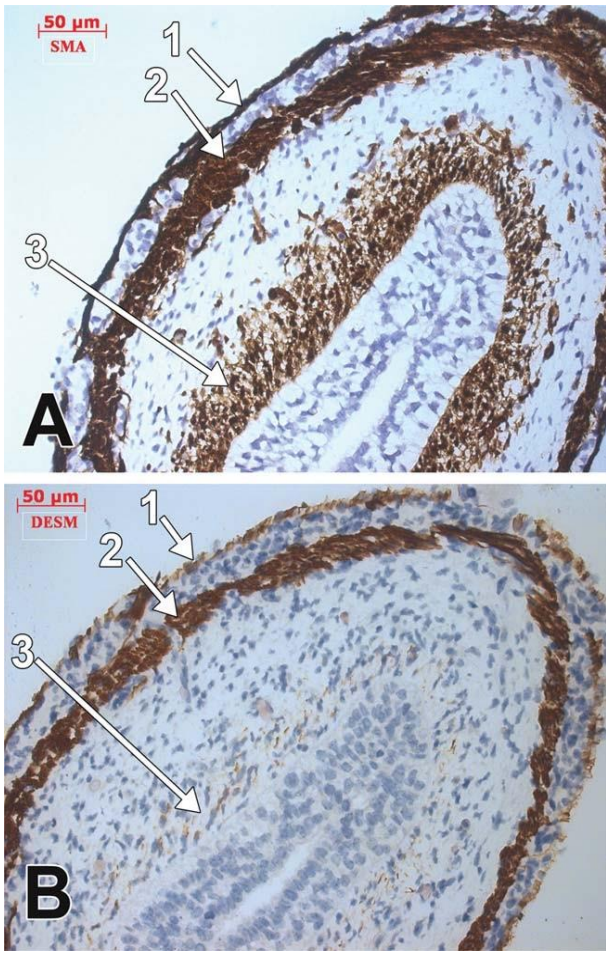

Figure 1.35. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with a-SMA (A) and desmin (B) antibodies. 1. longitudinal muscle layer; 2. circular muscle layer; 3. lamina propria.

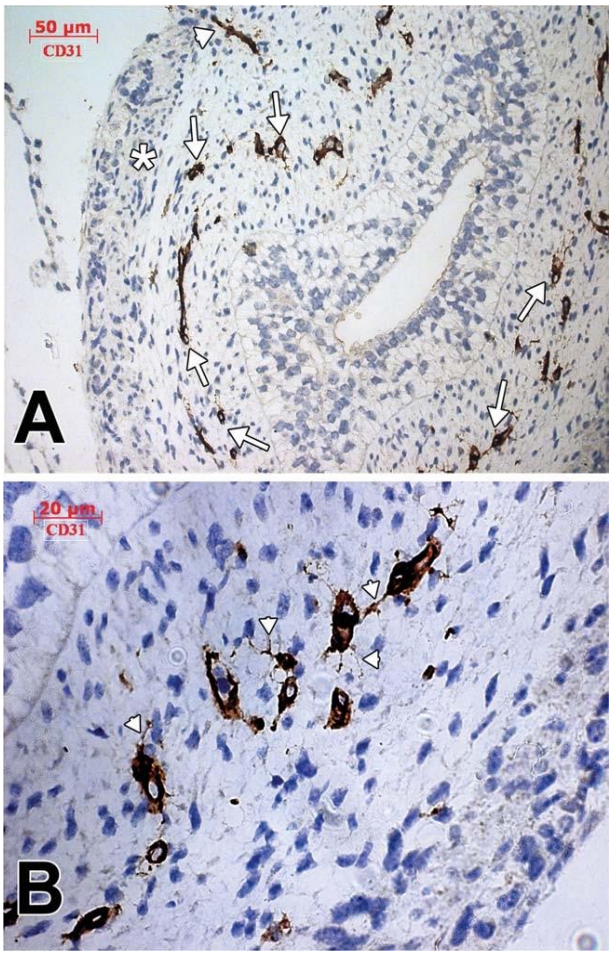

Figure 1.36. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD31 antibodies. A. The microvascular layer identified with CD34 antibodies between the lamina propria and the submucosa is positively labeled with CD31 antibodies (arrows). A penetrating microvessel is indicated (arrowhead). The circular muscle layer (*) lacks immune positive elements. B. ETCs label with CD31 antibodies and project into stroma moniliform dichotomizing processes (arrowheads).

Penetrating dichotomizing vessels were also identified. Endothelial cells projecting in the adjacent stroma thin moniliform processes were identified as endothelial tip cells (ETCs). The microvascular layer identified with CD31 antibodies was also CD34-positive; ETCs were also positively labeled by CD34 (**Figure 1.37**). However, labeling with CD34 antibodies identified an outer positive network, mostly corresponding to the circular muscle layer (**Figure 1.37**). Since in this location CD31 did not label any element, the network has been considered mesenchymal. Nestin-positive immune labeling was associated with the microvascular layer at the lamina propria – submucosa border (**Figure 1.38**), and with the muscular coats identified with desmin and a-SMA antibodies, but with the myenteric plexus layer between the circular and longitudinal muscle layers.

CD117/c-kit positive labeling was associated mostly with the myenteric plexus layer, where ICCs with long dichotomizing processes were found (**Figure 1.39**). These c-kit positive ICCs (ICC-MY) were configuring a complete circumferential network; they were sending long processes coursing circumferentially on the outer surface of the circular muscle layer, and processes which were penetrating in this muscle layer, with a radial or oblique course. Also c-kit positive cells and processes were found within the inner part of the circular muscle layer (ICC-IM). With DOG1 antibodies, a weak positive reaction was found in the circular and longitudinal muscle layers (**Figure 1.40**). A strong DOG1 positive immune labeling was found for the ICC-MY (cell bodies and processes) applied on the outer surface of the circular muscle layer, and for ICC-IM scarcely distributed within the inner part of the circular muscle layer (**Figure 1.40**).

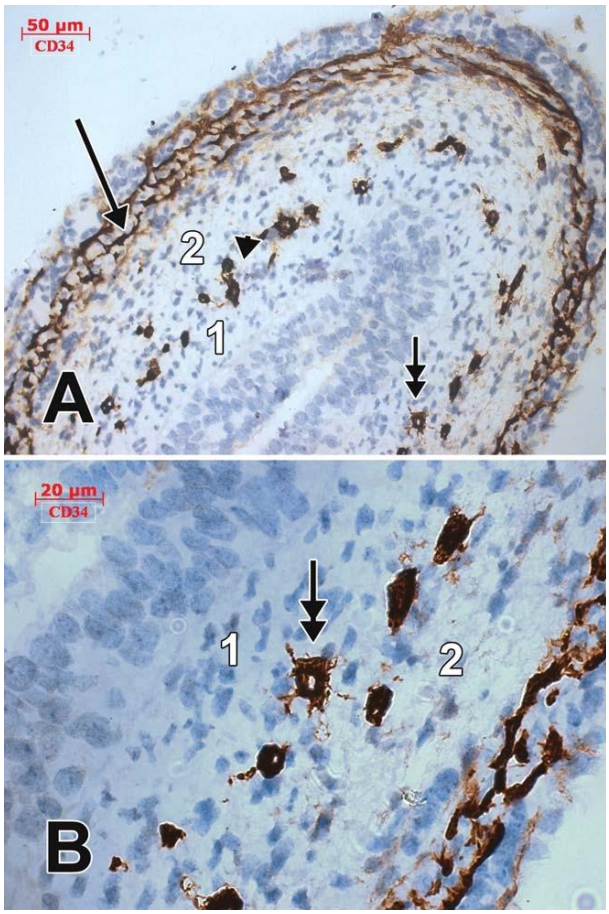

Figure 1.37. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD34 antibodies. A. A mesenchymal network is identified within the circular muscle layer (arrow). A different microvascular layer (arrowhead) lies between the lamina propria (1) and the submucosa (2); ETCs are indicated (double-headed arrow in A and B).

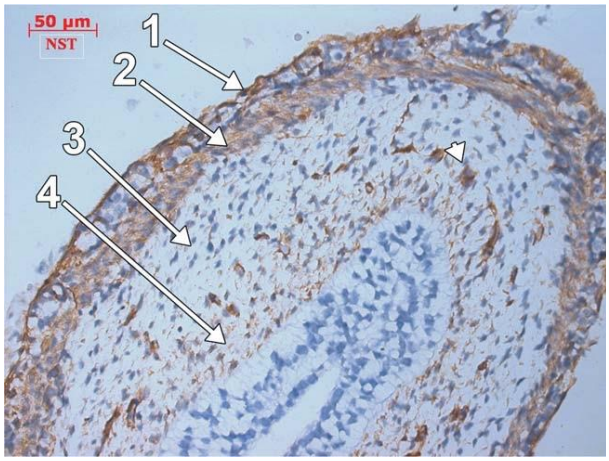

Figure 1.38. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with nestin antibodies. 1. longitudinal muscle layer; 2. circular muscle layer; 3. submucosa; 4. lamina propria. The microvascular layer at the submucosa/lamina propria border is indi- cated (arrowhead).

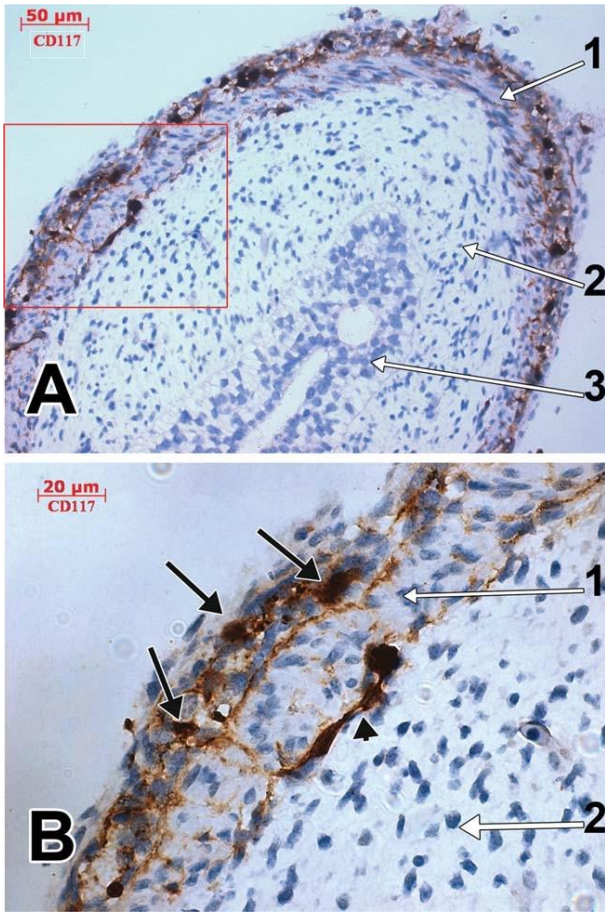

Figure 1.39. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with CD117/c-kit antibodies. Inset in A is evaluated at a higher magnification (B). CD117/c-kit positive interstitial Cajal cells correspond topographically to the myenteric plexus (B, black arrows) but also lay on the submucosal boundary of the circular layer (B, black arrowhead). 1. circular layer of tunica muscularis; 2. lamina propria; 3. epithelium.

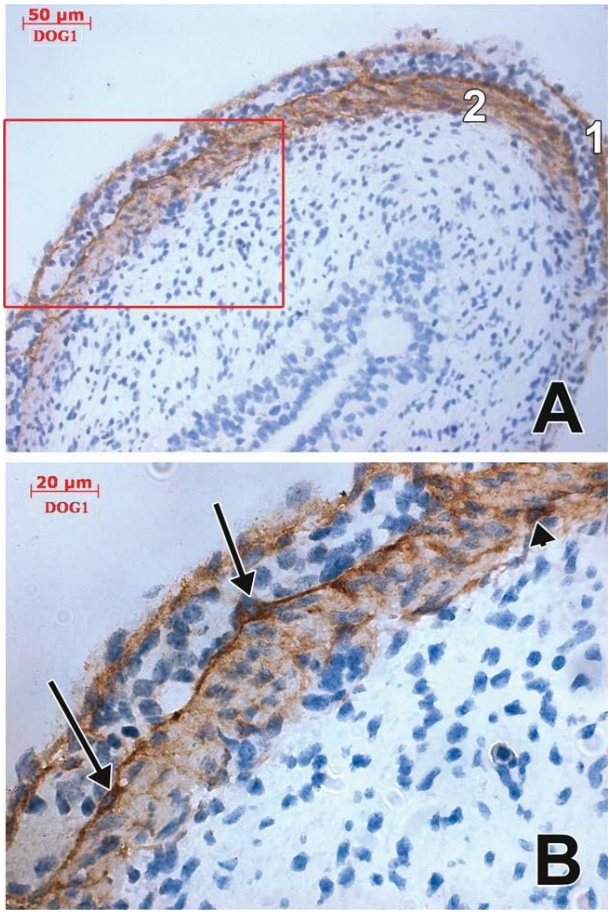

Figure 1.40. Oblique section of the esophagus wall in a 27 mm. human embryo, immune labeling with DOG1 antibodies. Inset in A is eval- uated at a higher magnification (B). 1. longitudinal layer of tunica mus- cularis; 2. circular layer of tunica muscularis. Immune positive cells are identified on the outer side of the circular muscle layer (arrows) and in the deep part of the circular muscle layer (arrowhead).

1.5.4. Discussion

In this study, evidence was brought on the esophageal microvascular developmental morphology in late stage embryos. Taking into account the CD31 and CD34 labeling, it appears that a microvascular layer, supplied by penetrating vessels, lies at the borderline between lam- ina propria and submucosa, and it is immature, as proven by the ongoing processes of sprouting angiogenesis supported by ETCs.

The layer can be important when investigations are conducted in angiogenesis dependent diseases, such as Barrett's esophagus (a premalignant condition of the lower esophagus), as it may provide further proliferating endothelial cells of new blood vessels. These cells may serve as an important source for diagnostic markers specific for the evolution of Barrett's epithelium (Auvinen et al., 2002). It is known that ETCs conduct sprouting angiogenesis (Stanescu et al., 2012; Phng et al., 2013; Rusu et al., 2013c); the ETCs processes invade the stromal compartment by a different stage migration, project filopodial, and moniliform processes, and are under the influence of the stromal cell compartment (Rusu et al., 2013a).

It was demonstrated that ETCs label with CD34 antibodies (Siemerink et al., 2012; Stanescu et al., 2012; Rusu et al., 2013a). This study shows that ETCs also label with CD31 antibodies. It is known that nestin, which is a type VI intermediate filament protein, labels various tissues during embryogenesis, such as muscle fibers, hepatocytes, and renal progenitors (Krupkova et al., 2010). Nestin also labels neural crest stem cells and endothelial cells in newly formed blood vessels (Rusu et al., 2013b). *This is consistent with our findings of nestin-positive labeling of the microvessels at the submucosa-lamina propria border. Conversely, immunoreactivity for a-SMA and nestin, found at the border of lamina propria and submucosa, may suggest the existence of an immature lamina muscularis mucosae*. Moreover, the external muscle coats nestin-positive labeling seemed not unusual, as long as transient nestin expression was found in immature esophageal smooth muscle cells in early stages of development (Su et al., 2011).

It is actually established that ICCs originate from mes- enchyme and not from the neural crest (Lecoin et al., 1996; Young, 1999; Timmermans, 2001; Wallace and Burns, 2005). ICCs and smooth muscle cells may have common precursors (Young, 1999; Timmermans, 2001). *Our results support, as second time evidence, the findings of Radenkovic et.al. (2010) who demonstrated that in late stage embryos the layer of ICC-MY is morphologically defined in esophagus. Moreover, c-kit and DOG1 labeling demonstrate that scarce intramuscular ICCs and networks are defined in this stage.* This is comparable with the ICCs topography in adult esophagus, where these can be found within the muscle bundles, a few in their proximity, but the majority are found at the periphery of the bundles (Faussone-Pellegrini and Cortesini, 1985).

It was established that ICC-MY act as primary pacemaker cells (Takaki et al., 2010; Rusu et al., 2011a), and intracellular calcium dynamics play an important role in the pacemaking (Takaki et al., 2010). Intracellular calcium oscillations periodically activate plasmalemmal channels, such as Ca^{21}-activated Cl^{2} channels (Takaki et al., 2010). Anoctamin 1 (Ano1, TMEM16A, DOG1) is a major component of the plasmalemmal Ca^{21}-activated Cl^{2}

channels (CaCCs) (Ferrera et al., 2010; Cipriani et al., 2011; Kunzelmann et al., 2011; Shimizu et al., 2013; Simon et al., 2013). *In this regard, the evidence of a sublayer of DOG1 positive ICC-MY is suggestive for a subpopulation of ICC-MY being qualified for pacemaking at this early developmental stage.*

Anoctamin-1 is a voltage-sensitive calcium-activated chloride channel, also known as transmembrane member 16A (TMEM16A) is a protein that, in humans. It is encoded by the ANO1 gene and expressed in smooth muscle and epithelial cells, especially in human interstitial cells of Cajal, throughout the gastrointestinal tract. Changes in ANO1 channel activity directly/positively correlate with ICC activity (Katoh and Katoh, 2003).

In the gastrointestinal tract, at the level of interstitial cells of Cajal, it plays an important role in epithelial chloride secretion mediating intestinal motility. Here, ANO1 some compounds like niflumic acid have been shown to block slow waves, which produce motility, in the human intestine (Kunzelmann et al., 2011).

This substance knockout mice fail to produce slow waves altogether but Carbachol has been shown to markedly activate the channel. In regard of this, the secretory diarrhea is a Carbachol overdose symptom (Sheridan et al., 2011).

On the other hand, Crofelemer, which works like an antidiarrhoeal, inhibits ANO1 channel. As a consequence to this, ANO1 activation is necessary for normal function of the ICC and its generated pacemaker activity in the smooth muscles of the intestine (Tradtrantip et al., 2009). In oesophageal squamous cell carcinoma and breast cancer progression its overexpression was reported (Britschgi et al., 2013).

Most of the recent studies are strengthened by a relatively large sample size but they do all have some limitations. On breast cancer, they show that the expression of Ano1 in progesterone-positive tumors was higher than in progesterone-negative tumors, especially in the estrogen-negative tumors, it remains to be determined whether Ano1 expression is regulated by the Estrogen/Progesterone signaling pathways (Hu et al., 2015).

There was recently reported that inhibition of histone deacetylase (HDAC) downregulated the expression of Ano1 in breast cancer cells. It has been reported that inhibition of HDAC silences progesterone-mediated signaling (Matsuba et al., 2014).

Further studies are required to identify whether progesterone signaling is involved in HDAC-mediated regulation of Ano1 expression. In addition, although the 11q13 amplification contributes to Ano1 overexpression in human breast cancer, it is unlikely the major contributor to Ano1 overexpression in progesterone -positive tumors, because the 11q13 amplification only occurs in approximately 15% of human breast cancer. However, we can not exclude the possibility that 11q13 amplification plays a role in Ano1 overexpression in progesterone -positive tumors, since 11q13 has been identified as a susceptibility locus for estrogen- and progesterone-positive breast cancer. Our finding can be strengthened by investigating the 11q13 status in the progesterone -positive cohort vs progesterone -negative cohort (Lambrechts et al., 2012).

The impaired relaxation of lower oesophageal sphincter during swallowing is an underlying characteristic of achalasia. Activation of nicotinic acetylcholine receptor (nAChR) is primarily involved in swallow-induced LES relaxation. It has been shown that interstitial cells of Cajal (ICC) play crucial roles in the regulation of gastrointestinal (GI) motility by functioning as the pacemaker of GI motility and the interface for neurotransmission to control

sensitive GI smooth muscle contractility. However, whether or not—and how—ICC are involved in swallow-induced LES relaxation has been unclear (Yoshihiro et al., 2019).

1.5.5. Final remarks

Further studies are however needed to explore the c-kit and DOG1 phenotypes dynamics during the fetal and perinatal periods, and use of double label immunohisto-chemistry could add useful information.

CHAPTER 2. THE PHOENIX OF BONE RESTORATION - FROM PATHOLOGY TO ANATOMY

2.1. State of the Art

Anatomy is the oldest scientific discipline of medicine. The first documented scientific dissections on the human body are carried out as early as the third century BC in Alexandria.

At that time, anatomists explore anatomy through dissections of animals, primarily pigs and monkeys (Bishop, 1995).

In 1559, Niccolo Massa wrote in his *Anatomiae Libri Introductorius* from 1536 that "it is very clearly apparent from the admonitions of Galen how great is the usefulness of a knowledge of the bones, since the bones are the foundation of the rest of the parts of the body and all the members rest upon them and are supported, as proceeding from a primary base. Thus if any one is ignorant of the structure of the bones it follows necessarily that he will be ignorant of very many other things along with them" (Palmer, 1981).

Practicioners from antiquity through the Renaissance discribed the form and function of the skeleton, as the hardest part of the body. Galen was the one who started the investigations of the skeleton following a certain pattern. He was primarily impressed with the hardness of the bone and saw its necessity for the structural integrity of the body. Than, he observed that "to protect the system completely, it was better for it to consist of many bones, and further, of bones just as hard as they are. Nature consequently did not merely entrust its defense to the skin, as she did for the parts in the abdomen, but first, before the skin was put on, she invested it with bone like a helmet" (Brain, 1986).

Generations after Galen relied heavily upon his knowledgement. Arround 1620, the Scottish physician John Moir could lecture to his students: "Bone is generated out of semen, fat and earth by the power of heat and the innate spirit".

In the eleventh century, persian polymath Ibn Sina (Avicenna) offered a humoral explanation of bone as primarily made of earth, based on the fact that bones were cold and dry, like the earth itself - "The bone is however moister than hair, because bone is derived

from the blood, and its fume is dry, so that it dries up the humors naturally located in the bones. This accounts for the fact that many animals thrive on bones, whereas no animal thrives on hair or at least it would be a very exceptional thing if hair ever did provide nourishment. The proof that bone is moister than hair is that when equal weights of bones and hair are distilled in a retort, more water and oil will flow and less "faex" will remain" (Strathern, 2005).

Avicenna also idea as a practical advice that the best way to gain knowledge of the skeleton was to see it separated from the rest of the body, became common practice in the Renaissance.

On the whole, however, medieval and early Renaissance anatomists had less to say about the skeleton than many other parts of the body beacuse it seemed deceptively simple and self-evident in ways that less visible structures did not.

There was not primarily learned physicians who were interested in the skeleton but surgeons and bone-setters - less learned practitioners who dealt directly with the ordinary and extraordinary health problems associated with broken bones (Levine, 2013).

Jacopo Berengario da Carpi addresses the problem by including all possible names at the end of the century: Greek, Arabic and Latin - "this is properly called the hand because from this part almost all handicrafts emanate. Between this and the second part is a juncture composed of many bones called in Arabic raseta and ascam and in Greek carpus." Courageously, Berengario wrote that despite many attempts he was unable to detect the famous rete mirabile in humans. He also noted that the nerves linked to the brain are solid structures, not hollow tubes, as advocated by Galen. His conclusions were based on a systematic dissection method that he called anatomia sensi (Parent, 2019).

Terminology was not the only problem facing Renaissance anatomists as they discovered that their descriptions diverged considerably from Galen's, because he had frequently taken the similarity between human and animal anatomy to be an exact correspondence. For example, Alessandro Achillini stated in 1520 that "in the larger hand there are thirty bones. There would be thirty-one if the ninth of Galen was included, but that, however, is a monkey bone" (Matsen, 1968).

Andreas Vesalius published in his masterpiece - On the Fabric of the Human Body - he could cite numerous errors of Galen in the number and shape of the bones, though he, too, continued to identify many animal parts as belonging to humans. Leonardo played with the confusion between human and animal anatomy by drawing a fanciful foot of a beat based on a human one an interesting reversal of the common trend (Margócsy, 2018).

During Renaissance anatomy theaters were filled with articulated skeletons, such as the one Vesalius prepared in 1546 and can still be seen today at the University of Basel. It was known by that time that bones had differing degrees of density, flexibility and motility.

Even though many medical practitioners did not puzzle over the skeleton to the same degree that they did with other parts of the body, they all recognized their cultural as well as scientific importance. Since the end of the sixteenth century, skeletons had become the quintessential image of anatomy but they also continued to be an image of death, in the form of a grim reaper brought to life by the skill of the anatomist (Boas, 1970).

The modern term orthopedics occured in the 1700s but orthopedic principles were beginning to be developed and used during primitive times. The Egyptians described ways to

recognize and manage common orthopedic conditions and the Greeks and Romans subsequently began to study medicine in a systematic manner. By the time, each of them greatly improved our understanding of orthopedic anatomy and surgical technique. During Middle Ages little progresses have been done. A much faster advancement was noted during the Renaissance, including the description of various injuries, improvements in surgical technique, and development of orthopedic hospitals (Swarup and O'Donnell, 2016).

Nowadays, orthopedic surgery is a rapidly developing field that has benefited from the works of numerous scholars and surgeons. This regards a better control of postoperative infections, introduction of novel technology, invention of x-ray in 1895 by Wilhelm Conrad Röntgen (Zimmerman and Veith, 1993).

In 1942, Austin Moore performed the first metal hip arthroplasty, subsequently advanced by the work of Sir John Charnley in the 1960s (Chillag, 2016).

Hugh Owen Thomas is considered the father of orthopedic surgery in Great Britain and a pioneer of modern orthopedic surgery. He advocated enforced REST as the best remedy for fractures and tuberculosis (Ponseti, 1991).

Thomas Splint invented a collar to stabilize a fractured femur and prevent infection, to treat tuberculosis of the cervical spine. His manoeuvre became a compulsory orthopedic investigation for fracture of the hip joint and a method of detecting hip deformity (Chillag, 2016).

Robert Jones is considered the father of the modern orthopaedic surgery because he organized and revolutionized the management and treatment of war wounds in World War I when he performed as Director of Military Orthopaedics in the British Army. He established specialized military orthopaedic hospitals for the after-care and rehabilitation of the wounded soldier. After the setting up of orthopaedic departments in the British teaching hospitals and the formation of the British Orthopaedic Association he hence the claim that he established orthopaedics as a specialty in its own rights (Cope, 1995).

This research direction has been reflected in publishing the following articles:

1. Forna N, Scripcaru A, Onofrei P, **Stan CI**, Tudor R, Popescu DC. Management of collapse tibial plateau fractures using a hydroxyapatite-tricalcium phosphate ceramic (atlantik®) and plate osteosynthesis. Rev Chim (Bucharest) 2019; 70(1): 254-258.
2. Scripcaru A, Berea G, Cotrutz CE, **Stan CI**, Puha B. Hemiarthroplasty in Complex Proximal Humeral Fractures Is uncemented methaphyseal corundum blasted titanium humeral stem an efficent alternative? Materiale plastice 2018; 55(4): 676-679.

2.2. Plate osteosynthesis in tibial fractures

2.2.1. Introduction

Proximal tibial fractures are grossly heterogeneous lesions associated with many complications and their prognosis is related to articular comminution with severity of collapse and degree of articular step-off, as well as to osteoporosis, soft tissue envelope and patient's comorbidities (Waddell et al., 1981; Blokker et a., 1984). The disadvantages of classic Open Reduction and Internal Fixation (ORIF) with laterally placed plates (skin necrosis, nonunions and infections) (Young et al., 1994; Mills and Nork 2002), prompted the introduction of biological techniques with Minimally Invasive Plate Osteosynthesis/MIPO using classic plates (Krettek et al., 2001) or the ideal locking plates with monoaxial angular stability type Less Invasive Stabilization System – proximal lateral tibia/LISS-PLT (Goesling et al., 2003).

The last acquisition in angular stability fixation tehniques and design was Locked Compression Plate/LCP with combi-holes and the new Polyaxial Locking Plates that allow screw angulation according to the pattern of comminuted fracture (Frigg et al., 2001;Haidukewych et al., 2008). One of the biggest challenges of the tibial plateau fractures is the successful management of the metaphyseal bone defects which are associated with massive comminution, osteoporosis and reduction techniques (Bucholz et al., 1989; Newman et al., 2008; Hodadadyan-Klostermann et al., 2002).

Autografts are still considered the golden standard in treatment of bone defects, even if the literature reports difficulties in graft harvesting and graft availability (Newman et al., 2008). The allograft usage involves the infection risk (viral or bacterial) and high costs, while it requires a bone bank with all facilities.

For over a century, scientists are trying to discover or synthesize inorganic products that could be used as bone substitutes; these products must provide long lasting bone biocompatibility and must provide bone healing abilities. These bone substitutes must solve the autograft and allograft disadvantages (Verne et al., 2002; Gisep et al., 2004; Ribeiro et al., 2006).

Bone grafting materials are those implants that promotes bone healing by one of the following actions: osteogenesis, osteoinduction and osteoconduction (El-Ghannam 20005; Grauer et al., 2005; Hidaka et al., 2006). A material is osteogenous when it contains living cells, able to form bone tissue (for example, autografts of living bone).

An osteoinductive material is a biological stimulus whichinduces local differentiation of mesenchymal cells from the soft parts in osteoblasts and osteocytes (for example, bone morphogenetic proteins/BMP). An osteoconductive material allows the apposition of a new bone on its surface, acting mainly as a support for the bone tissue (for example, hydroxyapatite, tricalcium phosphate) (Pryor et al., 2009). Autografts or cancellous autogenous bone grafts, as well as the vascularized cortical ones, can be osteogenous (since they contain living bone cells), osteoinductive (due to the protein matrix), and osteoconductive (due to the mineral bone matrix).

These properties also describe the ideal bone substitute (Xiong et al., 2014). Osseointegration is a term used to describe the biological interaction between the grafted material (graft/implant) and the host in the process of bone healing.

Osteoconductive materials became more important especially in bone pathology while they are used as bone substitutes. These substances have a composition similar to the bone mineral matrix and are biocompatible. Their main function is of bone tissue support, allowing bone apposition on their surface; thus, they are used mainly for treating the bone defects (Wongwitwichot et al., 2010). More recently, they are used as a vehicle for osteoinductive substances, augmenting bone formation (Xidaki et al., 2018).

While initially, only coral hydroxyapatite, calcium phosphate (Plaster-of-Paris) and then bioactive glasses (bioviotroceramics) were used as bone substitutes,nowadays we are using phosphocalcic cements and osteoconductive ceramic materials (Bauer and Muschler 2000; Larson 2007). Phosphocalcic cements (CPC) consist in one or more calcium phosphates (CaP) soluble in aqueous solutions. Many experimental and clinical studies have used phosphocalcic cements (Constantz et al., 1998; Frankenburg et al., 1998).

In the past 80 years, the ceramic materials (phosphocalcic products) were intensively investigated and used in bone repair (Bohner 2000).

The most important property of the phosphocalcic compounds is the water solubility, so as a compound is more resorbable as it is water soluble (e.g. β-TCP) when a compound is less soluble in water and in the bone matrix, it will be less or hard to be resorbed (e.g. HA). The most used compounds in the medical field are represented by the tricalcium phosphates (β-TCP), hydroxyapatite (HA) (Ohura et al., 1999) and biphasic tricalcium phosphates (BCP, a mixture of β-TCP and HA in a variety of ratios) (Wongwitwichot et al., 2010).

These materials are biocompatible and osteoconductive ceramics representing synthetic scaffolds which provide structural support for cells and newly formed tissue; these scaffolds act as extracellular matrix for natural process of tissue regeneration (Hench and Polak 2002). In the same time, the rate of degradation for scaffolds must be comparable with osseous apposition (Lee and Shin 2007).

One of the main disadvantages of biphasic tricalcium phosphate ceramics is their fragility with low fracture resistance which limits their use in cases with high strength; these macroporous ceramics are weaker in bending or torsion comparing to compression (Gisep 2002; Bignon et al., 2003). This is the reason for increasing the mechanical strength using osteosynthesis implants (Goff et al., 2013).

Atlantik® biphasic tricalcium phosphate substitute

Atlantik® (MedicalBiomat, France) is ceramic bone substitute, representing a mixture of 70% Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] and 30% beta-tricalcium phosphate [$Ca_3(PO_4)_2$].

This ceramic bone substitute (**Table 2.1**) is produced in various geometric blocks (70% total porosity, partially interconnected by 300-600 μm pores) as well as in granular form with a granule diameter of 0.5 mm, 1 mm, 2 mm and 4 mm (70% total porosity, with a minimum pore size 300- 600 μm and maximum size 2500-5000 μm.

Table 2.1. Technical characteristics of atlantik® bone substitute, adapted from Liao (Liao et al., 2000)

CHARACTERISTICS	BLOCKS	GRANULATES
CRYSTAL PHASES	HAO: $Ca_{10}(PO_4)_6(OH)_2$=70+5% TCP: $Ca_3(PO_4)_2$=30+5%	
DIMENSIONS	+0.5mm (except specific shapes)	0.5mm:300-600µm 1mm: 600-1200 µm 2mm: 1250-2500 µm 4mm: 2500-5000 µm
%POROSITY	70+2%	-70%
MACROPOROSITY	=300-600 µm	=50-1500 µm
COMPRESSIVE STRENGTH	10MPa	x
ENDOXINES	<20EU	

The granular form of Atlantik® must be used in areas with no or low mechanical stress while the blocks will be used in regions with maximal compression stress of 10 MPa. While a pore size larger than 100 µm is necessary, the optimal interconnection size is still debatable. However, the increasing of porosity content or size strongly decreases the mechanical properties.

Exponential functions are used to assess the strength- porosity dependence of ceramics (Bignon et al., 2003):

$$\sigma_R = \sigma_0 \text{'''exp('b'''p)}$$

Where σ_R represent the strength for a volume fraction of pores p (%), and σ_0 represent the strength of the material to porosity.

Observations of micro- and macropores were conducted with a scanning electron microscope (SEM).

In **Figure 2.1** and **Figure 2.2** there are revealed the macroporosity and microporosity, respectively, for Atlantik® substitute with a porogen particle size 300-600 µm (Bignon et al., 2003).

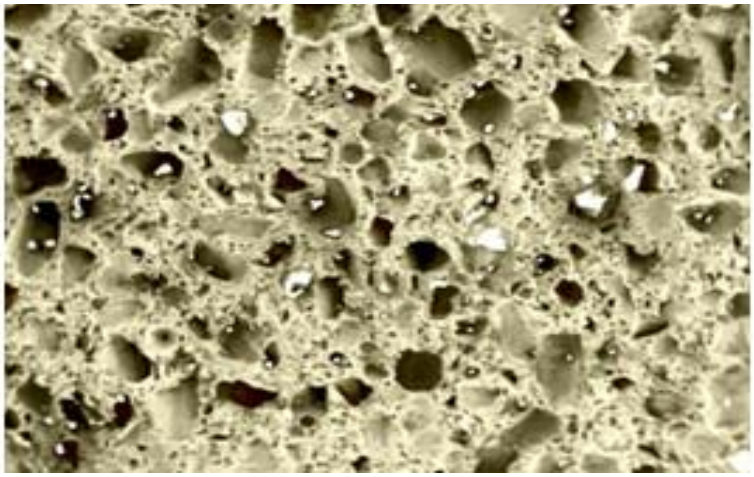

Figure 2.1. Atlantik® macroporosity for porogen particle size of 300-600 µm Adapted from Bignon (Bignon et al., 2003)

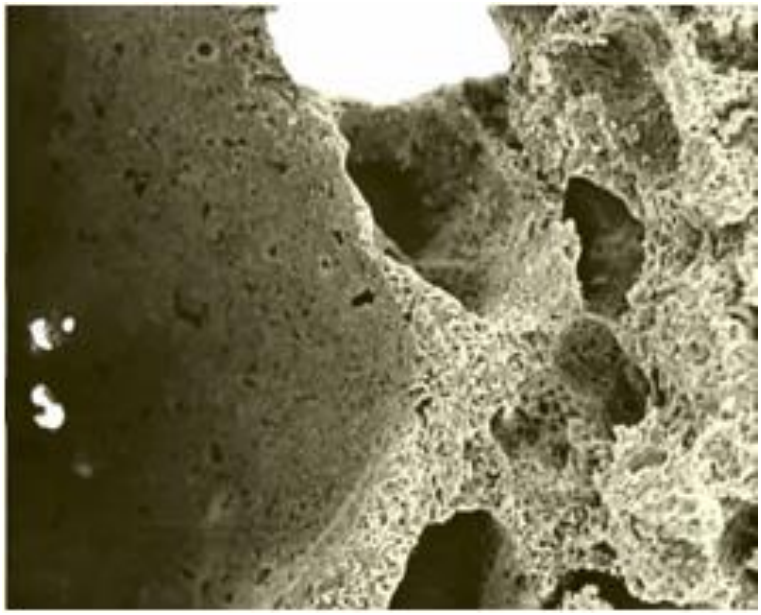

Figure 2.2. Atlantik® microporosity for porogen particle size of 300-600 μm Adapted from Bignon (Bignon et al., 2003)

The aim of this retrospective study is to assess the use of a macroporous biphasic synthetic bone substitute Atlantik® (MedicalBiomat, France) combined with plate osteosynthesis for management of complex tibial plateau fractures, while exhibiting the biocompatibility, quality and extent of osseous healing.

Personal contribution – published paper:

Forna N, Scripcaru A, Onofrei P, **Stan CI**, Tudor R, Popescu DC. Management of collapse tibial plateau fractures using a hydroxyapatite-tricalcium phosphate ceramic (atlantik®) and plate osteosynthesis. Rev Chim (Bucharest) 2019; 70(1): 254-258.

2.2.2. Materials and methods

Our retrospective study was realized between April 2015–December 2016 in Department of Orthpaedics of Universitary ”Sf. Spiridon” Hospital Iași and Vaslui County Hospital. A sample of 27 patients with acute tibial plateau fractures were evaluated, 12 males and 15 females with a mean age of 46.5 years (range 22-76 years). Leading causes of the fractures were high-energy traffic accidents and falls. The classification of fractures was realized according to Schatzker and we included 10 fractures type II, 4 fractures type IV, 8 fractures type V, 5 fractures typeVI. The imagistic exam included in all cases radiographs of the knee and tibia as well as CT scans with 3D reconstruction for articular comminution and collapse.

2.2.3. Results

All patients were operated with reconstruction of the proximal tibia fracture, augmentation of the bone defect with Atlantik® bone substitute in granular form and osteosynthesis with supportive plate (**Figures 2.3 and 2.4**).

Three patients with skin injuries were operated with temporary bridging external fixator while the conversion to a plate fixation and augmentation with bone substitute was realized after 7, 10 and 14 days respectively.

For all patients we have used a lateral curved incision with disinsertion of the anterior tibial muscle 1-2 cm from the tibial ridge for minimally invasive insertion of the plate. Reposition of the articular collapse was realized through a metaphyseal lateral cortical window using a special curved instrument and fluoroscopic control.

The bone defect was augmented with Atlantik® bone substitute according to the triad of osteoconduction:

a) direct apposition of the implant with the surrounding bone;
b) viability of the surrounding bone;
c) augmentation of the interface between surrounding bone and substitute by internal fixation with plates (we have used classic plates in 10 cases and locked plates in 17 cases).

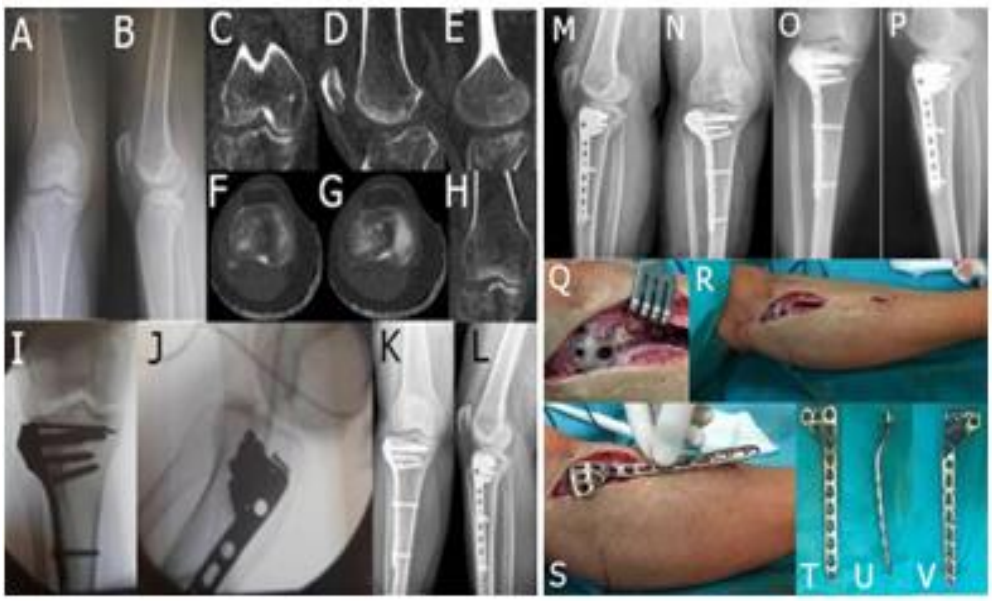

Figure 2.3. (A-V) Fracture of the lateral tibial plateau type II Schatzker. Plate and bone substitute. (A, B) preoperative X-rays; (C-H) CT exam; (I, J) intraoperative fluoroscopic image with excellent reduction of the articular surface, filling the bone defect with Atlantik® bone substitute, osteosynthesis with 2 Kirschner wires and a locked plate; (K, L) X-ray control at 1 month; (M, N) X-ray control at 3 months (radiologic evidence of decreasing granular aspect of bone substitute); (O, P) X-ray control at 12 months; (Q) intraoperative aspect after plate removal with cortical window and osseointegration of the bone substitute; (R, S) wounds aspect after plate removal with MIPO incisions and plate position; (T-V) aspect and design of a LCP-PLT plate with limited contact.

The patients were immobilized with a fixed orthosis for 2-3 weeks; they started walking without weight-bearing until 6-8 weeks (according to the radiographic results) and rehabilitation after removal the orthosis. Total weight-bearing was allowed at 10-12 weeks post-operatively.

The clinical and radiographic follow-up for all patients was recorded for a minimum period of 12 months (average 18,5 months, range 12-28 months). We recorded the quality of articular reconstruction as well as the stability of the construct, the time to fracture healing, the osseointegration of the bone substitute, the functional results and rehabilitation according to Neer Score.

The anatomical reconstruction of the articular surface was recorded with postoperative and follow-up X-rays for 20 patients, while in 7 patients we found minimal secondary displacement (less than 2 mm) due to fracture comminution and osteoporosis.

An uneventful union was present after an average 2.8 months (limits 2-4 months). The radiological aspects as well as the osseointegration of the bone substitute were recorded using 3 important aspects: the zone between ceramic and surrounding bone, the radiological density of the ceramic and ceramic biodegradation.

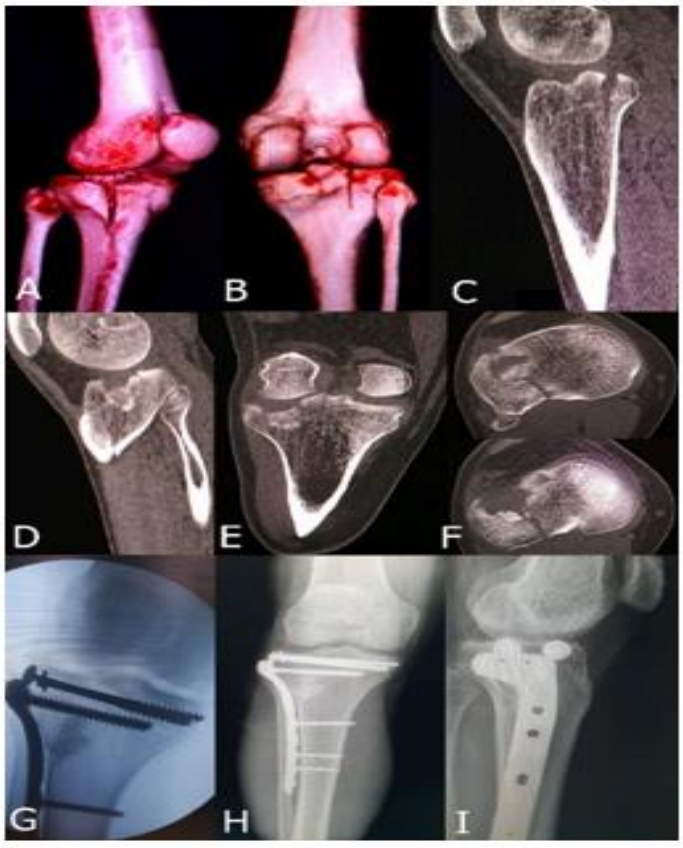

Figure 2.4. (A-I) Fracture of the lateral tibial plateau type II Schatzker. Plate and bone substitute. (A, B) CT exam with 3D reconstruction which reveals the fracture aspect; (C-F) sections of CT exam; (G) intraoperative fluoroscopic control with excellent articular reconstruction of the fracture: osteosynthesis with 2 cancellous screws and LCP-PLT plate, filling of the bone defect with Atlantik® bone substitute; (H, I) X-ray control at 1 month postoperative.

Postoperative X-ray showed radiolucent zones between the implanted ceramic and receiving tissue. Over time, the radiolucent aspect disappeared and new bone developed on the ceramics, due to osteoconductive properties. The mean interval for disappearance of radiological gap was 15 weeks, while the granularity disappeared at 5.5 months with apparent increasing in radiographic density and homogenization.

In the present series, we observed no reactions to ceramic implant, such as wound problems with excessive postoperative drainage, dermatitis, allergic reactions or infections; no obvious evidence of ceramic biodegradation was detected even after 2 years post implantation.

At the most recent follow-up, 6 patients developed secondary mild osteoarthritis signs but there were well tolerated in most of the cases. The mean Range of Motion (ROM) was 118° (range 10° -130°) while the final result was graded with the Neer Score (mean value 85 points, range 70-100 points); we have found 19 excellent and 8 satisfactor y results.

2.2.4. Discussions

Complex articular tibial plateau fractures are difficult to treat and they are associated with a high rate of complications. In difficult cases, primary total knee arthroplasty has potential advantages for elderly patients, while it can be technically challenging in younger patients (Stevenson et al., 2018).

The management of bone defects associated with comminution and osteoporosis represents a great challenge in clinical practice.

Bone grafting (autografts, allografts and synthetic substitutes) is an important treatment for bone defects while successful incorporation of grafted biomaterial into bone defects requires the ability and performance of material to promote new bone formation and provide a scaffold for osteogenesis. While the autograft techniques have limited resources and the allograft used is restricted due to the spreading of infectious disease, developing the ideal substitute is an actual trend in orthopaedics.

The characteristic of an ideal artificial bone material include safety, biocompatibility, excellent biodegrability, ideal porosity, good mechanical properties, osteogenesis, osteoinduction and osteoconduction (Costantino and Friedman, 1994).

Every bone substitute has strengths and weaknesses and no one has demonstrated all the mentioned requirements. Pore diameter and the porosity, which are connected, are important physical parameters for scaffolds since they allow adequate space for cell migration and expansion (Thompson et al., 1995).

A minimum pore size of 100 μm is considered optimal for bone ingrowth, while the pore size more than 200 μm allows the development of mature osteon (Bansal et al., 1995). Biphasic calcium phosphate (BCP) bone substitutes (HA-β-TCP in a variety of ratios) are considered the most promising alternative to autologous bone graft.

In the last years there were investigated a lot of chemical and physical characteristics with important effects in functionality in vivo and in vitro: porosity, grain size and roughness (Mate Sanchez De Val et al., 2016). These studies demonstrated that BCP are biocompatible, bioactive and osteoconductive. The treatment of the most difficult fractures of tibial plateau by plates osteosynthesis was improved with the use of bone substitutes.

In our institutions, until 2015, we have used for bone defects 2 types of macroporous BCP: Ceraform® (Teknimed, France - a mixture 65% HA, 35% β-TCP) and Eurocer® (FH Orthopedics, France - a mixture 55% HA, 45% β-TCP); we exhibited the efficacy of this ceramics, with a fast and good quality osseointegration, while the osteosynthesis was used in most of the cases.

In the present investigation with Atlantik® bone substitute, we found that grafted ceramic was well incorporated into surrounding host bone; the radiolucent gaps disappeared after a mean time of 15 weeks and these radiological changes represent direct bone apposition to the ceramic implant, due to the osteoconduction.

The difficulties of developing new bone substitutes using a single material, prompted the research for preparing composites biomaterials for repairing bone defects. A new biomedical composite with a good similarity to a human bone, a porous nano-hydroxyapatite/polyamide 66 (n-HA/PA66) has been developed in the last years (Yang et al., 2007).

Calcium phosphate-crystals became ideal drug delivery systems, minimizing the effective dose of the drugs and the side effects. A lot of studies presented the outstanding surface interaction properties of ceramics, which make them appropriate candidates for transporting antibiotics, hormones, bone morphogenetic proteins, vitamins, and oncological drugs.

The implementation and effectiveness of an iterative process aimed to quantify and enhance the anatomical fit of an osteosynthesis plate must regard a defined shape-based acceptance criterion while complying with basic clinical requirements and engineering limitations. This is based on employing virtual tools (a database of individual three-dimensional bone models, statistical analysis of the bone geometry, and proprietary software tools) to evaluate conformity between plate designs and bone shape. The conformity should be quantified by the mean distance between plate and bone. The enhancement was completed when the median mean distance of the population was below the shape-based acceptance criterion threshold (Tkany et al., 2019).

Bioresorbable materials have begun to be applied in the clinic, beeing primarily used in children with fractures. Until now there are few reports of their application for treatment of condylar fractures. This particular type of bioresorbable plates and screws for patients with condylar fractures, assisted by digital preoperative planning seem to have great perspectives (Yan et al., 2019).

2.2.5. Final remarks

The successful treatment of bone defects in complex tibial plateau fractures is difficult. Ceramic BCP (a mixture of HA and β-TCP in a variety of ratios) are considered the most promising alternative to autologous bone graft.

The retrospective study on 27 collapse tibial plateau fractures demonstrated that biphasic ceramic biomaterial Atlantik®, combined with supportive plate osteosynthesis, is an effective synthetic bone substitute due to a fast healing and good quality osseointegration with no mechanical failures or inflammator y reactions.

2.3. New perspectives in shoulder prosthesis

2.3.1. Introduction

Fractures of the proximal humerus in adults account for 4-5% of all fractures (Lind et al., 1989) and are the third most frequent fracture in the elderly following fractures of the proximal femur and distal radius (Court-Brown et al., 2006; Robinson et al., 2012).

While most simple fractures can be treated non- surgicaly, prosthetic hemiarthroplasty has become an accepted method of treatment for some subgroups of proximal humerus fractures, including 4-parts fractures, 3-parts fractures in elderly patients with poor bone quality, fracture-dislocation, fractures of the anatomic neck (especially in old aged people) and fractures affecting the joint surface of the humeral head (of head split type) which imply the destruction of over 50% of the humeral head (Moeckel et al., 1992; Robinon et al., 2003).

Four-parts fractures of the proximal humerus are difficult lesions and represent a challenge for the orthopaedic surgeon. Due to the vascularization impairment, both orthopaedic and surgical treatment lead to unfavourable results (Neer 1970; Hertel et al., 2004; Petrigliano et al., 2014; Neuhas et al., 2014), and the only accepted attitude in the case of elderly patients is arthroplasty (Compito et al., 1994; Antunasa et al., 2008). This type of surgery allows a good functional result for comminuted or irreparable articular fractures.

In young patients motivated for a thorough post-operative rehabilitation, hemiarthroplasty is an alternative solution to osteosynthesis, especially in the case of high-energy trauma, where the risk of osteonecrosis is increased (Ballmer and Hertel 1998).

The hemiarthroplasty in proximal humeral fractures was sustained by Neer in the 1970s (Neer, 1970) who presented excellent results in around 90% of his patients. The next studies with this innovative technique reported results strongly related upon anatomic union of the tuberosities around the implant (Movin et al., 1998; Boileau et al., 2002; Kontakis et al., 2008). Despite the patient satisfaction after hemiarthroplasty with the pain - free shoulder, some restriction in active elevation (Prakash et al., 2001; Krause et al., 2007; Gronhagen et al., 2007), prompted the proposal of reversed total arthroplasty for the treatment of 3 -or 4 - parts fractures of the proximal humerus in elderly subject (Sirveaux et al., 2004; Cazeneuve et al., 2014).

The aim of this experimental study is to reveal the design and characteristics of unipolar shoulder prosthesis type Arrow (Groupe FH®, France) as well as to evaluate the outcome of the proximal humerus fracture treated with this implant.

Personal contribution – pablished paper:

Scripcaru A, Berea G, Cotrutz CE, **Stan CI**, Puha B. Hemiarthroplasty in Complex Proximal Humeral Fractures Is uncemented methaphyseal corundum blasted titanium humeral stem an efficent alternative? Materiale plastice 2018; 55(4): 676-679.

2.3.2. Material and methods

This experimental study was conducted on 8 patients with 3- or 4- parts proximal humeral fractures. We used an unipolar shoulder prostheses type Arrow (Groupe FH®, France) and we analyzed the outcome of the treatment of using this implant with an uncemented metaphyseal corundum blasted humeral titanium stem; the patients were operated at the Department of Orthopaedics and Traumatology of the Universitary Clinic Hospital ”Sf. Spiridon” Iași and at the Emergency County Hospital Piatra Neamț, between 2014 - 2016.

- *Prosthetic design*

The Arrow platform system asures for hemiarthroplasty a stem and a humeral head (**Figure 2.5**). The titanium humeral stem (**Figure 2.5**) is corundum blasted in the metaphyseal part (allowing bone ingrowth) and a smooth diaphyseal part (that can be cemented or uncemented). The shape of the humeral stem ensures a high stability of the prosthesis due to the good press fit metaphyseal fixation and preservation of the bone stock of the humerus. The lateral fin avoids stem rotation. The lenght of the stem between 120 and 170 mm prevents varus or valgus malposition.

The humeral head has an anatomic design and is made of cobalt chrome. The possibility of placing the head centered or off centered helps reproduce the anatomical medial and posterior offset of the humerus. The shape of the head covers the stem plate leaving no space between the collar and the head, thus increasing the articular surface. The head comes in 6 sizes (40, 44, 46, 48, 50 and 54) with a thickness between 12 and 18 mm.

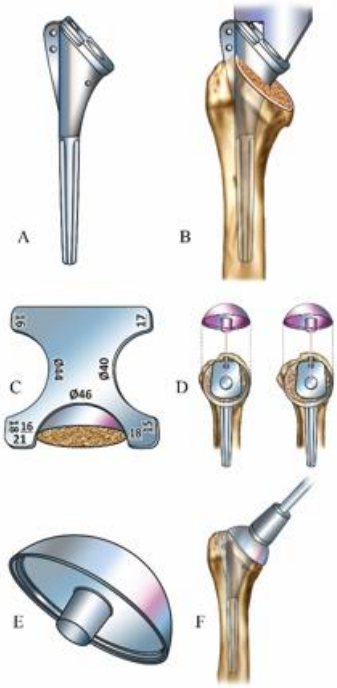

Figure 2.5. (A-F) Arrow Platform System for unipolar hemiarthroplasties; (A-B) - Metaphyseal corundum blasted titanium humeral stem; C -The diameter and thickness of the articular head is measured with the humeral head template; D -A centered or an off-centered trial humeral head is used in order to cover the bone as much as possible; (E -F) - The definitive cobalt chrome humeral head is impacted onto the humeral stem.

The study consisted of a retrospective analyses of 8 female patients with complex proximal humeral fractures (6 with 4-parts and 2 with 3-parts), with an average age of 62.5 years (range 56-76). The mean interval between the accident and the surgery was 14 days (range 5 - 28 days).

Surgical technique

All the patients were operated with general anesthesia, in a beach chair position and upper limb free, through a deltopectoral access, without or with minimal desinsertion of the deltoid (**Figure 2.6E**). The deltoid and cephalic veins were retracted laterally and pectoral medially while not damaging the coracoacromial ligament.

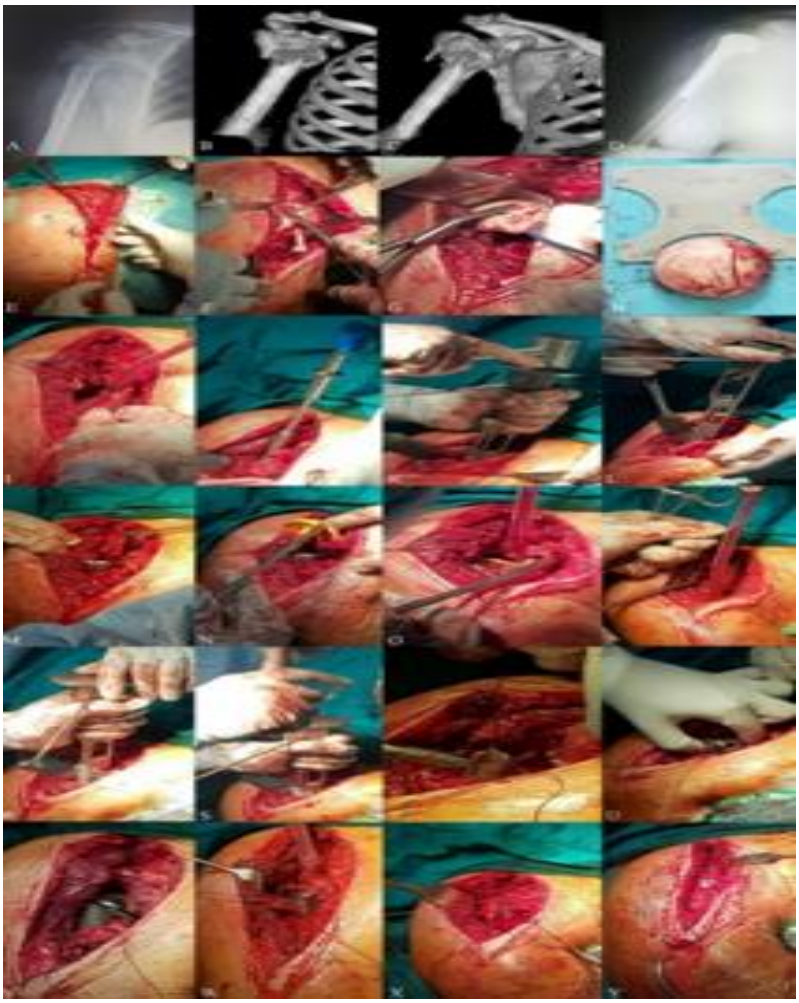

Figure 2.6. (A –Y) – Hemiarthroplasty for a 3 - parts proximal humeral fracture using unipolar uncemented Arrow prosthesis (4 weeks after accident); A - Preoperative x-ray; (B-C) - CT images with 3D reconstruction; D - Postoperative x-ray; E - Deltopectoral approach; F - Sectioning of the tendon of the long head of the biceps; G - Humeral head removal; H - Humeral head measurement with the humeral head template; I - The tuberosities are tagged with heavy sutures and retracted; J – Manually diaphyseal reaming with increasing size of reamers; K, L - Manually preparation of the humeral metaphysis with increasing size rasps and reproducing the retroversion (20-30 degrees); M, N, O -Attaching the trial humeral head, performing the reduction and assess glenohumeral stability ; P -Temporary reduction of the tuberosities below the head of the modular prosthesis; R, S, T - Introducing the definitive humeral stem; U - Impact the definitive humeral head onto the humeral stem; V- Reduction of the humeral prosthesis; W, X - Sutures of the tuberosities; Y – Wound closure

The axillary and the musculocutaneus nerves were protected. The bicipital groove was a critical landmark while the fracture line between the greater and lesser tuberosity is located posterior to the groove. In cases with 3-parts fractures, the lesser tuberosity was osteotomized for creating a 4-parts fracture. After the sectioning of the tendon of the long head of the biceps (routinely tenodesis of the humeral insertion of the pectoralis major muscle), the humeral head is removed and the tuberosities are tagged with heavy sutures (**Figures 2.6G – I and 2.7E - F**) Next, the humeral channel is exposed and prepared with a sequential reaming until there was a sensation of reaming into the cortex (**Figure 2.6J**).

Then, the metaphysis is progressively prepared using increasing size of trial rasps. The retroversion of 20-30 degree of humeral component was realised using anatomical landmarks and by aligning the retroversion shaft attached to the metaphyseal rasp handle and the forearm (**Figures 2.6K – L and 2.7G -H**). After introduction of the last rasp and trial humeral head, reduction was realized verifying height and the stability of the prosthesis (**Figures 2.6M –O and 2.7I -J**). Temporary reduction of the tuberosity below the head of the modular prosthesis was realized with towel clips for verifying the position and acceptable adjustment to the stem collar (**Figures 2.6P and 2.7K**). At the end we replaced the trial rasp with the definitive corundum blasted titanium humeral stem and the definitive centered or off-centered humeral head was impacted onto the stem (**Figures 2.6R – U and 2.7L**).

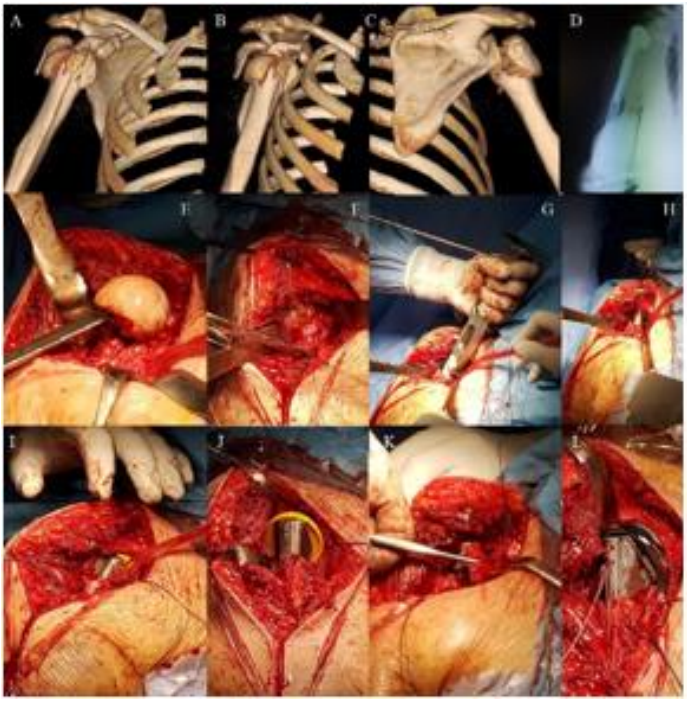

Figure 2.7. (A – L) - Hemiarthroplasty for a 4 part proximal humeral fracture using unipolar uncemented Arrow prosthesis; (A – C) – CT images with 3D reconstruction; D – Postoperative x-ray; E - Humeral head removal; F - The tuberosities are tagged with heavy sutures and retracted; (G – H) - Manually preparation of the humeral metaphysis with increasing size rasps and reproducing the retroversion (20-30 degrees); (I – J) - Attaching the trial humeral head, performing the reduction and assess glenohumeral stability; K - Temporary reduction of the tuberosities below the head of the modular prosthesis; L - Introducing the corundum blasted humeral stem and impacting the definitive humeral head onto the stem;

The final reduction of the prosthesis was realized (**Figure 2.6V**) and both tuberosities were fixed with 2 horizontal cerclage sutures followed by 2 vertical figure in eight sutures (**Figures 2.6W - X**). A final check of the stability of the repair and range of motion was realized. Wound closure was realized with aspirative drain (**Figure 2.6Y**). Perioperative control radiographs were realized in order to confirm the position of the implants and of the tuberosities.

The patients shoulders were immobilized postoperative in an internal shoulder rotation orthesis with 30 degrees arm abduction for 30-45 days.

Postoperative rehabilitation started after first week with pendular exercises and passive anterior flexion exercises. Active rehabilitation was started after the 6th week while strengthening exercises only after 12 weeks. All pacients underwent clinical and radiographical examination with a mean follow-up of 14 months (range 12-20 months).

Despite numerous adapted shoulder scores (Haragus et al., 2018), clinical assessment was performed using Constant - Murley score (which includes pain, mobility, activity and strength) (Constant et al., 1987) and active joint amplitudes in the operated shoulder. Radiological evaluation was conducted with special attention to the position of corundum blasted titanium stem (with osseointegration), as well as to the healing and position of the tuberosities.

2.3.3. Results

While all our patients had minimal pain with improving everyday comfort, the mean Constant - Murley score was 74, (limits 34-92) (**Table 2.2**). In evaluating the active range of motion at the final follow-up we found the following means : abduction 98° (limits 40 - 140); anterior elevation 112° (limits 60-150); external rotation with the elbow at the side 26° (limits 0-30) and internal rotation at 90 degrees abduction 51° (limits 0- 60).

Table 2.2. Clinical results at the final follow-up

Results	Mean Value	Average
Constant – Murley score	74	34-92
Abduction	98	40-140
Anterior elevation	112	60-150
External rotation with the elbow at the side	26	0-30
Internal rotation and 90 degrees abduction	51	0-60

From the radiographical evaluation we found abnormal tuberosity positioning and healing problems in 2 patients. Tuberosity healing was good in the other 6 patients while we found 3 cases with periprosthetic ectopic ossifications.

The osseointegration of the uncemented metaphyseal corundum blasted humeral titanium stem was optimal, with no partial or complete radiolucency around the humeral component.

Two patients had significant limitations of movement that were treated conservatively with long-term rehabilitation. There were no infections or implant instability.

2.3.4. Discussion

In the 1970s, Beddow and Alloy were using a prototype reverse shoulder arthroplasty in Liverpool for patients with rheumatoid arthritis but did not publish the results. In 1987, Grammont introduced the reverse total shoulder arthroplasty to treat rotator cuff tear arthropathy (Grammont et al. 1987). The Grammont prosthesis reverses normal shoulder anatomy by replacing the humeral head with a cup and the scapular glenoid fossa with a ball. Previously, all shoulder arthroplasties reproduced the normal anatomy. This new reversed design allows more control of shoulder motion by the deltoid muscle in rotator cuff–deficient patients.

Reverse total shoulder arthroplasty was approved by the US Food and Drug Administration in 2004, and the number of RTSAs performed annually increased (Kim et al., 2011).

However, an important limitation of this nonanatomical prosthesis is its inability to restore active internal and external rotation. This is caused mainly by design limitations of the prosthesis (Wilde and Walch 2006) producing mechanical impingement and malfunctioning of the rotator cuff remnants (Seebauer et al., 2005).

A second prosthetic limitation of this reversed principle rises from its hinged rotation (the humeral component rotates around the glenosphere) instead of a spinning rotation (the humeral head rotates on the spot) as seen in an anatomical setting. This type of rotation requires more room, because without it, a conflict between the humeral and glenoid parts can occur. Best known is the scapular notching in which the humerus is in conflict with the infraglenoid tubercle (Boileau et al., 2005; Wilde et al., 2010; Middernacht et al., 2008).

Anatomic total shoulder arthroplasty is most commonly performed for degenerative osteoarthritis in patients older than 60 years (Wiater et al., 2009). Other indications for total shoulder arthroplasty include inflammatory arthritis, humeral head avascular necrosis with secondary glenohumer- al arthritis, Charcot arthropathy, and postinfectious arthritis (Merolla et al., 2008).

The first generation of total shoulder arthroplasty was monoblock and limited. The second generation (introduced by Neer) had a modular humeral head and ingrowth coating on the stem. The third generation of total shoulder arthroplasty design allowed anatomic adjustment of the humeral head offset (Sanchez-Sotelo, 2011).

In case of 4-parts fractures of the proximal humerus, the results are influenced by many factors: age of the patient, quality of bone, the moment when the surgical intervention took place, deficiencies in the surgical technique, post-surgical complications, post-surgical rehabilitation.

A favorable and functional prognosis can be anticipated for a younger patient without pre-surgical neurological deficit or post-surgical complications and with a satisfactory aspect of the shoulder after 6 weeks.

The results are weaker in the larger group of old-aged patients (often poorly motivated and suffering from other associated conditions), especially in those who show neurological deficit, a post-surgical complication which requires reintervention or an excentrically-placed prosthesis with retracted tuberosities (Bonnevialle et al., 2016).

The altered geometric configuration created with reverse shoulder arthroplasty allows reconstruction of rotator cuff– deficient shoulders. Patients experience a significant improvement in range of motion and markedly decreased pain.

As for the time of the surgical intervention, it has been noted that early prosthetic (less than 4 weeks after the trauma) leads to a better prognostic in terms of functionality (Bosch et al., 1996). As the lesion grows older the recovery of function and mobility rate of the prosthetic shoulder become weaker (Dimakopoulos et al., 1997).

Furthermore, the results can be influenced by the incomplete solving of the anatomical and surgical issues: damage in the circumflex nerve, early or secondary migration of the tuberosities as a consequence of a deficient reinsertion (Boileau et al. 1999).

Despite the increase using of reverse shoulder arthroplasty (Bonnevialle et al., 2016) or introducing new type of shoulder prosthesis (Tomoaia et al., 2008; Herle et al., 2010), displaced 3-parts or 4-parts proximal humeral fractures are best treated with hemiarthoplasty (especially in elderly). The crucial aspect is to understand the key concepts of well performing this demanding operation.

Revision surgery for shoulder prostheses remains among the most difficult tasks in shoulder surgery. The poor results in function and longevity of unconstrained prosthesis for revision of failed hemiarthroplasty or total shoulder arthroplasty led many authors to use reverse ball and socket prosthesis for selected cases (Gagey et al., 2001; Valenti et al., 2014).

Not many surgical options are available for failed shoulder prosthesis, especially when worsened by a loss of glenoid bone stock or an irreparable rotator cuff lesion (Valenti et al., 2014).

Petersen used an anatomic Neer-type prosthesis in their series of 158 cases operated in a 15-year period. They reported 19% of excellent results, 41% of satisfactory results, 28% of unsatisfactory and 12% of poor results. The high rate of bad results (40%) explains why this treatment is not the preferred one (Petersen and Hawkins 1998).

Arhens et al. reported 60% of recurrence of anterior instability and 40% of recurrence of posterior instability in revision of instability after unconstrained arthroplasty (Arhens et al., 2001).

Using a reverse prosthesis with a center of rotation lateral to the level of the glenoid (Encore, Houston, Texas), Frankle reported encouraging results, in a series of sixty patients (Frankle et al., 2005). The gain was 35.9° in external rotation at 0° of abduction compared with the 11.2° in the study reported by Sirveaux with a Delta III (Sirveaux et al., 2004). The first hypothesis is that a more anatomic center of rotation improves the tension in the remaining subscapularis or infraspinatus or teres minor muscles, recruiting some anterior and posterior fibers of the deltoid, which participate to external and internal rotation (Valenti et al., 2014).

Shoulder instability and reduced range of motion are two common complications of a total reverse shoulder arthroplasty. Morphometrical approach is proposed to estimate how the glenoid component positioning can influence the stability and the range of motion of a reverse shoulder prosthesis. On virtual simulations, the influence of the glenosphere positioning could be investigated. The most suitable configuration could be used to find, with no in vivo experiments, the correct position of a reverse shoulder prosthesis depending on the different dimensions and shape of the bones of each patient (Ingrassia et al., 2019).

Polyethylene wear measurement of reverse total shoulder arthroplasty (rTSA) is restricted to in vitro, in silico, and retrieval analysis, with no method for the quantification of in vivo wear of well-functioning implants. The model-based radiostereometric analysis is a measurement tool for in vivo rTSA wear using a phantom setup. This novel technique allows for the in vivo measurement of polyethylene wear without the requirement of marker beads or baseline radiographs, expanding the potential for in vivo wear measurements to larger populations and retrospective analysis (Van de Kleut et al., 2019).

2.3.5. Final remarks

Hemiarthroplasty for 3 or 4 parts proximal humerus fracture provides good results, a high subjective satisfaction rate with absence of pain and improving the patients everyday comfort. The challenge in this difficult surgery is correctly establishing the height and retroversion of the stem as well as the strong suture of the tuberosities which allow anatomic union around the implant. The uncemented metaphyseal corundum blasted titanium stem type Arrow assures a perfect osseointegration with optimal stability of the prosthesis.

CHAPTER 3. MORPHOFUNCTIONAL AND APPLIED DEVELOPMENTAL RESEARCH ON THE ENDOCRINE SYSTEM

3.1. State of the art

The morphofunctional research on the growth, development, and metabolism in vertebrates provides an overview of vertebrate endocrinology and it was the starting point of studying human endocrinology. The concept of developmental study of the endocrine system aims to strengthen the bridge between medical and comparative anatomists, molecular endocrinologists, biologists, molecular biologists, biochemists, researchers, and physicians by addressing the benefits that they can derive from this association (Schreibman et al., 1992).

The structure and biological function of growth hormone (GH) and prolactin (PRL) family as well as the extrinsic, genetic, and humoral factors that influence human growth, rise a particularly interest.

Recent discoveries in molecular endocrinology have significantly increased our understanding of endocrine diseases, as well as that of the medical management of these complicated disorders. Developmental endocrinology translate all the scientific informations regarding endocrine morphology, physiology and development into an understanding of the clinical pathogenesis, diagnosis, and treatment of these diseases.

Developmental studies are constituting an invaluable guide to the practical clinical management of endocrine pancreatic and gonadal disorders (Eugster and Pescovitz, 2002).

This story begins with a practice that came to be known as “organotherapy,” in which patients were treated with extracts of various endocrine glands. It has made its way from the

fringe to the mainstream of the medical world in 1891, when George R. Murray successfully treated a myxedematous patient with an extract of sheep thyroid (Murray, 1891; Schwartz, 1999).

Another upholder of the organotherapy was Henry R. Harrower (1883–1934), who leaded practitioners and he eventually created The Harrower Laboratory in Glendale, California. There were produced, marketed, and distributed organotherapy products on an industrial scale. "Harrower's Monographs on the Internal Secretions", "Practical Hormone Therapy" and "Practical Organotherapy—The Internal Secretions in General Practice" are his main written work (Schwartz, 1999).

This literature include theories to explain the purported benefits of various "pleuriglandular" extracts, advocated for conditions ranging from reproductive failure to epilepsy, nephritis, "mental deterioration," and many others. He particularly favored the theory that because dysfunction of any of the endocrine glands can cause dysfunction of many others, "organotherapy contemplates the giving of extracts of many glands" (Harrower, 1922).

Harrower and his colleagues formed an organization known as The Association for the Study of Internal Secretions in 1916 (Wilhelmi, 1988).

Probably the most famous follower of this practice was Harvey Cushing, a giant of academic medicine. He took full advantage of the opportunity afforded by his Presidential Address to lambaste the practice of organotherapy. Cushing wrote that "what is there to say of a pluriglandular complex except to acknowledge an abysmal ignorance? Surely nothing will discredit the subject in which we have a common interest so effectively as pseudoscientific reports which find their way from the medical press into advertising leaflets, where, cleverly intermixed with abstracts from researchers of actual value the administration of pluriglandular compounds is promiscuously advocated for a multitude of symptoms, real and fictitious" (Cushing, 1921).

Next great step consisted in the isolation of the insulin from pancreatic extracts by Banting, Macleod, Collip, and Best, helping to usher in the modern era of endocrinology. The isolation of a specific hormone for therapeutic purposes marked a sea change that, along with the condemnation of Cushing and others, heralded the marginalization of organotherapy and its advocates. Than, the "Association for the Study of Internal Secretions" was renamed the "Endocrine Society", thenceforth to be presided over by academic devotees of the scientific method. It evolved rapidly into an organization led by academics who championed rational treatment rooted in proven pathophysiological concepts of endocrinology (Bliss, 1982).

In 1869, a German medical student, Paul Langerhans, had discovered two systems of cells in the pancreas: the acini, which he knew produced the pancreatic digestive secretions, and another system whose function was unknown to him. These cells looked to Langerhans like tiny clusters of cells, or islands, floating among the acini (Langerhans, 1868). In 1901 Eugene Opie, an American pathologist at Johns Hopkins University, made the association between the degeneration of these cells, which had been named the "islets of Langerhans," and the onset of diabetes. Through the experimental efforts of these and many other researchers, the stage was set for the discovery of insulin—the hormonal antidiabetic secretion of the islets of Langerhans—in the first decades of the 20th century (Sakula, 1988).

The link between pancreatic secretions and diabetes was first shown in 1889 by Oskar Minkowski and Joseph von Mering, while they investigated the effect of pancreatic secretions on the metabolism of fat. They performed a complete pancreatectomy on a laboratory dog, only to discover that the animal developed a disease indistinguishable from diabetes (Luft, 1989).

Returning to the origin, the goal of identifying neurocircuits that control feeding and energy balance is now being realized. The discoveries in the field have the potential to completely change our understanding not only of how pathological weight gain occurs but also how the biologically defended level of body fat stores becomes elevated in obese individuals and other endocrine disorders. Especially in diabetes mellitus, until these and other fundamental questions are answered, the pothole-ridden landscape of diets, supplements, and other ineffective therapies that passes for today's treatment don't look so different from the practice of organotherapy a century ago, will continue (Guyenet and Schwartz, 2012; Sohn et al., 2013; Sternson et all., 2013; Krashes et al., 2014).

Evolutionary disorders can arise at many levels between parents and offspring, between mates, or between phenotypically divergent genders, morphs, or ontogenetic stages that share the same underlying genome. Current studies show that artificial selection for high and low testosterone levels in bank voles, *Myodes glareolus* demonstrate how the pleotropic effects of testosterone on gene expression in the brown anole, *Anolis sagrei*, may help to reduce genetic correlations for traits that are shared between the sexes, thereby facilitating the evolution of sexual dimorphism and the resolution of intralocus sexual conflict (Cox et al., 2016).

Type 2 diabetes mellitus has to be considered as a gender-associated disease: sex differences play in fact a key role in the onset as well as in the progression of the disease and a higher mortality for cardiovascular diseases is detected in diabetic women with respect to men. The results obtained from all the available animal models studies appear to only partially address this issue so that the search for more precise information in this respect appears to be mandatory (Franconi et al., 2008).

This research direction has been reflected in publishing the following articles:

1. Avram I, Lupașcu FG, Confederat L, Constantin SM, **Stan CI**, Profire L. Chitosan microparticles loaded with antidiabetic drugs - preparation and characterization. Farmacia 2017; 65(3): 443-448.
2. Bălășescu E, Rusu MC, Vrapciu AD, Mirancea N, Manoiu VS, **Stan CI**. Early onset of podocytes apoptosis - a tem study in streptozotocin-induced diabetic rats. Rom J Morphol Embryol 2014; 55(1): 71-75.
3. Sava A, Motoc AGM, Stan CI. Electron microscopic aspects of the effects of certain prostaglandin analogs on mouse testes. Rom J Morphol Embryol 2015; 56(2): 771-775.

3.2. Advances in endocrine pancreatic function research

3.2.1. Introduction

Diabetes mellitus is a chronic metabolic disorder resulting from a defect in insulin secretion, insulin action or both. This disorder claims four million lives every year and it is a leading cause of blindness, kidney failure, heart attack, stroke and amputation (Alberti et al., 2006; Buse 2011).

The current oral treatment options for type 2 diabetes mellitus (T2DM) include sulfonylureas, glinides, biguanides, thiazolidinediones and alpha-glucosidase inhibitors, drugs which are often associated with serious side effects (Bennett et al., 2012; Derosa and Maffioli 2010; Thule et al., 2014).

Metformin is a biguanide drug used as first-line therapy in type 2 diabetes mellitus treatment. However, metformin has a low degree of biovailability (50-60%) and short and variable half life time (0.9-2.6 h) which requires repeated administration of high doses to maintain effective plasma concentration (Hajjar et al., 2013; Mansoory and Jain 2012). Unfortunately, at higher doses, metformin is often associated with several side-effects such as lactic acidosis, diarrhea, nausea, vomiting and flatulence (Scheen et al., 2013).

Glybenclamide is a drug which belongs to third generation sulfonylureas with enhanced potency and increased duration of action (Dora et al., 2010; Pompermayer et al., 2007) but with reduced bioavailability (45%) attributed to its poor dissolution properties (Nagpal et al., 2012). It is administrated in doses of 2.5-5 mg/day as monotherapy or in combination with metformin (500 mg/day) as Glucovance, Bidiab, Glibomet and Gliformin. Metformin/glybenclamide fixed-dose combination should be avoided in the elderly and those with renal or hepatic impairment (Lupaşcu et al., 2015). This combination can also cause gastrointestinal side effects, hypoglycaemia and weight gain and it increases the risk of severe and prolonged hypoglycaemia (Lamos et al., 2012).

In order to increase the pharmacokinetic and safety profile of metformin and glybenclamide, new binary drug microparticles based on chitosan were developed. Chitosan is a biopolymer very suitable for biomedical and pharmaceutical applications based on its properties such as biodegradability, low toxicity, low immunogenicity and good biocompatibility (Dash et al., 2011).

Cell death types are defined by morphological criteria. Apoptotic cell death is characterized by rounding up of the cell, retraction of pseudopodes, chromatin condensation, karyorrhexis, and blebbing of plasmalemma that remains undamaged until later stages of apoptosis (Kroemer et al., 2005; Duprez et al., 2009).

In human diabetic samples, apoptosis involves epithelial cells of the proximal and distal tubules, endothelial cells, and interstitial cells (Kumar et al., 2009); however, as Mulay commented, podocyte apoptosis is often suspected but it is rarely detected (Mulay et al. 2013).

Podocyte loss is a key event in glomerular disorders (Lasagni et al., 2013). Diabetic podocytopathy is characterized by proteinuria and albuminuria, glomerular hypertrophy and hyperfiltration, thickening of the glomerular basement membrane (GBM) with altered matrix

composition, the reduction of the nephrin protein in the slit diaphragm, and effacement of foot processes of podocytes (Gruden et al., 2005; Li et al., 2007; Ziyadeh et al., 2008; Mandache and Penescu 2012).

Eid proved that exposure of mouse podocytes to high glucose levels causes apoptosis, about one third of the cells becoming apoptotic by 72 hours through the generation of reactive oxygen species via sequential upregulation of CYP4A and the NADPH oxidases Nox1 and Nox4 (Eid et al., 2009).

Tissue degeneration in diabetes mellitus begins in early stages; however, there is limited evidence on cell injuries in these stages (Haligur et al., 2012). It was however identified by immunohistochemistry, in streptozotocin-induced diabetes, a caspase-dependent mechanism of the renal tubular apoptosis (Haligur et al., 2012).

As it was hypothesized that in diabetic nephropathy podocyte apoptosis is a key event, we aimed to better characterize morphological signs of apoptotic cell death (including podocyte apoptosis) in kidneys from rats with early streptozotocin-induced diabetes by a transmission electron microscopy (TEM) study.

We have also purposed to to develop new binary polymeric systems based on chitosan in order to achieve an improvement of the pharmacokinetic and pharmacological profile of two of the most used oral antidiabetic drugs - metformin and glybenclamide.

The aim of our research in this field is to find a cure for diabetes mellitus, by a morphopathological approach. We envision a world in which advances in endocrine science, education and care promote optimal health and well-being. In this future:

- ***Interdisciplinary teams accelerate and translate scientific discoveries to prevent and cure endocrine disorders.***
- ***Science and technology drive personalized clinical care.***
- ***Basic science is respected and valued for the role it plays in fundamental breakthroughs.***
- ***Technology increases the pace of scientific discovery.***
- ***Providers and patients actively influence healthcare policy.***
- ***Global collaborations, innovations in therapies and care models, and new technologies expand access to care and eliminate health disparities.***
- ***Endocrine researchers and clinicians are sought after sources of reliable information for patients, policymakers, and the media.***

We propose a reappraisal of the various animal models for a study of type 2 diabetes mellitus that would take into consideration a gender perspective.

Personal contribution – published papers:

1. Avram I, Lupașcu FG, Confederat L, Constantin SM, **Stan CI**, Profire L. Chitosan microparticles loaded with antidiabetic drugs - preparation and characterization. Farmacia 2017; 65(3): 443-448.
2. Bălășescu E, Rusu MC, Vrapciu AD, Mirancea N, Manoiu VS, **Stan CI**. Early onset of podocytes apoptosis - a tem study in streptozotocin-induced diabetic rats. Rom J Morphol Embryol 2014; 55(1): 71-75.

3.2.2. Materials and methods

3.2.2.1. Study of the chitosan microparticles loaded with antidiabetic drugs

- **Materials**

Chitosan medium molecular weight (CS), metformin hydrochloride, glybenclamide, acetic acid, sodium tripolyphosphate (TPP), dimethylsulfoxide (DMSO) were purchased from Sigma Aldrich Company.

- *Preparation of chitosan microparticles loaded with antidiabetic drugs*

The antidiabetic drugs (metformin, glybenclamide) were loaded into chitosan microparticles using ionic gelation method. Briefly, the drugs were dissolved in minimum volume (0.5 mL) of proper solvent (distilled water for metformin hydrochloride and DMSO for glybenclamide). The drug solutions were added into 3 mL of chitosan solution 1% in acetic acid. The mixture was stirred at room temperature for 3 h and then was dropped through a syringe needle (26 G) into 20 mL of TPP solution 2% in distilled water under stirring (325 rpm). The mixture was stirred again (200 rpm) at room temperature for 24 h. The formed beads: chitosan-metformin (CS-M), chitosan-glybenclamide (CS-G) and chitosan-metfomin-glybenclamide (CS-MG) were separated from TPP solution and washed three times with distilled water and then dried at room temperature. In order to obtain high loading efficiency and stable microparticles, three concentrations for each drug have been used (30 mg, 22.5 mg and 15 mg), which means that the ratio between antidiabetic drug and chitosan was 1:1, 0.75:1 and 0.5:1 (w/w).

- *Chracterization of chitosan microparticles loaded with antidiabetic drugs.Fourier transform infrared (FT-IR) spectroscopy*

FT-IR spectra of chitosan and chitosan microparticles loaded with antidiabetic drugs were recorded using a Biorad FT-IR spectrometer FTS 575C in the range between 4000 cm^{-1} and 500 cm^{-1}, after 32 scans at a resolution of 4 cm^{-1}. The spectra processing was carried out with the Horizon MB^{TM} FTIR Software.

- *Particle size measurements and morphology*

The size of the microparticles (in wet and dry state) was measured using Zeiss (Axiotech) optical microscope (5 times magnification). The scanning electron microscopy technique (SEM) using a Desktop SEM (Phenom, The Nederlands) was chosen to study the morphology of the microparticles.

- *Swelling degree*

The swelling degree (SD) of the polymeric systems was performed in distillated water and simulated gastric fluid (SGF) at pH 1.6, at 37°C and it is based on measuring the microparticles weight as a function of time (9). A sample of dried micropaticles (W_1) was place in distillated water and simulated gastric fluid respectivelly (SGF). At different times microparticles were removed from water and SGF respectively, dried quickly and carefully with filter and weighted (W_2). The experiments were performed in triplicate and average values were calculated. The swelling degree at different times was calculated using the following formula:

$$SD\ (\%) = W_2\text{-}W_1/W_1 \times 100$$

where: W_1 = the weight of the dried microparticles; W_2 = the weight of the swollen microparticles at different times.

- *Loading efficiency*

The loading efficiency (LE %) of the antidiabetic drugs into chitosan microparticles was evaluated using UV spectrophotometric method (UVIKNO XL, BIOTECH Instruments) (6, 10). The content of drug (metformin, glybenclamide) in the TPP solution after removing the beads was calculated by measuring the absorbance of the solution at 233 nm (for metformin) and 300 nm (for glybenclamide) respectively using the standard curve for each drug. The loading efficiency (%) was calculated using the following formula:

$$LE\ \% = C_1/C_0 \times 100$$

where: C_0 = initial concentration of the antidiabetic drug; C_1 = antidiabetic drug concentration in the TPP solution.

3.2.2.1. Study of the in streptozotocin-induced diabetic rats

Five adult Wistar rats weighting 350–400 g were used. The animals were adequately kept and fed. The streptozotocin (STZ) powder (Sigma-Aldrich Gmbh, Munich, Germany) was diluted in distilled water 1 mL/20 mg. The animals were injected with streptozotocin 55 mg/kg of the body weight intraperitoneally under general anesthesia with diethyl ether inhalation. Body weight and blood glucose concentration were measured before injection, three days, and weekly thereafter.

For measurement of the blood glucose concentration, we have used Accu-Chek Active (blood glucose monitoring system by Roche Diagnostics GmbH, Mannheim, Germany) and Accu-Chek Active blood glucose test strips. Blood samples were taken from the tail vein. The body weight and blood glucose were measured weekly. After preanesthesia with ether, the animals were euthanatized after three weeks by intracardiac injection of a veterinary euthanasia drug (0.2 mL of T-61, Intervet, Kirkland, Quebec, Canada).

All procedures were approved by the Institutional Bioethics Committee.

Kidney samples were prepared for TEM, as previously described. The Formvar coated grids were examined in a Philips electron microscope EM 208S (acceleration voltage of 80 kV) and snapshots were taken using a video camera Veleta and the iTEM Olympus Soft Imaging System.

3.2.3. Results

3.2.3.1. Study of the chitosan microparticles loaded with antidiabetic drugs

- *Fourier transform infrared (FT-IR) spectroscopy*

The presence of the antidiabetic drugs in the polymer matrices has been proven by the FT-IR spectral data (**Figure 3.1**). The spectra of chitosan micropaticles revealed the following characteristic bands: 1636 cm^{-1} (-CO-NH-NH_2), 1456 cm^{-1} (CH_2), 1040 cm^{-1} (C-O-C) and 1375 cm^{-1} (CH_3). The presence of metformin in the polymer matrix was proved by the following spectral bands: 3367, 3294, 3150 (N-H), 1558, 1541 (-NH_2), 1684 (-C=N), 1080, 1063, 1040 (C-N) and 2974, 2943 (-CH_3) cm^{-1}. The spectral bands at 3365, 3292, 3153 (N-

H), 1624, 1558 (C=O), 1165 (SO_2) and 737 (C-Cl) cm^{-1} were attributed to glybenclamide and the cross linking agent (TPP) was identified by spectral bands at 1219 cm^{-1} (P=O) and 895 cm^{-1} (P-O-P).

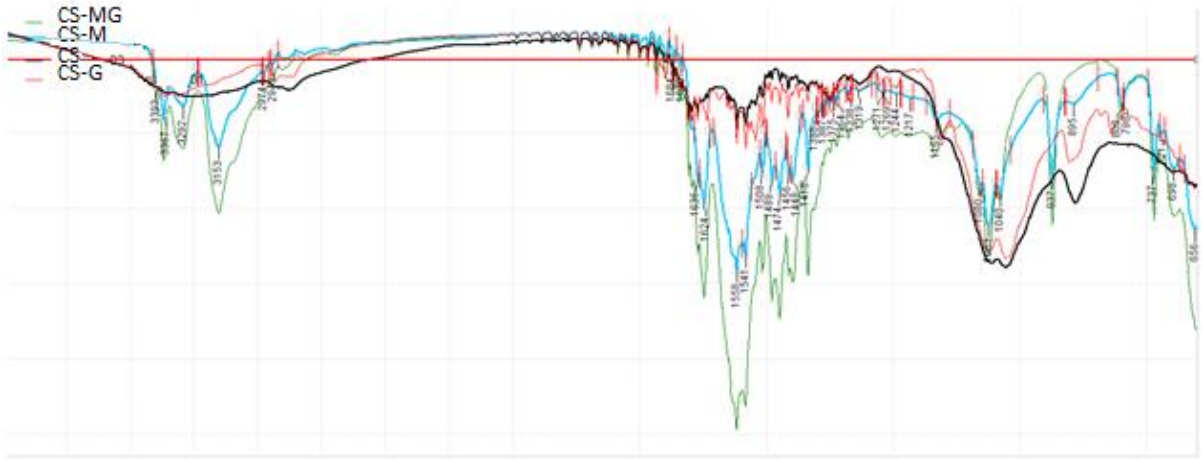

Figure 3.1. TheFT-IR spectra of the chitosan (CS) and of the loaded chitosan microparticle (CS-M, CS-G, CS-MG)

- *Particle size measurements and morphology*

The size of the chitosan loaded microparticles in wet and dry state and the data about their stability are presented in **Table 3.1**. Stable microparticles were successfully formed at all chitosan-drug ratio used. This parameter is very important because it influence other characteristics of the beads such as swelling degree and loading efficiency. The size of the microparticles ranged between 500-710 μm in wet state and between 300-480 μm in dry state. It was also observed that chitosan-metformin-glybenclamide microparticles are a bit bigger than chitosan-metformin and chitosan-glybenclamide microparticles respectively.

Table 3.1 The characteristics of the chitosan-drug microparticles

Antidiabetic drug	**Ratio Drug: CS (w/w)**	**Particle size (μm)**	
		Wet	**Dry**
Metformin	1:1	506.85±12.2	306.4±8.9
	0.75:1	686.92±9.30	319.2±10.5
	0.5:1	647.18±23.4	348.1±6.4
Glibenclamide	1:1	651.82± 15.8	368.3±10.9
	0.75:1	644.67 ±10.4	357.3± 15.7
	0.5:1	639.87±14.9	379.4±8.6
Metformin/ Glibenclamide	1:1	715.91±18.7	482.4±13.5
	0.75:1	709.45±16.2	479.1±11.4
	0.5:1	710.13±19.6	482.5±6.3

The scanning electron microscopy (SEM) revealed that chitosan microparticles (CS) have a regular, spherical shape with smooth surface, whereas upon loading the compounds, the shape becomes irregular with rough surface (**Figure 3.2**).

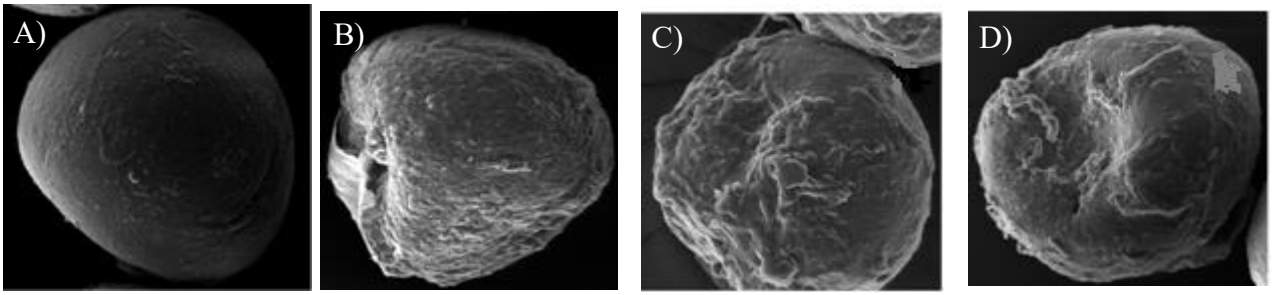

Figure 3.2. SEM micrographs for CS (A) and CS-M (B), CS-G (C) and CS-MG (D) systems

- *Swelling degree*

In distilled water the chitosan-metformin (CS-M) microparticles showed a higher swelling degree (304-380%) compared to chitosan (179%), while for chitosan glybenclamide (CS-G) systems the swelling degree was lower (53-106%) (**Figure 3.3A-C**).

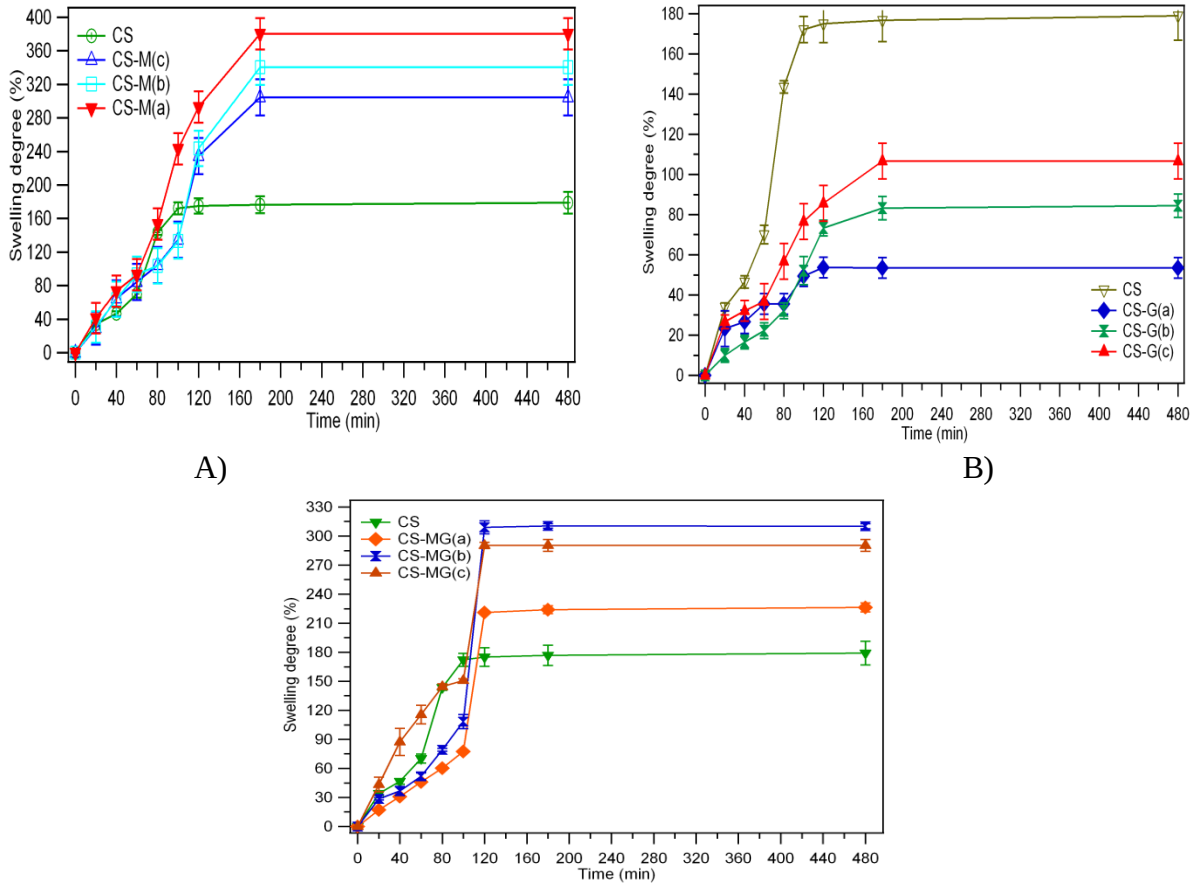

Figure 3.3. The swelling degree of CS and CS-drugs: CS-M (A), CS-G (B), CS-MG (C) at different concentrations: 30 mg (a), 22.5 mg (b) 15 mg (c) in distilled water

The binary systems (chitosan-metformin-glybenclamide) showed the highest swelling degree, which ranged between 226% (30 mg:30 mg) to 310 % (22.5 mg:22.5 mg) compared to chitosan (179%). The dynamic equilibrium was reached after 3 h and it remained at a constant value for about 8 h.

Also it was observed that the swelling degree of unitary and binary systems was higher in SGF compared to the values recorded in distilled water (**Figure 3.4 A-C**).

For CS-M the swelling degree ranged between 530% (CS-Mc) and 590% (CS-Ma) while the values recorded for CS-G ranged between 116% (CS-Ga) and 239% (CS-Gc). For binary systems (CS-MG) the swelling degree ranged between 490% (CS-MGc) and 540% (CS-MGa).

- *Loading efficiency*

The loading efficiency (LE) of the antidiabetic drugs in the chitosan microparticles, at different concentrations is shown in **Figure 3.5**. As it can be observed, glybenclamide is efficient loaded into the matrix of chitosan, regardless of the used concentration (30 mg, 22.5 mg, 15 mg).

For CS-G, the loading efficiency ranged between 96% (15 mg) and 98% (30 mg). For metformin the loading efficiency is inversely proportional to the used concentration, the highest value being obtained at a concentration of 15 mg (40%).

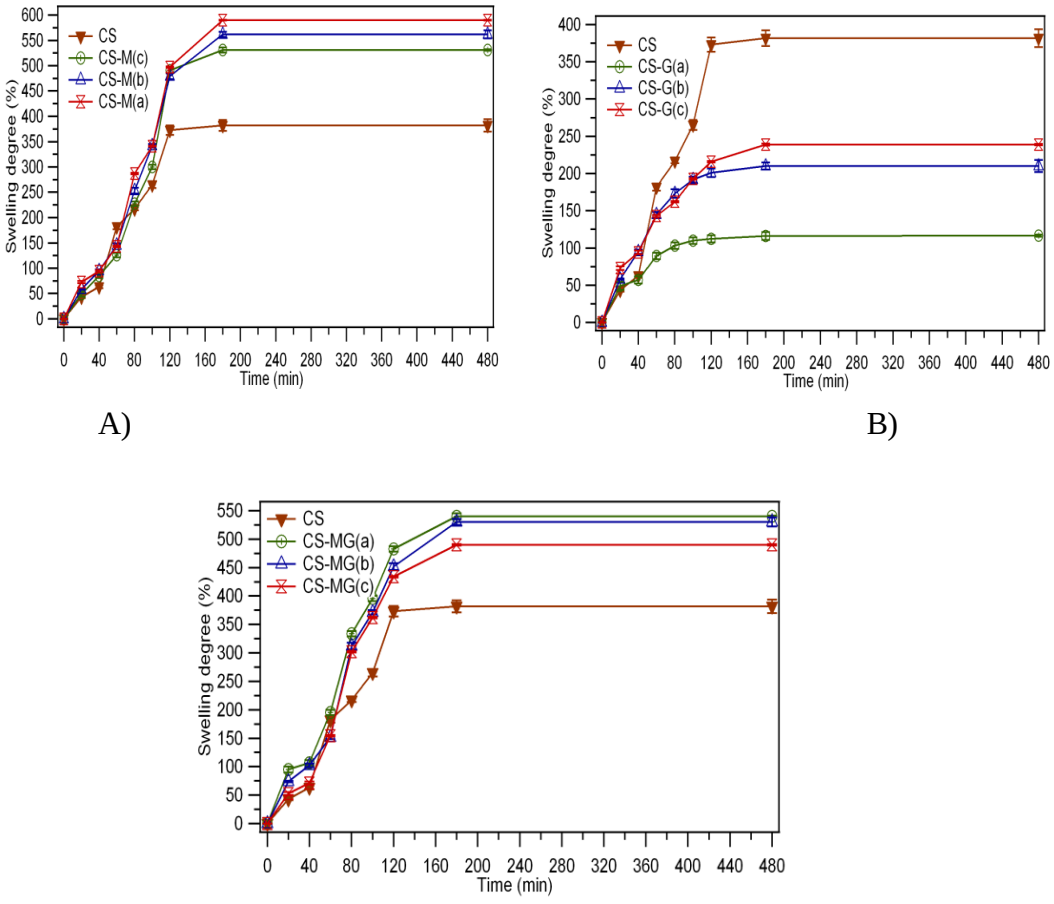

Figure 3.4.The swelling degree of CS and CS-drugs: CS-M (A), CS-G (B), CS-MG (C) at different concentrations: 30 mg (a), 22.5 mg (b) 15 mg (c) in distilled water

This can be explained by the hydrophilic properties of metformin, which facilitate the release of the drug from the polymer matrix to the aqueous medium in which the the cross-linking process is made. For binary systems (chitosan-metformin-glybenclamide), the loading efficiency was higher than the unitary systems (chitosan-metformin and chitosan-glybenclamide respectively) and inversely proportional to the used concentration. The highest percentage of loading efficiency for metformin (51%) and glybenclamide (98%) was recorded at a concentration of 15 mg of each drug.

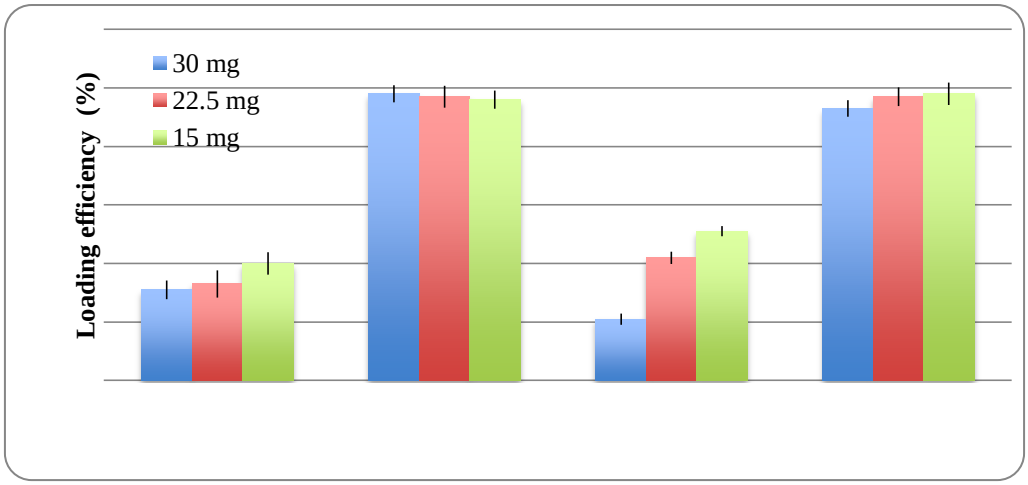

Figure 3.5.The loading efficiency (%) of metformin, glybenclamide, metformin-glybenclamide in chitosan polymer matrix at different concentrations.

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3.2.3.2. Study of the in streptozotocin-induced diabetic rats

On ultrathin cuts, we adequately identified the main components of the filtration barrier: endothelial cells (ECs), glomerular basement membrane (GBM), and podocytes (P). Foot processes of podocytes were applied on the GMB that was thickened, but uniform.

We identified two morphologically distinctive types of podocytes, “dark” and “light” (**Figure 3.6**). The “light” podocytes were larger and peculiarly rich in Golgi complexes (**Figure 3.6C**); they had extremely rare foot processes emerging directly from the cell body, and applied on the GBM. These “light” podocytes were thus considered as positive for foot processes effacement.

Both types of podocytes that werefound had deeply invaginated nuclei but those in “light” podocytes were euchromatic, while those in “dark” podocytes were rather heterochromatic with condensation of chromatin (**Figure3. 6**).

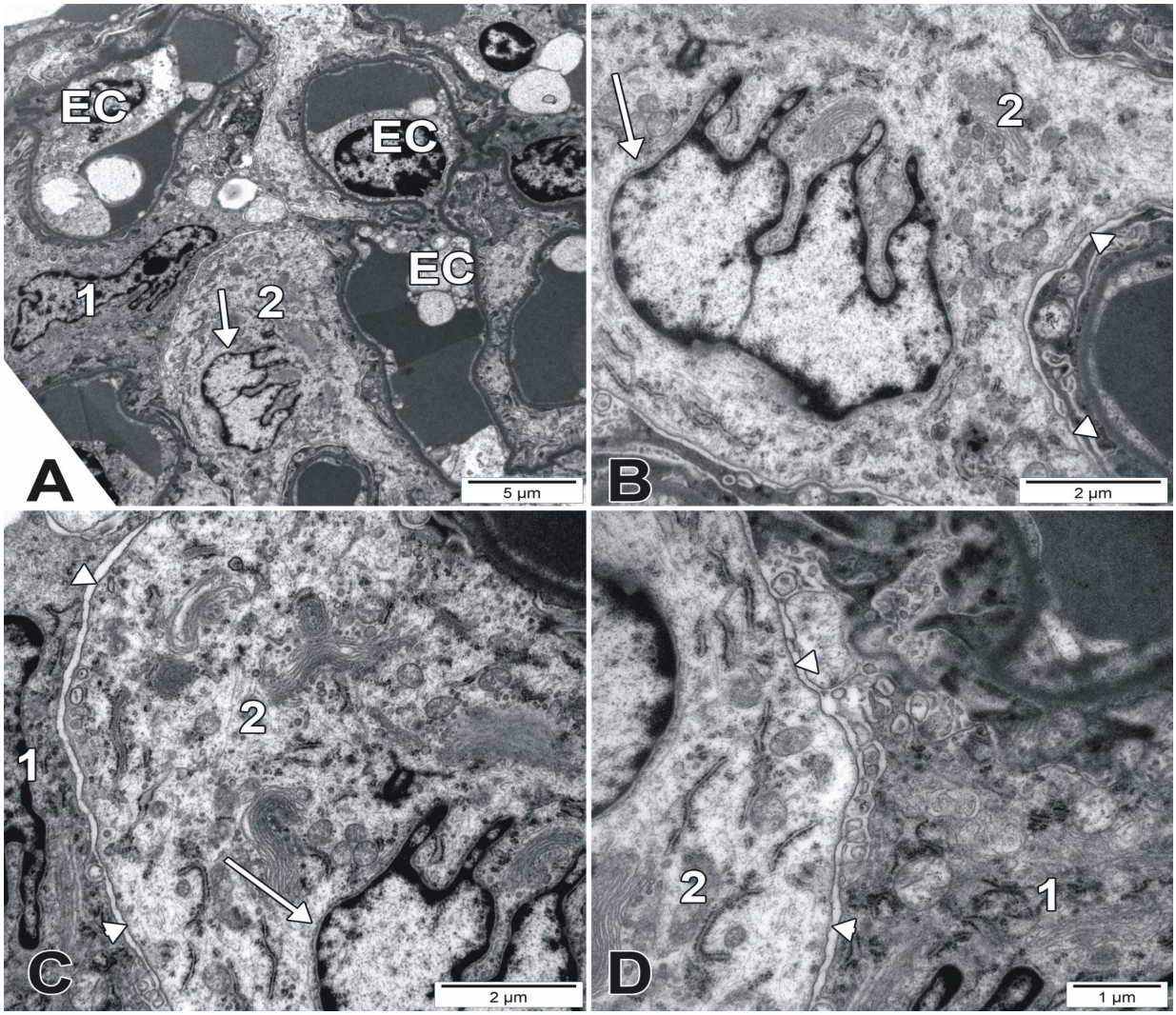

Figure 3.6. Ultrathin cut of diabetic rat kidney. Within a renal glomerulus, "dark" (1) and "light" (2) podocytes are found (A), both presenting deep nuclear invaginations; however, the nucleus of the "dark" podocyte is also positive for karyopyknosis, while the nucleus of the "light" podocyte (arrow, A–D) is euchromatic. Almost complete foot processes effacement (arrowheads, B–D) was found in the "light" podocyte. EC: Endothelial cell.

Loss of cell processes with rounding of cells and chromatin condensation, belong to the morphological pattern of apoptosis, suggesting that the two types of podocytes were in different stages of apoptotic processes.

This staged pattern of podocytes morphologies was reinforced by intermediate morphologies of podocytes, with most foot processes effaced, and presented peculiar nuclear morphologies – partly normal, euchromatic, and partly positive for karyopyknosis and shrinkage (**Figure 3.7**).

We also found swollen podocytes with large cytoplasmic vacuoles and effaced foot processes. Slit diaphragms, seemingly unaltered, were double or single (**Figure 3.8**). Swollen mitochondria, whorls, and lipid inclusions were occasionally found within podocytes. On the opposite side of the GBM, mitotic ECs were found, as demonstrated by the evidence of centrioles (**Figure 3.9**).

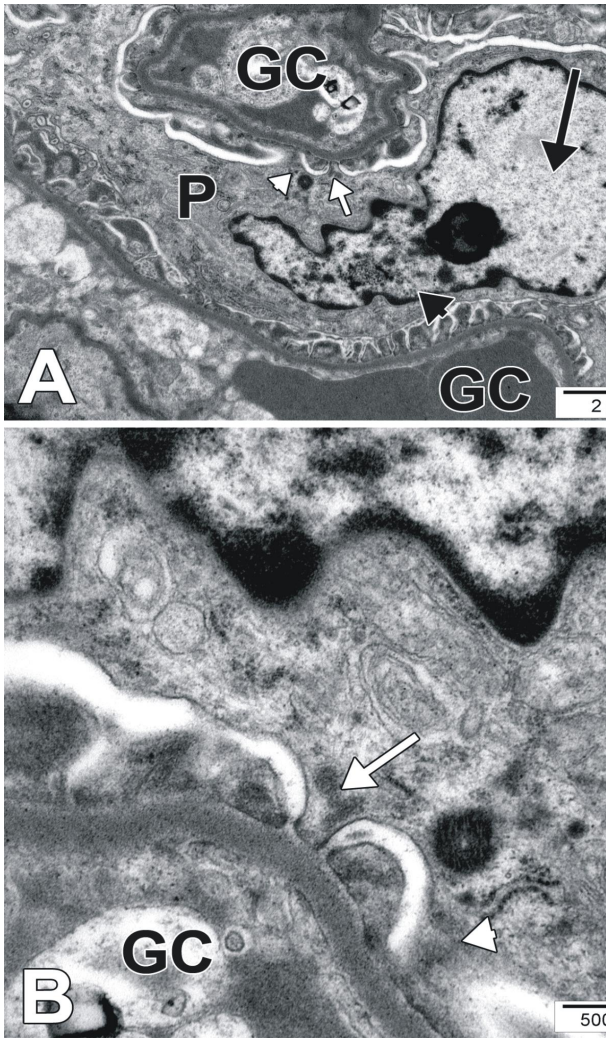

Figure 3.7. Ultrathin cut of diabetic rat kidney. (A) A podocyte (P) is identified between glomerular capillaries (GC). The nucleus of the podocyte is partly euchromatic (black arrow) and partly apoptotic (black arrowhead), with karyopyknosis and blebbing. The podocyte displays few foot processes (white arrow and arrowhead in A, detailed at higher magnification in B).

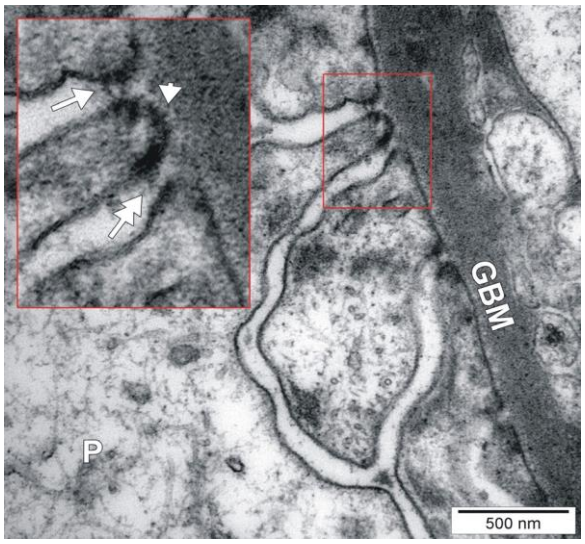

Figure 3.8. Ultrathin cut of diabetic rat kidney. A podocyte (P) foot process projects on the glomerular basement membrane (GBM). Cell–matrix interactions (arrowhead) are indicated. On opposite sides of the foot process double- (arrow) and single- (double-headed arrow) slit diaphragms are identified.

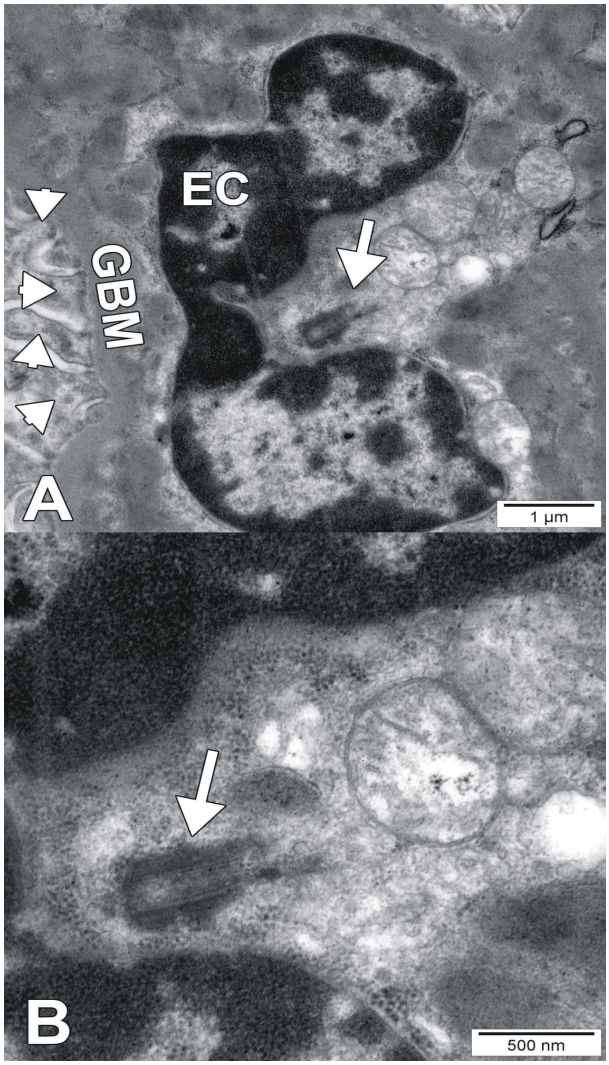

Figure 3.9. Ultrathin cut of diabetic rat kidney. (A) On each side of the glomerular basement membrane (GBM) foot processes of podocytes (arrowheads) and an endothelial cell (EC) are identified. The EC mitosis is suggested by the EC centriole (arrow). (B) The centriole (arrow) is detailed at higher magnification.

3.2.4. Discussions

In a previous TEM study on diabetic mice (18 months of open hyperglycemia) were not identified apoptotic features of podocytes, being presumed that they were eliminated in the urine. The authors speculated a staged morphological evolution of podocytes, beginning with podocytes hypertrophy and swelling. The peculiar rich content of podocytes in Golgi complexes was also found in that experiment, suggesting the pattern to be unrelated to the duration of the disease. Swollen podocytes were thought to occur as a later aspect of cell injury. These morphological aspects, as well as the lipid inclusions and effacement of foot processes were also found in the present study. However, in that study the nuclear morphology of podocytes was overlooked. Attention was paid to the damaged processes of podocytes, in order to support a hypothesis of podocyte atrophy (Mandache and Penescu, 2012).

If in TEM a podocyte process is not identified on the GBM, a cell process located within the urinary space and not applied on the GBM cannot be firmly diagnosed as being a podocyte process.

The density of podocytes is reduced in diabetic nephropathy, and podocyte apoptosis and detachment play pivotal roles in this event. However, available data are scarce and a time

sequence of events is unclear; viable podocytes detachment as well as podocytes detachment because of their apoptosis being concurrent theories (Spurney and Coffman 2008). *We found here early signs of podocyte apoptosis in cells with effaced foot processes; these disappear later on during the apoptotic process. Podocytes apoptosis and detachment appeared us as concomitant processes, and may not succeed in a temporal sequence.*

Viable podocytes were assessed in TEM in the urine of streptozotocin-induced diabetic rats, and further grown on culture media (Petermann et al., 2004). *If foot processes effacement is considered as an apoptotic event, according to our theory, the differences between in vivo and in vitro conditions should be accounted for podocytes evolution, toward death or survival.*

Podocyte apoptosis in the experimental diabetic glomerulopathy becomes detectable after four months of disease and is preceded by glomerular hypertrophy that induces compensatory podocyte hypertrophy, and associates podocytopathy and proteinuria. Podocyte apoptosis finally leads to podocytopenia and drives progression to expansion of the mesangium and glomerulosclerosis. Mesangial and endothelial cells loss are known phenomena associated with diabetic nephropathy (Menini et al., 2007). Our TEM results raise discussions on this pathogenic sequence, as signs of ongoing podocyte apoptosis and foot processes effacement were assessed at three weeks of disease duration. Jung et al. suggested recently that apoptosis occurs differentially in diabetic nephropathy, being faster in hypertrophic glomeruli (Jung et al., 2012).

Podocytes lacking insulin receptors are known to have increased apoptosis (Rask-Madsen and King, 2010). Insulin regulates the expression of vascular endothelial growth factor A (VEGF-A) in podocytes via the insulin receptor (Hale et al., 2013). Podocytes are the main source of VEGF in renal tissue, with paracrine and autocrine activity on endotheliocytes and podocytes (Fogo et al., 2009). Mesangial cells also produce VEGF and express VEGF receptors (Iijima et al., 1993). Lack of VEGF-A in podocytes leads to loss of all major cell types in the glomerulus: endotheliocytes, mesangial cells and podocytes (Eremina et al., 2003; Eremina et al., 2006), but not exclusively. It is known that VEGF stimulates vascular endothelial cell proliferation (Khan et al., 2011). In this regard, evidence of proliferating endotheliocytes in our samples may denote that even though there is podocyte loss at three weeks of diseases, the remaining podocytes continue to exert their influence on endotheliocytes. A possible role for adiponectin can be speculated here, as time as it was shown that it suppresses the VEGF-stimulated endothelial cell migration (Mahadev et al., 2008) and glomerular endothelial cells were found positive for constitutive adiponectin (Tang et al., 2010).

Pyknosis and karyorrhexis are common features of both apoptosis and oncosis (Van Cruchten et al., 2002). Karyorrhexis is not pathognomonic for apoptosis. Ischemic cell death is characterized by swelling; oncosis is cell death withswelling that is usually accompanied by karyolysis (Majno and Joris 1995).

The morphological appearance of necrosis is frequently that of oncosis; oncosis (cell swelling), together with swelling of organelles and plasmallema rupture are main morphological traits of necrosis (Kroemer et al., 2009). The Nomenclature Committee on Cell Death (NCCD) limits the use of the term “oncosis” as it overlaps with necrosis, and with a partial apoptosis evolving into necrosis, and indicates that the term “necrosis” should be

kept for “historical reasons”. The NCCD recommends not to usethe term of “apoptonecrosis”. In these regards, we kept the term of “podocytes apoptosis”, even though a degree of oncosis in podocytes with effaced foot processes was estimated.

This study partly reached its goal, identifying podocytes with early apoptotic changes, with foot processes effacement and karyopyknosis. However, there are limitations of the study, as in TEM it is quite difficult to perform a quantitative analysis and thus the observed podocytes alterations cannot be firmly related to diabetes

Further molecular studies should clarify whether or not different cell death modalities act in eliminating podocytes. The relative contribution of programmed versus mitotic cell death to podocyte loss is unknown. Murine double minute-2 (MDM2)-dependent mitotic catastrophe was found mediating podocyte loss in adriamycin nephropathy.

The main aim in the drug therapy of any disease is to attain the desired therapeutic concentration of the drug in plasma or at the site of action and maintain it for the entire duration of treatment. Drug targeting means delivery of the drug-loaded system to the site of interest. Drug carrier systems include polymers, micelles, microcapsules, liposomes and lipoproteins to name some. Different polymer carriers exert different effects on drug delivery. Chitosan comes from the deacetylation of chitin, a natural biopolymer originating from crustacean shells and it is a biocompatible, biodegradable, and nontoxic natural polymer with excellent film-forming ability. It is also able to react with polyanions giving rise to polyelectrolyte complexes. In the regard of these features, chitosan has become a promising natural polymer for the preparation of microspheres/nanospheres and microcapsules. The techniques employed to microencapsulate with chitosan include ionotropic gelation, spray drying, emulsion phase separation, simple and complex coacervation (Mitra and Dey, 2011).

Previous studies have reported that braykinin B2 receptor (Bdkrb2) involves in high glucose-induced renal and podocytes injuries. However, there have been some studies with contradictory results that Bdkrb2 has a protective effect on hyperglycemia-induced injuries in vivo and in vitro. The post-transcriptional regulatory mechanism of microRNA (miR) in high glucose-treated podocytes by targeting Bdkrb2 signaling in vitro suggested that miR-204-3p may play a protective role in high glucose-induced apoptosis and dysfunction in podocytes through down-regulation of Bdkrb2 (Han et al., 2019).

3.2.5. Final remarks

New binary polymeric systems based on chitosan-metformin-glybenclamide have been developed and physico-chemical characterized in order to improve the pharmacokinetic and pharmacological profile of the used antidiabetic drugs. These polymeric systems showed improved swelling degree compared to chitosan and unitary polymeric systems, chitosan-metfomin and chitosan-glybenclamide. Also the loading efficiency of the antidiabetic drugs was higher for binary systems compared to unitary systems, which means that these formulations could be a good therapeutic alternative for the management of diabetes mellitus treatment.

Further ultrastructural studies should combine immunohistochemistry to firmly relate the partly apoptotic phenotype of podocytes we found here with the molecular machinery of apoptosis.

3.3. From developmental endocrinology to clinical research

3.3.1. Introduction

Initially, prostaglandins were highlighted in the seminal plasma and then in the rest of the male and female genital tract (Abatiello et al., 1975; Frungieri et al., 2007). Numerous actions have been identified, parti- cularly on the female genital tract (luteolysis, myometrial contractions, etc.) and therefore many prostaglandin analogs were synthesized as Meteneprost, Luprostiol, Gemeprost, etc., which are used for their ocitocic action in human therapy (Ruan et al., 2011; Purohit et al., 2012). Additionally, many prostaglandin analogs with luteolytic action (Fluprostenol, Prostalene, etc.) are used in veterinary medicine (Murta et al., 2014; Volkmann et al., 2011). Prostaglandin analogs, firstly used in obstetrics and gynecology, are now widespread in both sexes, especially in the treatment of gastric and duodenal ulcers, glaucoma, etc. (Tanaka et al., 2002; Alam et al., 2009; Freitas and Fardilha2014). Therefore, we tried to highlight the effects of repeated administration of Cloprostenol and CIPG isopropyl ester (both prostaglandin F2α analogs) for the male gonad.

Personal contribution – published papers:

Sava A, Motoc AGM, Stan CI. Electron microscopic aspects of the effects of certain prostaglandin analogs on mouse testes. Rom J Morphol Embryol 2015; 56(2): 771-775.

3.3.2. Materials and methods

In our experiment, we used Cloprostenol and CIPG isopropyl ester, synthesized in National Institute for Chemical-Pharmaceutical Research and Development (ICCF), Bucharest, Romania. We used three groups of white, male mice, aged 50–80 days, kept in standard laboratory conditions, which received the same feed. Each group included 12 mice.
The first batch was the control group and received no substance at all.

The second batch received 25 μg/kg of Cloprostenol dose per body/day, intraperitoneal administration (a single dose per day) on a daily basis for a four weeks period of time.

The third batch received a 25 μg/kg CIPG isopropyl ester dose per body/day intraperitoneal administration (a single dose per day) on a daily basis for a four weeks period of time.

After 7, 14 and 28 days of treatment, we sacrificed four animals in each of the batches by cutting their carotid arteries. Fragments taken from the testicle were immediately fixed by means of immersion in a 2% glutaraldehyde solution in 0.1 M sodium cacodylate buffer at pH 7.2 for six hours at 40C. The fragments were de- hydrated in alcohol solutions of a gradual concentration from 50% to absolute alcohol, and afterwards immersed in propylene oxide and then immersed in EPON 812 in gelatin capsules. Polymerization was performed in the thermostat.

Semithin sections were performed with Reichert Om-U2 ultramicrotome and caught on the blade, stained with Toluidine blue, examined and photographed with the Zeiss Axio Scope microscope on Kodak film. After selecting the fields for the electron microscopy examination, thin sections were obtained in the same ultramicrotome, which were caught on Fornvar pre-treated grids. The thin section contrasting was performed by means of uranyl acetate and lead citrate. The examination and the photography were performed with a Philips C100 electron microscope (Philips, Eindhoven, The Netherlands).

3.3.3. Results

With respect to the control group, we observed a fragment of a Leydig cell with smooth endoplasmic reticulum during the ultrastructural examination. We have also noted structures, which were electron-opaque, relati- vely homogeneous, bound by a membrane unit corres- ponding to lysosomes (**Figure 3.10**). In the depression of Sertoli cells, we observed 1st and 2nd order spermatocytes, spermatides and maturing sperm (**Figure 3.11**). At all sacri- ficed animals did not show any changes of the male gonad.

The batch subjected to our experiment showed the first changes after a week of treatment. The cross section revealed the height increase of endothelial cells.

The swollen endothelial cells showed a more abundant cyto- plasm with irregularly shaped nuclei, with a shrunken aspect, dense chromatin, demonstrating a toxic effect on the endothelium (**Figure 3.12**).

In some capillaries, the con- gestion of erythrocytes that clump together “in rouleau” formation is important, demonstrating a slow flow (**Figure 3.13**).

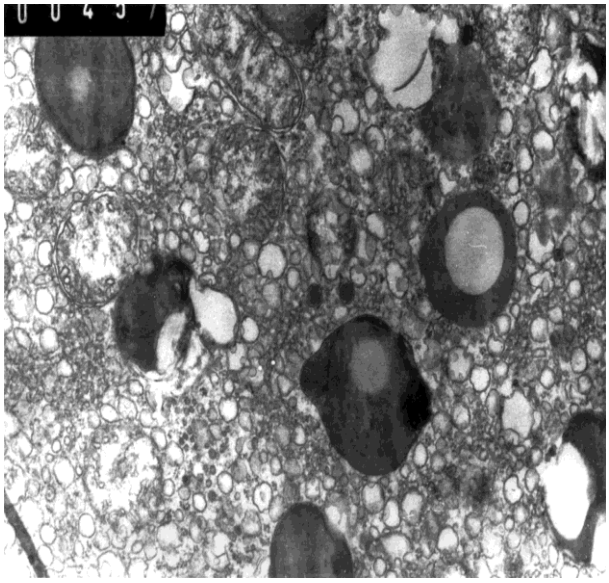

Figure 3.10. Mouse testicle control group after two weeks of treatment, ×15 000.

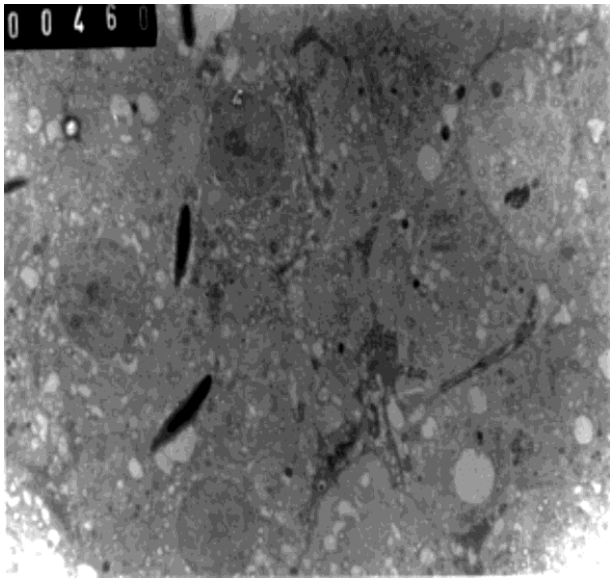

Figure 3.11. Mouse testicle control group after two weeks of treatment, ×5700.

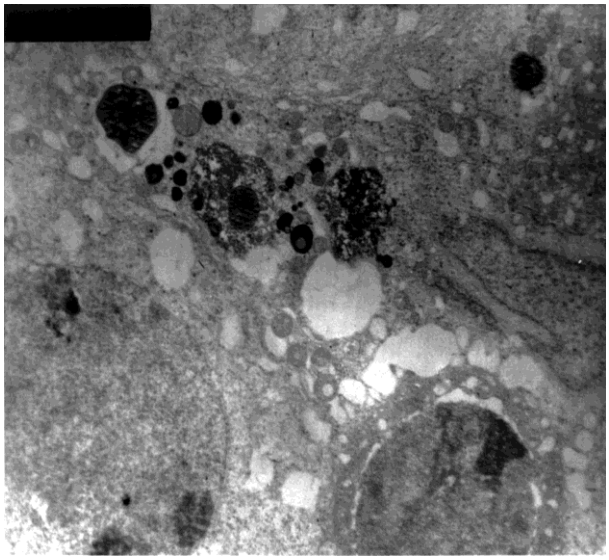

Figure 3.12. Animal treated with Cloprostenol after one week of treatment, ×5700.

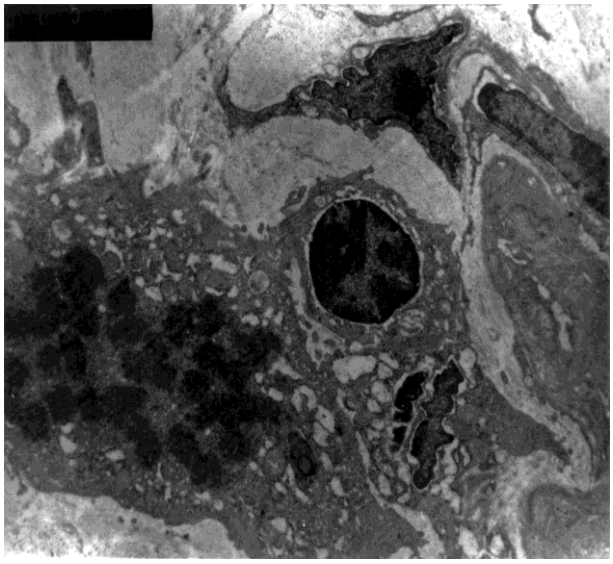

Figure 3.13. Animal treated with Cloprostenol after one week of treatment, ×5700.

Two weeks after the treatment, we have mainly selected areas where the interstice was displayed as being enlarged and with a hyaline content. Electron microscopic in these areas, the interstice display cells with ultrastructural features of a macrophage highly loaded with residual bodies, an ultrastructure which characterizes an intense process of phagocytosis (**Figure 3.14**). The moderate electronic density of these bodies could advocate in favor of the phagocytosis of apoptotic bodies.

Furthermore, the interstice displayed a large number of fibroblasts. In addition to fibroblasts, fibrocytes occur without obvious changes. Capillaries displayed very thin endothelium areas, sometimes even showing discontinuities (widening of intercellular spaces – **Figures 3.15 and 3.16**).In the seminiferous tubules, inside the adluminal area, the frequency of structuring sperm is small. Spermatids are rare while in the cytoplasm of Sertoli cells lysosome-like dense bodies frequently occur (**Figure 3.17**).

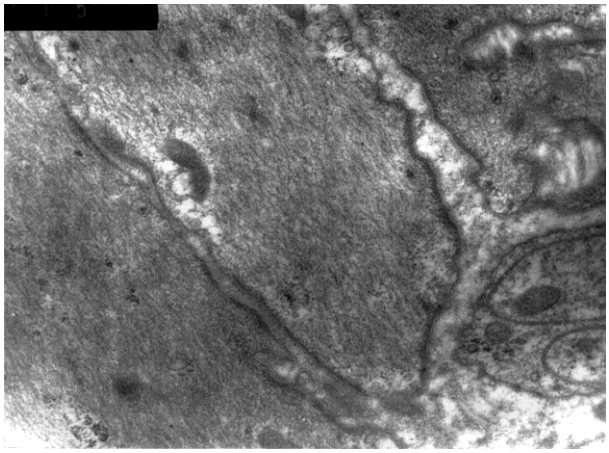

Figure 3.14. Animal treated with Cloprostenol after two weeks of treatment, ×15 000.

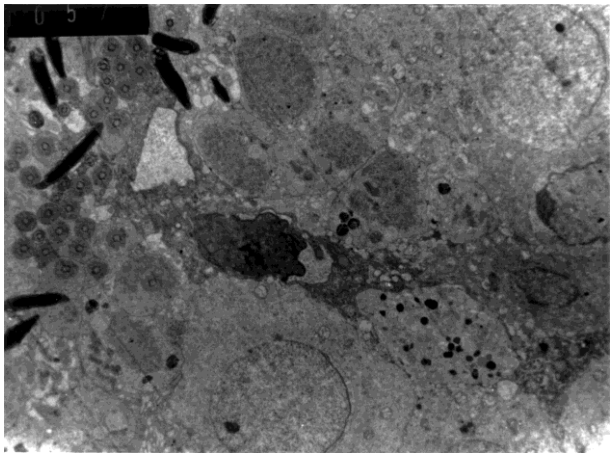

Figure 3.15. Animals treated with CIPG isopropyl ester, after two weeks of treatment, ×3400.

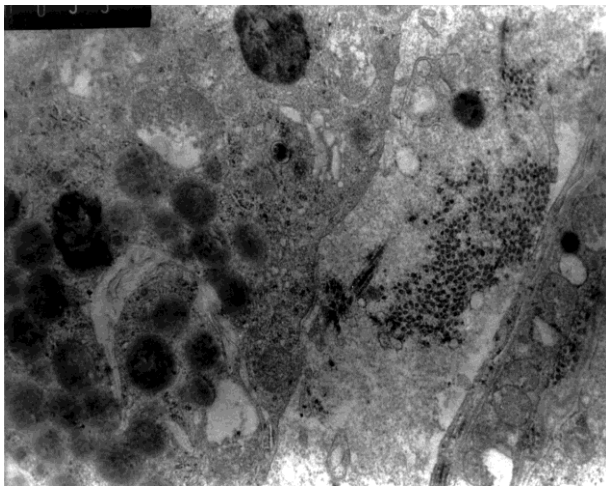

Figure 3.16. Animals treated with CIPG isopropyl ester, after two weeks of treatment, ×9100.

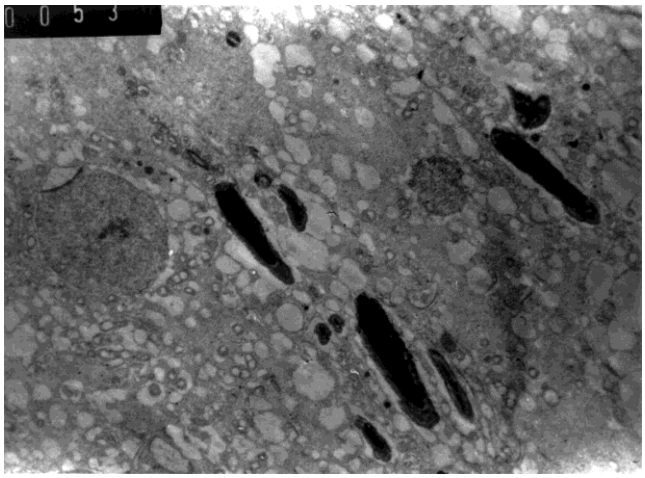

Figure 3.17. Animal treated with Cloprostenol after two weeks of treatment, ×3400.

After four weeks of treatment, the changes observed after two weeks are more visible. The lumen of capillaries contains erythrocytes that clump together "in rouleau" formation; the endothelial cells have a well-represented cytoplasm rich in organelles (**Figure 3.18**). The Leydig cell is rich in mitochondria and includes a smooth endoplasmic reticulum and rare lipid inclusions (**Figure 3.19**).

After four weeks of treatment, there also was evidence of spermatocytes with condensed cytoplasm and nuclei with a high chromatin condensation, ultrastructural aspects suggesting the early stage of apoptosis (**Figure 3.20**). Among spermatides and in relation to extensions of the Sertoli cells, we noticed structuring sperm with obvious acrosomalvacuole. Nevertheless, it was impossible to observe the sperm with acrosome formation and with a chromatin condensation level that is appropriate for this stage of maturation (**Figure 3.21**).

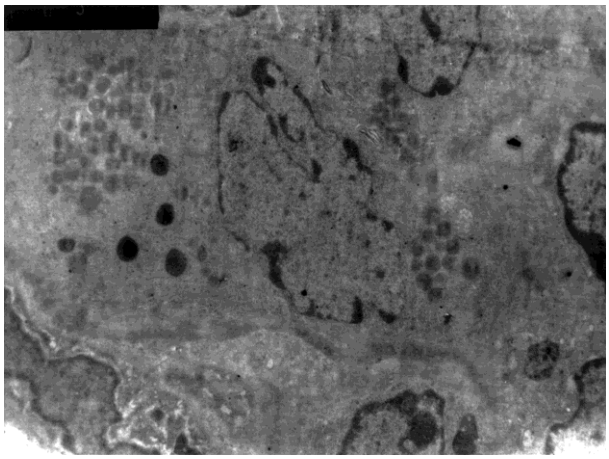

Figure 3.18. Animal treated with CIPG isopropyl ester after four weeks of treatment, ×5700.

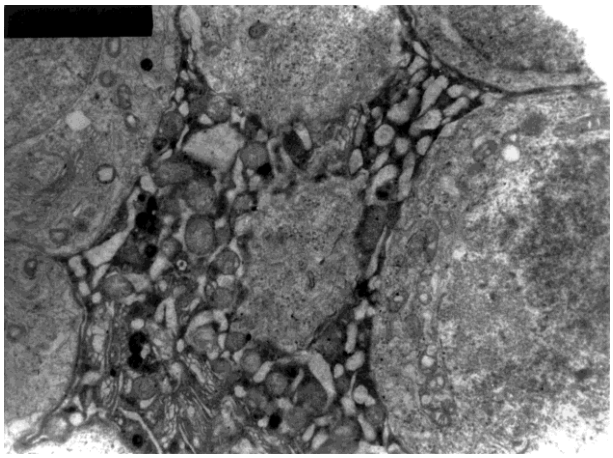

Figure 3.19. Animal treated with CIPG isopropyl ester after four weeks of treatment, ×7100.

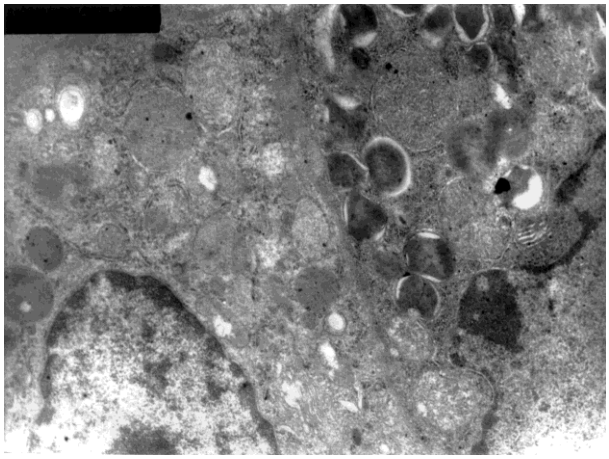

Figure 3.20. Animal treated with Cloprostenol after four weeks of treatment, ×9100.

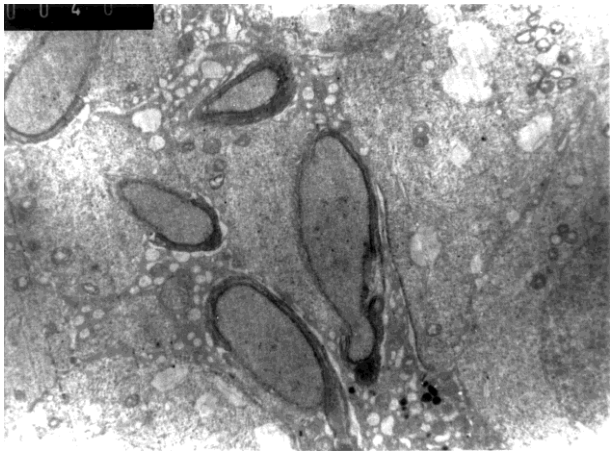

Figure 3.21. Animal treated with Cloprostenol after four weeks of treatment, ×4500.

3.3.4. Discussion

Our results are similar to the results of other researchers regarding the inhibitor action of the prostaglandins on the spermatogenesis. In this way, Abatiello proved that the prostaglandins F1α and F2α have an important action in decreasing spermatogenesis during the meiotic phase (Abatiello et al., 1975). *Histopathological examination revealed increased numbers of exfoliated immature germinal cells in the seminiferous tubules and epididymi of prostaglandin- treated animals.* The effects produced by prostaglandin F2α are similar with the effects produced by the anti-spermatogenic factor indenopyridine CDB-4022 (Selvaraju et al., 2010; Kumar et al., 2014).

The seminiferous tubules were devoid of spermatozoa and contained only Sertoli cells, spermatogonia, sperma- tocytes and occasionally spermatids; several multinucleated giant cells were observed in the lumen of the tubules. The Leydig cells were atrophied (Jarad et al., 2011).

Although some researchers suggest the fact that the testicle has reduced effects to induce inflammatory reactions (Korfanty et al., 2014; Saeed et al., 2009; Venza et al., 2001), our results on the testicle show the capacity of the testicle to sustain an inflammatory action by important influx of macrophages (Zhang et al., 2006; Farhat et al., 2011).

It is important too, to emphasize the effects of the prostaglandin F2α on the microcirculation on the level of the testicle. Our data suggest an accumulation of fluid in the interstitial space at the same time with the growth of the diameter of the blood capillaries as well as a decrease of the endothelial cells from the capillary wall. After four weeks of treatment, one can notice even the destruction of some capillary vessels (Parent and Fortie, 2005).

The effects of the prostaglandin F2α and of their analogous on the microcirculation are controversial. In this way, some authors describe a vasoconstrictor action of some prostaglandin F2α on the kidney microcirculation. The primary sites of action of 8-*epi*-prostaglandin F2α are in preglomerular and possibly mesangial smooth muscle, where it exerts potent constrictor effects. These actions result in reductions in renal perfusion and

probably in glomerular capillary surface (Lazarus et al., 2004; Kotarska et al., 2013; Zhang et al., 2013). In rat renal circulation, the constrictor effects of 8-*epi*-PGF2 are caused by its activation of thromboxane receptors (Schell et al., 2010). The same vasoconstrictor effect on the cutaneous (Zerani et al., 2011) or pulmonary (Rowley et al., 2005) microcirculation is noticed by some authors.

Other researchers emphasize a vasodilator effect of prostaglandin F2α on the synovial or ophthalmic micro- circulation (Caton and Crowshaw 1988; Einarsson 1981; Hild et al., 2001). Singh and Dominic demonstrated that the administration of prostaglandin F2α caused marked suppression of spermatogenesis and significant reduction in the weights of the testis, epididymis and accessory sex glands (Singh and Dominic 1986), like Winnall, too (Winnall et al., 2007). Our results suggest a slight vasodilator effect of prostaglandin F2α on the testicular microcirculation accompanied with a retraction of the endothelial cells from the capillary wall. Similar results were obtained by Takahashi and Sada (Takahashi et al., 1992; Sada et al., 1987).

Another interesting aspect of our study is related to a possible action inducting the apoptosis exerted by the prostaglandin F2α on the mouse testicle, just like other results from the medical literature. Thus, Zannoni report the apoptotic action of the prostaglandin F2α on the porcine corpus luteum while Wang ascertain the same action on the rodent corpus luteum (Zannoni et al., 2007; Wang et al., 2003). The mechanisms through which the prostaglandin F2α sti- mulates the apoptosis are not yet elucidated, even some theories are proposed (Pointis et al., 2010).

P38 mitogen-activated protein kinase (MAPK) plays a crucial role in regulating signaling pathways implicated in inflammatory processes leading to diabetic nephropathy (DN). A recent study shows that at the end of the 8-week experiment, renal pathological changes and the activation of the p38 MAPK signaling pathway and inflammation in the diabetic kidney were attenuated. This molecule significantly prevented type 2 diabetes induced kidney injury characterized by renal dysfunction and pathological changes. The protective mechanisms are complicated but may be mainly attributed to the inhibition of the p38 MAPK signaling pathway and inflammation in the diabetic kidney (Peng et al., 2016).

The first clinical sign of diabetes is polyuria. The prostaglandin E-2 (PGE(2)) receptor EP3 antagonises arginine vasopressin (AVP)-mediated water reabsorption and its expression is increased in the diabetic kidney. The contribution of EP3 to diabetic polyuria and renal injury is recently discovered to take place by inhibiting expression of aquaporins and that it promotes renal injury during diabetes. It may prove to be a promising target for more selective management of diabetic kidney disease (Hassouhneh et al., 2016).

3.3.5. Final remarks

The prostanoid analogs we used, Cloprostenol and CIPG isopropyl ester, have similar actions on male gonad in mice. These analogs induced significant changes in the evolution of the spermatogenesis and spermiogenesis. In relation to the treatment duration there were cellular changes suggesting apoptosis in different stages. With regard to spermiogenesis, the ultrastructural aspects indicate a decrease of the sperm structuring processes, especially in the acrosomal apparatus and chromatin. Referring to aspects related to the interstice, capillary

endothelium had a surprising ultrastructure suggesting an active transcytoplasmic transport under the action of particular present stimuli. The accumulation of interstitial fluid as an important influx of macrophages is obvious. The Leydig cells suggest a stimulation of the androgen synthesis according to the increasing duration of treatment and of the administered dose.

CHAPTER 4. THE PROPHECY OF SURVIVAL - ADVANCES IN TUMOURS FOLLOW UP

4.1. State of the Art

Colorectal cancer (CRC) remains a leading cause of morbidity and mortality in many developed countries. Globally, CRC contributes to 8.9% of all cancers, is the third leading cause of cancer death in men and women in the United States and the second leading cause of cancer-related death in the Western world (Siegel et al., 2014). Selection of the most beneficial treatment in CRC remains a challenge and is hindered by a lack of predictive and prognostic markers.

The first known attempt to find markers for malignancy was made 2000 years ago and is described in an Egyptian Papyrus where breast cancer was distinguished from mastitis (Hussain and Camilleri 2007). Incidentally the first tumor marker in modern medicine was identified by Bence-Jones, who in 1846 detected a heat precipitate in samples of acidified urine from patients suffering from "Mollities ossium" (Sharma, 2009).

The first modern tumor marker used to detect cancer was Human Chorionic Gonadotropin (HCG) the substance doctors look for in pregnancy tests. A high level of HCG in the blood may be a sign of a cancer of the placenta called Gestational Trophoblastic Disease (GTD). This cancer continues to produce HCG. Cancer Antigen-125 was introduced in 1983 by Bast for ovarian cancer (Barak et al., 2004). At present there are hundreds of tumor markers available and currently studies are going on to determine the accuracy of tumor marker in cancer detection, measurement of tumor treatment response and determination of recurrence (Babu et al., 2012).

The discovery of tumor markers occurred after the demonstration of tumor-specific transplantation antigens in chemically or virally induced tumors in syngenic rodents. The history of currently used tumor markers began in the 1940s, the first discovered being alpha-fetoprotein in 1956, followed by that of carcinoembryonic antigen in 1965. Since then the range of tumor markers has widened continously.

While analysis of individual genes in predicting response to therapy and disease prognosis has on occasion proven useful, it is becoming increasingly apparent that response to therapy and disease progression are largely driven by complex, multifaceted pathways; hence, analysis of any one single marker is unlikely to accurately predict response or progression with sufficient resolution and consistency. The use of pharmacogenetic profiling in CRC to predict clinical response, progression, and toxicity is still a developing field. To

make progress, there must be more coordinated evaluation of these markers before genetic information can become a routine part of clinical CRC treatment (Wilson and Ladner 2007).

The rejuvenation of the cancer stem cell hypothesis is another exciting area of research that must be pursued. A growing body of evidence suggests that cancers develop from a small subset of cells with self-renewal properties analogous to organ stem cells that acquire epigenetic and genetic changes required for tumorigenicity or may represent proliferative progenitors that acquire self-renewal capabilities (Clarke and Becker 2006; Wilson and Ladner 2007).

The continuing evolution of high-throughput technologies such as microarray gene profiling, proteomic profiling, and the newly developed metabolomics field will improve the resolution and sensitivity with which we can detect malignant markers (Eschrich et al., 2005).

Quantitative and qualitative assessment of prognostic factors in patients has particular importance, both preoperative, but most especially postoperative. The following paramters are of primary importance: intra and extramural invasion, the distance between the tumor and the mesorectum edge, the involvement of the lymph nodes, of the blood vessels, the peritoneum and of the sphincter complex. Weighing all factors, we have to take the best decision regarding the necessity of pre and postoperative neoadjuvant therapy as well as the decision on the appropriate surgical techniques (Bosari et al., 1992, Hall et al., 1992, McDougall et al., 2002). The VEGF pathway plays a major role in tumor growth and angiogenesis. The formation of new blood vessels carrying oxygen, nutrients, growth factors, and hormones is an important factor required for proliferation of solid tumors.

High-resolution magnetic resonance brings relevant data on the normal anatomical aspects of the rectum as well as on the possible pathological changes. The magnetic resonance imaging (MRI) investigations, in our study, bring us important information about the location and extent of the tumor, if it affects the mesorectum, its relations with the peritoneum, with surrounding fascia and organs, highlighting the rectal arteries and the neoformation vessels.

Solid tumors are known to progress through two distinct phases of growth – the avascular phase and the vascular phase. Endothelial cells change their dormant state in fast growing state, as a result of the signals received from the tumor cells and the associated inflammatory cells and there is known a large number of components which induces angiogenesis.

The transition from the dormant avascular state to the vascular state, wherein the tumor possesses the ability to invade the surrounding tissue and the metastasis to distant parts of the body depends upon its ability to induce new blood vessels from the surrounding tissue to sprout towards and then gradually penetrating the tumor, thus providing it with an adequate blood supply and microcirculation. In order to accomplish this neovascularization, it is now a well-established fact that the tumors secrete a number of diffusible chemical substances into the surrounding tissues and extracellular matrix (McDougall et al., 2002).

For these reasons, the microvascular intratumoral density was suggested as a criterion for prognosis in different types and locations of cancer, being used in evaluating the evolving of the breast cancer (Bosari et al., 1992; Hall et al., 1992), renal cancer (Sabo et al., 2001; Lidgren et al., 2005), rectal cancer (Sökmen et al., 2001), bladder (Bakkar et al., 2003; Goddard et al., 2003) or prostate cancer (Bono et al., 2002; Du et al., 2003).

4.2. Colorectal evaluation, from an anatomic perpective

4.2.1. Introduction

Colorectal cancer has become one of the most common forms of malignancy and of these, about a third of cases are localized in the rectum. Rectal cancer has a worse prognosis because it is characterized by a rate of local recurrence and a higher metastasis presence at the moment of diagnosis (Sagar, 1996). The fundamental principle of the curative treatment of these tumors remains surgery, but even that has undergone major changes over time as now is considered to be necessary a multidisciplinary team. The main purpose is individualizing the therapeutic strategy, consistent with patient and tumor characteristics (Salerno, 2006; Cervantes, 2007). Both preoperative, but especially postoperative, particular importance has quantitative and qualitative assessment of prognostic factors of patients, and in this respect intra and extramural invasion, the distance between the tumor and the mesorectum edge, the involvement of the lymph nodes, of the blood vessels, the peritoneum and of the sphincter complex, plays a primary role. Weighing all these factors, we have to analyze the best decision regarding the necessity or otherwise of the pre and postoperative neoadjuvant therapy as well as the decision on the appropriate surgical techniques (Torkzad, 2010; Smith, 2008; Ayuso, 2010).

Colorectal neoplasia has an increasing incidence among the population, and this fact compels in achieving an early diagnosis and treatment protocols. High-resolution magnetic resonance brings relevant data on the normal anatomical aspects of the rectum as well as on the possible pathological changes. The magnetic resonance imaging (MRI) investigations, in our study, bring us important information about the location and extent of the tumor, if it affects the mesorectum, its relations with the peritoneum, with surrounding fascia and organs, highlighting the rectal arteries and the neoformation vessels. We evaluated the degree of the extramural invasion of the blood vessels [extramural vascular invasion (EMVI) score] and thus, the impact of this determination in diagnosis, treatment and prognosis of colorectal neoplasia setting.

According to American Joint Committee on Cancer (AJCC), the most common method in this respect is the one that takes into account the extent of the tumor, the affecting or not of the lymph nodes and the presence of metastases – TNM staging. The EMVI score is another method used for staging cancer, besides TNM. It defines the presence of malignant cells in the blood vessels, outside its own vascular tunic, near the tumor. This score is long recognized as an independent predictor for local and systemic recurrence as well as for long-term survival (Milliaris et al., 1995, Herbst et al., 1998, Sabo et al., 2001). Also, this score is used as an indicator for oncological therapy. The accuracy of approximately 52% of the EMVI score can be greatly increased by correlating it to MRI because in the early stages of cancer it is difficult to assess whether a vascular structure is visualized or not. The accuracy increases in advanced stages where vascular structures are invaded, as they have the same native density as the tumor and it captures intramural contrast (Lidgren et al., 2005).

Neoplastic growth is characterized by uncontrolled proliferation well above the apoptosis rate, as well as loss of cell differentiation. For this point of view a neoplasm means

a cell growth disorder evidenced by excessive cellular proliferation. Determination of this proliferation can be used in the positive diagnosis and prognosis of neoplastic lesions, and from this point of view, Ki67 is a valuable marker because is not influenced by other comorbidities such as melitus diabetes, cardiovascular disorders, congenital malformations. Overexpression of this marker may suggest a disturbance of cell division, resulting in the appearance of an intensely-growing tumor. The marker is also used to determine the degree of aggressiveness and the metastatic tumoral potential (McLeod and Murray, 1999; Jankowski et al., 2008).

The mechanisms by which Ki-67 is involved in cell cycle control are not fully elucidated, but there is a hypothesis that increased values of Ki-67 would act by inhibiting the cell cycle by inducing cellular self-stabilization (Jankowski et al., 2008).

Immunohistochemical markers CD-34 and Ki-67 appeared relatively recent in the studies. Both markers are sensitive to the assessment of tumor aggressiveness. In normal tissues are very low expressed, and abundant in tumoral tissues. Neoangiogenesis refers to the growth of new capillary vessels from those that already exist. These vessels are strictly orientated to the tumor. This phenomenon is necessary for the tumoral metastasis and, if it does not occur, it is not realized; there are studies on important batches of patients showing a relation of determinism between microvascular density and metastatic risk. The expression of these markers is correlated, in the same studies, with the patient prognosis, being directly proportional to it. The same was observed in breast cancer, stomach cancer and brain cancer (Miettinen et al., 2005; Vieira et al., 2005; Nieto et al., 2007; da Silva et al., 2008; Li et al., Ma et al., 2010).

The data show that expressions of CD-34, Ki-67 markers are inversely proportional to the degree of tumor histologic differentiation and with tumor staging depending by degree of local extension. At the same time, it is widely accepted that angiogenesis processes have a determining role in the metastasis process and patient prognosis by fascial tunnels organized arround bloodvessels. These two immunohistochemical markers are of high sensitivity and precision, but, however, their expression can not play the role of organ diagnosis because they can not localize the tumor.

A 6-month, serial evaluation can play the role of a marker of cancer progression or regression and signifies a new tool in assessing the prognosis of these tumors.

The purpose of the study is to evaluate the immunohistochemical expression of the Ki-67 marker in colorectal adenocarcinomas. Intratumoral concentrations of the Ki-67 antigen are correlated with the tumour proliferation rate and thus the prognosis of the patients.

This research direction has been materialized by publishing the following articles:

1. Hînganu D, **Stan CI**, Ciupilan C, Grigorovici A, Bulimar V, Dima-Cozma C, Hînganu MV. Anatomical and Immunohistochemical Evaluation of Colorectal Cancer. Rev Chim (Bucharest) 2019; 70(1): 236-238.
2. Hînganu D, Eva I, **Stan C**, Hînganu MV. Morphological aspects of the rectal neovascularization in colorectal cancer – anatomical-surgical and imaging implications. Rom J Morphol Embryol 2016; 57(1): 161-165.

4.2.2. Materials and methods

4.2.2.1. Immunohistochemical study

The study was conducted on a group of 28 patients diagnosed with rectal cancer. We made the histopathological and immunohistochemical evaluation of patients in the study group. The evaluation was performed on the sections stained with hematoxylin-eosin, and for mucinous forms, on sections stained with Alcian blue. We selected the cases for the immunohistochemical examination, the techniques being applied to low differentiated colorectal carcinomas. Ki-67 is expressed throughout the cell cycle except for the G0 phase, which is why Ki-67's intratumor concentration values correlate with the tumor proliferation rate and thus the prognosis of the patients.

The nuclear accumulation of the Ki-67 protein was assessed semi-quantitatively: negative (-) which means lack of coloration or presence in less than half of the cells and positive (+) which means the presence of nuclear staining in over 50% of tumor cells. In turn, the positive response was assessed low positive, moderately positive and strongly positive (+, ++ and +++).

4.2.2.2. Imagistic study

The study was conducted on a group of 10 patients, colonoscopic diagnosed with rectal cancer, who underwent preoperative contrast MRI to establish tumor localization and resection possibilities. The MRI images acquired on these patients were compared with normal anatomical imaging procured on a control group of 15 patients. Of the 10 patients in the study group, seven were male, aged 46–57 years and three females aged 55–62 years. Of these, five were diagnosed with cancer of the higher portion of the rectum in T2, T3, T4 stages, three were diagnosed with medium rectal tumors in T3 stage and two with lower rectal cancer, T1 and T2 stages.

The MRI sequences obtained are consistent with others found in the literature literature (Mercury, 2006; Mercury, 2007):

- T2 fast (turbo) spin echo, made from one pelvic wall to the other (transverse);
- Axial, with a wide field throughout the pelvis;
- T2 fine sections in the axial plane – made along its axis, that include rectal tissue with 3 mm thickness;
- Coronal sections with high spatial resolution that allow the visualization of low rectal tumors, the levator ani muscle and of the anal mechanism sphincter;
- High resolution sections that go up to 5 mm above the edge of the tumor in order to be able to view a possible a lymphatic tumor invasion in the mesorectum.

The MRI examination was performed using the area antennas after following the protocol: images weighted in T2 FSE (fast spin echo) and T1 SE (spin echo) and ESF (edge-stopping function) in three orthogonal planes – axial, coronal and sagittal – with the following parameters: TR (repetition time) 400 ms, TE (echo time) 130 ms, 400×500 matrix 28 mm FOV (field-of-view) sections of 4 mm exam T1-weighted images natively and FSE after administration of intravenous paramagnetic contrast MIP (maximum intensity

projection) and 3D reconstructions followed. For the study, we used a Philips Achieva 1.5T MRI machine. A paramagnetic dye was injected: Magnegita 500 μM/mL solution for injection (Gadopentetate dimeglumine). The MRI dosage used for adults was of 0.5 mL/kg. The injection of the contrast (30 to 40 mL of Gadolinium) was carried out with a MRI specific injector (MEDRAD Spectris®) at a rate of 0.8 mL/s. For the acquisition of bolus MRI angiography, we used sequences followed by successive stages: the EFGRE (enhanced fast gradient-recalled echo) 3D sequence used with the following parameters: 42 cm, matrix 256×192 (reconstructed by interpolation matrix 512×512), TE 1.5 ms, TR 6 ms, TI (inversion time): 25 ms, angle: 25°, section thickness: 4 mm (rebuilt in pieces that overlap 4 mm× 2 mm). The total MRI acquisition time for the angio- graphy was of 95 seconds (five seconds between each step to move the table).

4.2.3. Results

4.2.3.1. Ki-67 evaluation in colorectal cancers

Most of the studied cases were moderate and low differentiated colorectal adenocarcinomas (G2 and G3). Approximately 60% were in advanced stages (T3), with perirectal adipose tissue invasion.

In G2 adenocarcinomas (**Figure 4.1**), tumor cells delineate glandular, irregular cavities sometimes with papillary excrescences of different shapes and sizes. The nuclei are large, elongated, hypercromatic, placed on 3-4 layers that occupy the entire thickness of the epithelium.

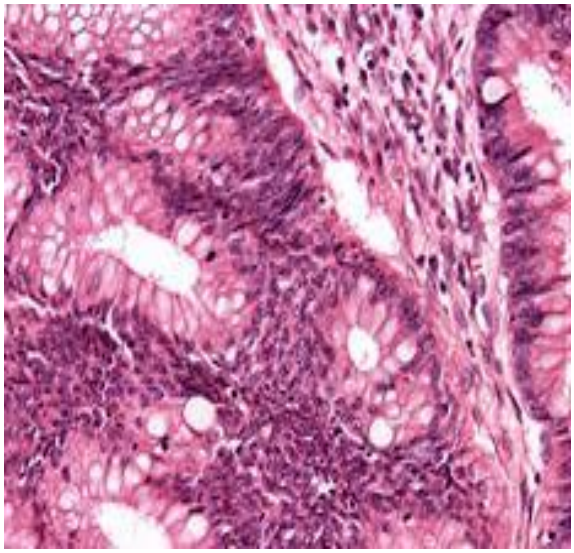

Figure 4.1. Moderately differentiated colorectal carcinoma. H & E staining, x40.

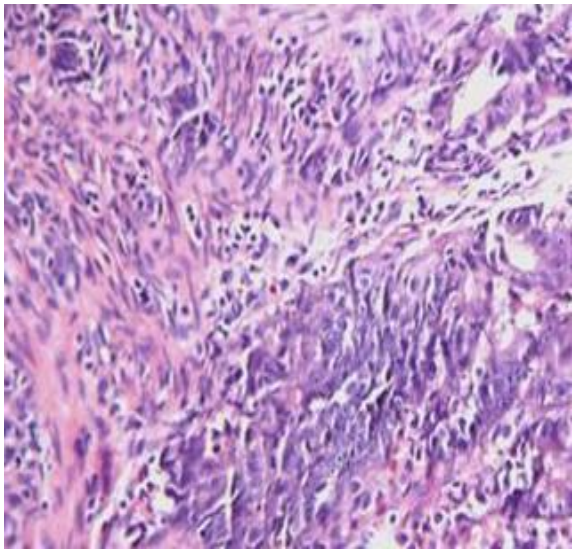

Figure 4.2. Low differentiated colorectal carcinoma. H&E staining, x20.

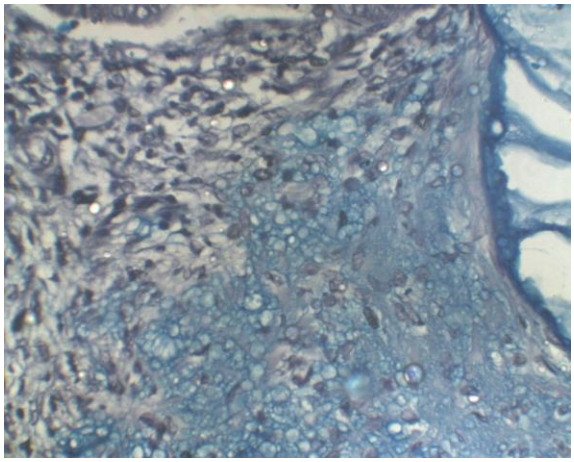

Figure 4.3. Low differentiated colorectal carcinoma. Blue Alcian staining, x40.

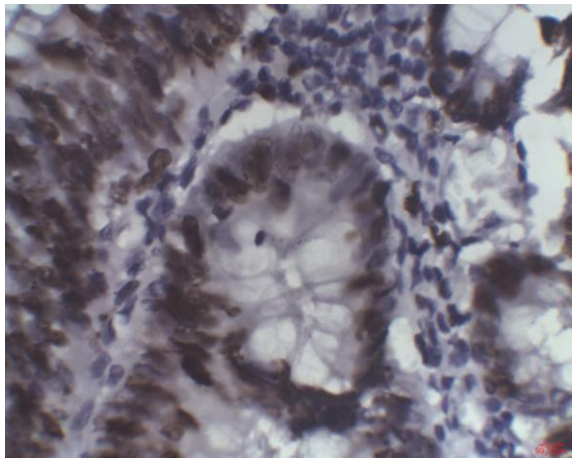

Figure 4.4. Strong nuclear reaction of Ki-67 on sections of colorectal cancer (+++)

There are areas of ulceration and infection with the presence of stromal inflammatory infiltrates. In G3 adenocarcinomas, tumour cells are usually small with hyperchromic nuclei. The stromal volume is increased and there are frequent ulceration areas, the stromal inflammatory infiltrate being abundant (**Figure 4.2**).

Of all the cases of studied colorectal tumours, we considered mucinous carcinomas the cases where the mucinous component represented more than 30% (**Figure 4.3**).

Immunohistochemical exam in sections stained for Ki-67 has shown that the topography of reaction is strictly nuclear (**Figures 4.4, 4.5, 4.6**), but we could not accurately identify the nuclear localization depending on the division stage.

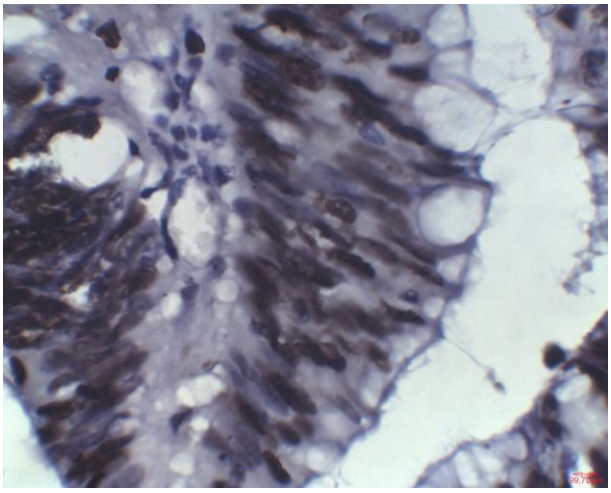

Figure 4.5. Nuclear Reaction of Ki-67 in the Cellsof a colorectal cancer. Ob. X60.

In the tumor there are Ki-67 poor stained areas, probably due to the completion of phase (interphase) and degradation of nuclear staining.

The lack of expression of studied markers in neighboring non-tumor tissues, including Ki-67, suggests that cancerous tissue proliferates in a pathway that is not correlated with adjacent tissues.

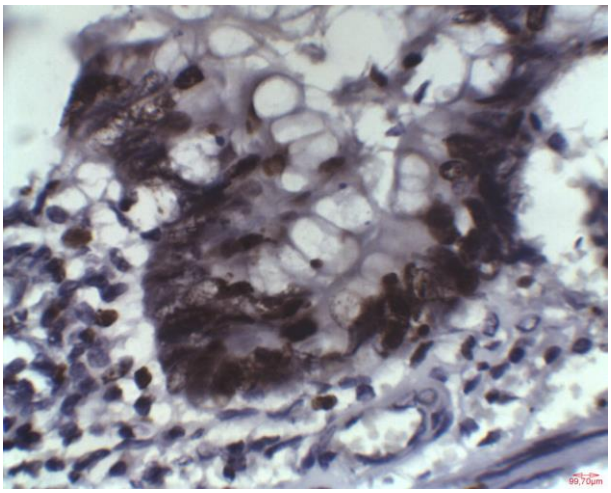

Figure 4.6. Intense nuclear (+++) reaction of Ki-67on sections of colorectal cancer

In cases with a strong positive response to Ki-67 (+++), prognosis is worse, with invasion of the perirectal tissues, regional lymph nodes and metastases. In these situations there is a direct correlation between the expression and accumulation of the Ki-67 protein and the local, regional or distant extension.

We have not noticed any correlation between the intensity of the reaction and the age and gender of the patient or the exact location of the primitive tumor.

4.2.3.2. Imagistic study

We used the EMVI score correlated with MRI imaging in order to establish the staging of colorectal carcinomas.

The criteria taken into account by the EMVI score were correlated with the images obtained in patients from the study group. In each of the evaluated patients, we found both, arterial and venous blood vessels in 0 stage – EMVI correlated with T2 or T3 stage tumor. In T3 B stages, we could objectify suggestive images for stage 4 EMVI near and distant from the tumor. Thus, T2 in stages (**Figure 4.7**), proximal and homolateral of the tumor, we have objectified the vascular paraneoplastic invasion appreciated by the caliber of the blood vessels, the degree of tortuosity, the appearance of their walls (nodule) and heterolateral the vessels appearing to be normal, especially in the early T1 and T2 stages. In T1 stages, on sections made at 3 mm, the paraneoplastic changes of their walls and their content lessen and even disappear at 6 mm maximum, both homo and contralateral of the tumor. In T2 and especially in T3 stages, the vascular paraneoplastic changes seem to interest long vascular portions with an important caliber, going even to the origin vessels (the trunk of the rectal middle artery, the internal iliac artery of the tumor and, especially, the veins of this path) (**Figure 4.8**).

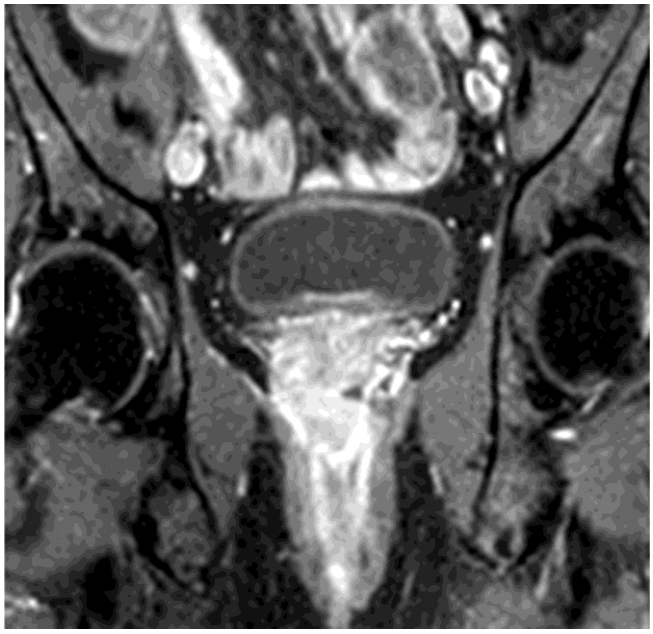

Figure 4.7. Neoformation vessels in the inferior right rectal artery, in straight angle, with enlarged perivas- cular lymphadenopathy – stage 2 EMVI.

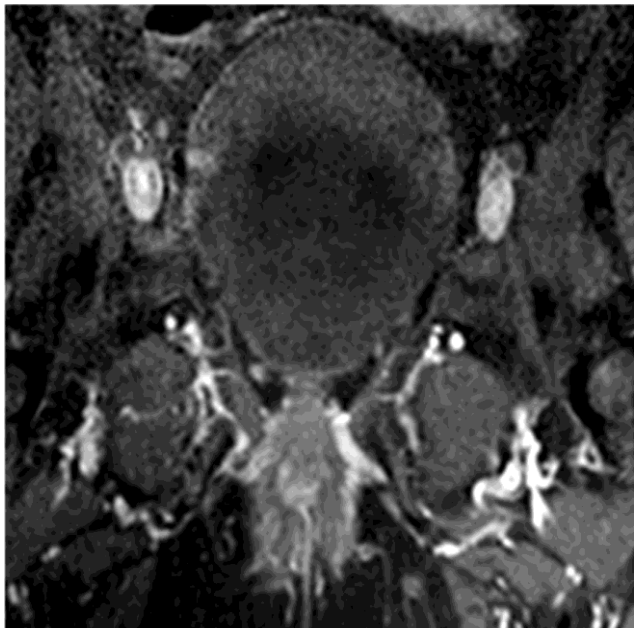

Figure 4.8. Middle rectal vein in middle rectal cancer, with the equivalent signal outlet to the tumor, specific for stage 4 EMVI.

The remote damage of the blood vessels (internal iliac vein) is correlated in most cases with secondary determinations of the liver **(Figure 4.9**). We believe that the distance paraneoplastic impairment of the vessels (the trunk of the middle rectal artery) signifies a high probability of microscopic liver determinations.

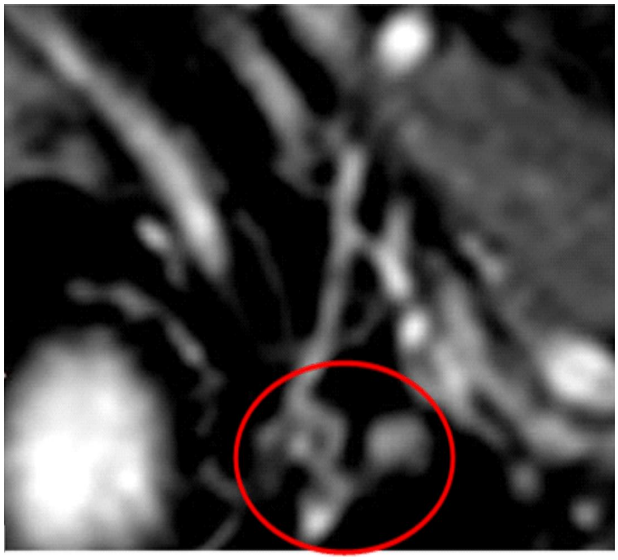

Figure 4.9. Neovascular degenerative process, on the branches of inferior left rectal veins, with the appearance of a central core and two satellite sanguine cores, typical for arterial paraneoplastic processes – stage 2–3 EMVI.

The aspect of tumor margins shows tumoral invasion in small veins, which goes out of the intestinal lumen and can produce nodules in the venous wall, distinct from desmoplasia (**Figure 4.10**). The presence of tumoral signal in the vascular structures is a landmark for tumoral presence (**Figure 4.11**). Affected vessels increase their volume and their inside signal is a medium gray.

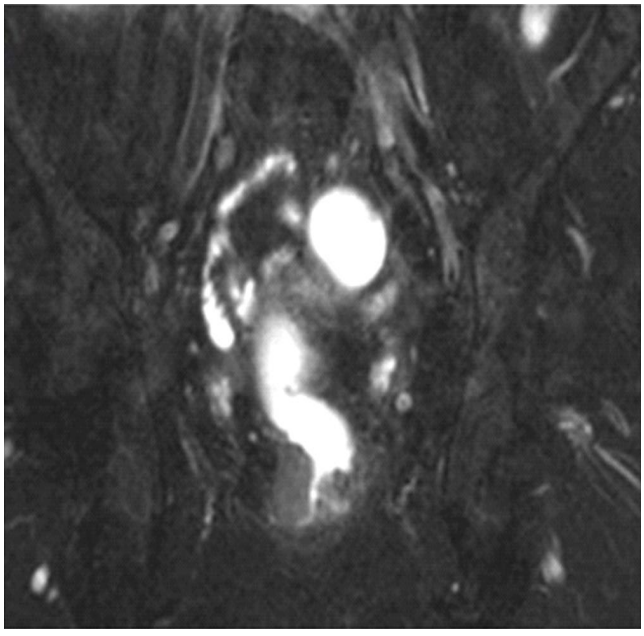

Figure 4.10. Pseudo-inferior rectal artery stenosis in the left, with similar changes on the contralateral inferior rectal artery, both intramural nodule type, specific to stage 3 EMVI.

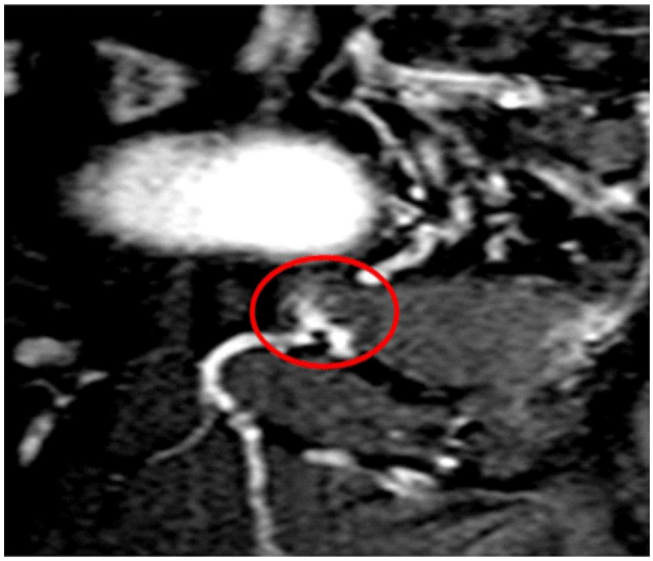

Figure 4.11. Left middle rectal vein in sagittal section, in a middle rectal cancer with intraparietal invasion – stage 4 EMVI.

Extension of tumor inside the vessels causes a smooth, nodular or an irregular appearance of vessels (**Figure 4.12**).

In none of the 15 cases of MRI investigated patients who refuted rectal neoplasia could we amend vascular corresponding EMVI score (**Figure 4.13**). Moreover, in these patients we have not discovered any benign formations, which can be considered preneoplasic condition, such as polyps, diverticula, or autoimmune diseases.

Of primary importance in determining the localization of the tumor, is the fact that the extramural invasion of the blood vessels in the lower rectal tumors is cranio-caudal and those of the upper and middle rectal tumors are mostly cross bared. This explains the rapid blood-stream metastasis of the lower rectum tumor. In this situation, the medium or large distance vascular invasion is precocious.

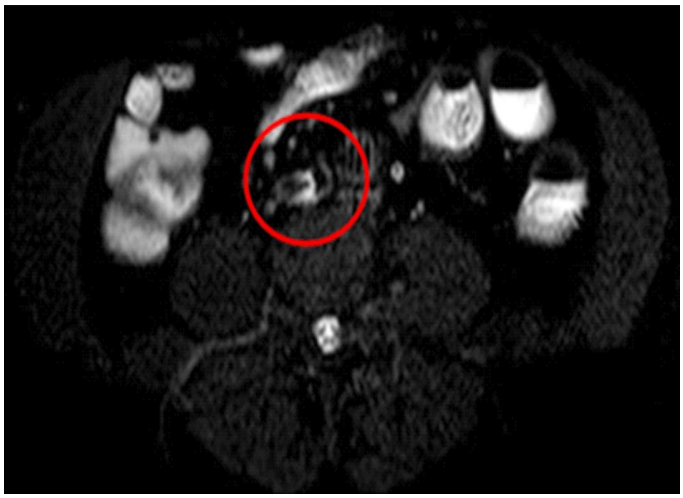

Figure 4.12. Neoformation vessels on the right inferior rectal artery, in MRI with multicenter condensing processes, characteristic for stage 3 EMVI.

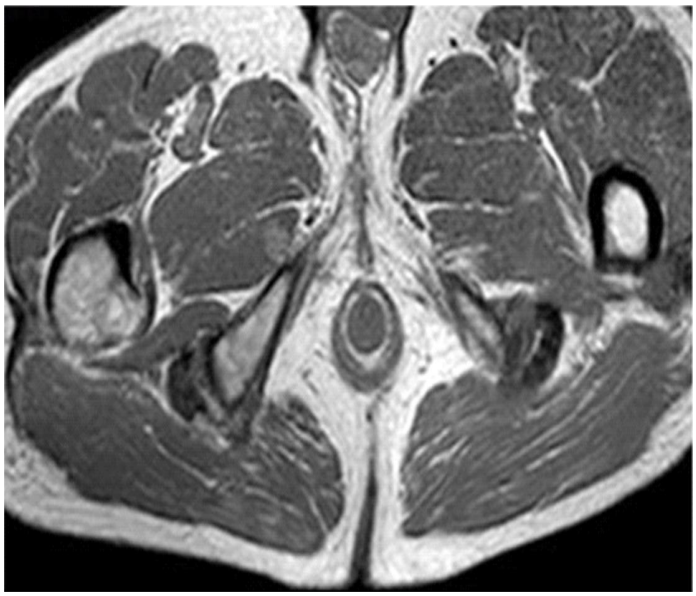

Figure 4.13. Normal aspect – stage 0 ENVI.

Upper and middle rectal neoplasms produce a pre-cocious extramural invasion in proximity.

The most used directions in extramural vascular invasion in various locations suggest the fact that they follow the vascular anastomoses between the three rectum territories. Given these considerations, it may be proposed an EMVI staging system based on MRI imaging.

4.2.4 Discussions

Ki-67, marker of cell proliferation is associated with a low prognosis in colorectal cancer patients. Its expression is increased in patients with Dukes C or D colorectal cancer versus those in stage A or B. The Ki-67 along with other clinical-pathologic markers could be useful in the prognosis of patients with colorectal cancer.

The growth rate of cancer cells depends on their proliferative activity and cell death rate. Researchers evaluated the expressions of Ki-67, PCNA, MCM in 55 patients diagnosed with colorectal carcinomas and showed that the expressions of the three antibodies are directly correlated with the presence of metastatic lymph nodes but are independent of the age and location of the tumor. It can be concluded that their positive expressions in the mass of the main tumor are directly proportional to pathological stage and histological differentiation gradient (Katarzyna et al., 2008).

Neoplastic growth is characterized by uncontrolled proliferation well above the cell death rate, as well as loss of cell differentiation. For this reason a neoplasm means a cell growth disorder evidenced by excessive cellular proliferation. Determination of this proliferation can be used in the positive diagnosis and prognosis of neoplastic lesions, and from this point of view, Ki-67 is a valuable tool. Exaggerated expression of this marker may suggest a disruption in the regularity of cell division, resulting in the appearance of an intensely-growing tumour.

The Ki-67 marker is also used to determine the degree of aggressiveness and the metastatic potential of the tumour.

The Ki-67 monoclonal antibody recognizes an antigen located in cell nuclei in all cell cycle phases except G_0. The Ki-67 staining method is simple and can be used as a technique to estimate the proliferative activity, as a prognostic marker as well as a screening test in individuals with high risk (Mullerat et al., 2003; Oshima et al., 2005).

The value of this protein's expression has a normal limit in the free mucosa, being somewhat higher in adenomas, and the maxima in adenocarcinomas (Saleh et al., 1999).

Researchers found a correlation between the histological degree of malignancy and the depth of tumor infiltration in the wall (Dziegiel et al., 2003). Determination of Ki-67 may subdivide a group of patients with similar clinical symptoms into diagnostic and prognostic subgroups.

There were described several methods for identifying tumor vessels based on the ability of endothelial cells of vascular tissue to release antigens which can serve as markers on tissue included in paraffin, CD-34 considered to be the best marker for neovascularization (De la Taille, 2000).

Modern management of colorectal cancer is multidisciplinary, with a major focus on preoperative assessment. This has become possible with the advancement of both medical imaging and immunohistochemical techniques. The formulation of a certain diagnosis and therapeutic protocol is a common element for aspiring multidisciplinary effort made by surgeons, oncologists and pathologists. In order to achieve preoperative staging of rectal cancer, it should be known local extension of the tumor and systemic dissemination of cancer cells. Formulation of a therapeutic protocol and prognosis of colorectal cancer depend on tumor stage, tumor type, invasion of lymph nodes, as well as whether or not the sphincter apparatus and neighborhood fascia are involved.

Neoangiogenesis occurs independently of malignant transformation, involves growth factors, extracellular matrix enzymes, endothelial cell migration and proliferation, lumen formation and anastomosis with other vessels, in relation to phenotypic and tumor genetic changes. Some authors have experimentally demonstrated that neoplastic cell populations release angiogenic molecules *in vivo*, before their neoplastic transformation reaches the level of formation of a solid tumor. Tumor growth and transformation of tumor cells into an angiogenic phenotype are associated with increased secretion of angiogenic molecules, such as fibroblast growth factor, FGF and endothelial proliferation factor VEGF. In addition, it has been shown that a tumor develops a pronounced angiogenic phenotype when angiogenesis inhibitors (anti-angiogenic factors) are suppressed during tumorigenesis, such as the case of thrombospondin, which normally helps keep the vessels in a non-angiogenic status (Park et al., 2014).

Initially, was used antibody measurement for CD31, CD34 markers and for growth factor VIII, but was recently used the quantification of CD105 endothelial cell marker expression (Ottaviano, 2015; Goldis, 2015). It seems, however, that this marker is not specific only to vascular endothelium (Agaki, 2001).

Neovascularization that crosses the tumor mass develops rapidly and presents itself as a disorganized chaotic network with tortuous, thin walls; as a result, the irrigation of different tumor segments is differentiated, with deficiencies in some segments which can lead to tissue

necrosis. In this context it was found that the tumor aggressiveness is much higher in the periphery than in the tumor center, in terms of microvascular density per mm^2 (Romani, 2006).

Structural features of the vascular network in tumors are: flattened endothelium, no differentiation between arteries and veins, poor structural stability, lack of parietal contractile elements, lack of receptors, incomplete formation of basal membrane, lack of lymphatic formation (Corines et al., 2018).

Most of the studies (Brouwer et al., 2018) show a connection between tumoral angiogenesis and metastasies but, there are differences caused by the method used for immunological staining of vessels; vimentine has a lower specificity then $VIII^{th}$ factor and this then CD34. Also there are significant differences between the degrees of differenciation, low, intermediary and high.

P53, also known as "genome guardian" is one of the most affected tumor suppressor gene, in the case of colorectal neoplasms. Mutations in this gene, appeared in the process of carcinogenesis, located on chromosome 17 occurs in more than 50% of carcinomas during the process of tumorigenesis. These mutations are linked with altered DNA replication and progression to cancer. The involvement of this gene in the occurrence of recurrences and prognostics of colrectal cancer is questionable (Kahlenberg, 2003).

Some studies on groups of patients make from p53 gene and its mutation a marker of relapse and life expectancy (Kahlenberg, 2000), while other studies have not found the existence of such a role for the gene. Regarding the ordinary response to radio and chemotherapy p53 mutation showed a high degree of resistance to them. This mutation does not respond to 5-fluoro-uracil and methotrexate.

Molecular biology studies carried out on parts of resection of colorectal carcinoma correlate p53 gene mutations with polyp-carcinoma sequence. This neoplastic process begins with the activation of oncogenes (Ki-RAS), inactivation of tumor suppressor genes (p53 and Ki-RAS DCC), followed by suppressor gene mutations during progression from adenoma to carcinoma. These mutations type "missense" is performed simultaneously with the loss of wild-type allele, with a prolonged half-life, which makes these changes to be detectable immunohistochemically (Levine, 1991). Cases in which over 45% of proximal colon cancers develop "de novo", ignoring the polyp-carcinoma sequence are due to genomic instability associated with a defect in mismatch repair of DNA.

Since 1988, it has been shown that the immunohistochemical determination of Ki-67 may have clinical application in the selection of patients with colorectal cancer who may benefit from radiotherapy and/or chemotherapy, particularly those with unresectable or locally recurrent tumors (Shepherd et al., 1988).

Research on colorectal carcinomas using immunohistochemical techniques with Ki-67 protein is simple and relatively easy to apply. The results are reproducible using the MIB 1 antibody with which excellent stains can be obtained, even on the classically fixed paraffin blocks 1 (Uzmaet al., 2008). *Ki-67 expression is high in non-mucosal adenocarcinomas, highly or moderate differentiated, and is low in those that are low differentiated with mucin secretion. This last type of tumour corresponds to an advanced Dukes stage. Thus, there is a close association between the histological grade, type and stage of the tumour.*

In T2 transversal section, the radiologic anatomy of the rectum shows the rectal lumen followed by musculara propria (which is formed sometimes from two distinct layers: internal and longitudinal), then the tunica sub- mucosa, with high MRI intensity and muscularis mucosae, with low MRI intensity, which delimit the rectal lumen. The muscular layer have irregular grooves steering, but uniform in size, because of the blood and lymphatic vessels which penetrate the rectal wall.

Around the musculara propria, with low MRI intensity, we can see an area of high MRI signal intensity, which belongs to perirectal fat. This adipose tissue contains blood vessels with reduced MRI intensity, blood vessels and lymphatic nodes and also conjunctive septa. Finally, we observe another zone with reduced MRI signal, which surround the rectum and the perirectal fat. This is fascia mesorectum. Any changes of these anatomic and radio-logical issues should be suspected and investigated like presenting paraneoplastic origins.

The blood and lymphatic vessels have a rich density to their home area (posterior and mesorectal), which decrease as we head towards anterior. This distribution is consistent with vascular predisposition of forming a rectal cancer, especially on the rear wall or possibly on the side. Evolution to anterior of a rectal tumor is the second step in neoplastic evolution and is influenced by the anatomy of the pelvis. This occurs because of two aspects:

1. The universal law of bilateral symmetry, which applies for the median unpaired organs;
2. The distance from the center of the tumor, which have been performed various types of sections (3 or 5 mm).

MRI assessment contains a number of protocols which must be individualized according to need of information that are intended to be purchased, to quality of clinical and paraclinical information on the cases, and to local conditions. If high-quality images are needed, about the relationship of tumor to nearby organs, a MRI 2D T2 protocol with sections obtained in sagittal, axial and oblique will be used.

In case of obtaining inconclusive images, to better highlight both the caliber and rectal coating layer it can be used the rectal administration of ultrasound gels or contrast agents. This maneuver allows more accurate delineation of tumor poles, reduce the potential artifacts and allow a better assessment of synchronous tumors (Slater, 2006; Jeong et al., 2014).

The establishment of a normal vascular pattern of rectum was obtained by comparison of angio-MRI results of patients who do not show degenerative lesions at the level of this organ to those obtained by MRI with contrast in neoplasia and those obtained by arteriography performed on resection pieces.

Major problems arise in a correct preoperative differentiating of a tumoral stage 2 by tumoral stage 3. This is a critical issue because the rectal tumor, starting with stage 3, it requires most often a preoperative radiochemotherapy (Sebag-Montefiore, 2009; Sauer, 2012; Alberda et al., 2014; Holman et al., 2017). However, recent studies question the correctness of the assessment prognostic and therapeutic value of colorectal cancers using only information provided by MRI (Al-Sukhni, 2013). For this, a group of researchers developed a local method for staging colorectal cancers corroborating investigations using MRI to determine the extramural venous invasion using the EMVI score, which envisages the

histology of the tumor cells in the own vascular endothelium (Mercury, 2007; Taylor, 2008; Bhangu et al., 2014).

The intramural and extra-mural invasion of blood vessels by a rectal tumor is an important score for prognosis in evaluating patients being correlated with histological demonstration on vascular resection specimen. The extramural vascular invasion corresponding to EMVI score 3, 4 will be stage T3, T4 (Smith, 2008; Troja et al., 2015; Fleshman et al., 2015).

Venous invasion of colorectal cancer leads to the presence in blood circulation of tumor cells, which goes in the portal circulation, which results in the appearance of distant metastasis by hematogenous spreading (Krasna, 1988; Talbot, 1980; Bokkerinket al., 2015; Fleshman et al., 2018).

4.2.5. Final remarks

EMVI score significantly improves the accuracy of determining a proper oncological treatment after surgery. This study raises serious questions over the current staging of colorectal cancers because changes in caliber and trajectory of rectal vessels appear early.

Starting with EMVI 2 score, the anatomical radiological changes that occurs seems to be caused by metastatic dissemination processes and not just by the neovasculogenesis. Extra- mural vascular invasion is depending on the tumor location: superior rectal location has a transversal but also caudal invasion; medium rectal location has a transversal, but also a vertical invasion; inferior rectal location has vertical but also transversal invasion. Atypical invasion directions may suggest the presence of synchronous tumors or one extremely aggressive.

Studies are required on extensive groups of patients in order to make the EMVI score a routine method in the diagnosis and prognosis of colorectal neoplasia.

Increased expression of the Ki-67 marker is generally associated with a low prognosis of survival, especially in undifferentiated or low differentiated cases of colorectal carcinomas. The Ki-67 along with other clinical-pathologic markers could be useful in the prognosis of patients with colorectal cancer.

SECTION II. PLANS FOR FUTURE RESEARCH

I. *Quantitative* and *qualitative* study of fasciae

An important direction of my future research relates to further investigations of fasciae as *muscles dependencies through quantitative* and *qualitative* ultrasound *determinations* and other advance radiologic techniques.

This should take us to the next level of understanding of their dynamics in adaptation to mechanical functions as well as to biochemical conditions which might have real improvements in terms of injury prevention, athletic performance and sports-related rehabilitation. This consensus reflects the state of knowledge regarding the role of fascial to provide an overview of the contemporary state of knowledge regarding the fascial system from the *microlevel* (molecular and cellular responses) to the *macrolevel* (mechanical properties). Another method to approach the morphofunctional features of the fascias is to weight and scale the responses of them to altered loading (physical exercise), injuries and other physiological challenges including ageing. These highlight the contemporary view of interventions that target fascial tissue in sport and exercise medicine. A coordinated effort of researchers and clinicians combining mechanobiology, exercise physiology and improved assessment technologies will assure the advancing in this field.

The fascial system builds a three dimensional continuum of soft, collagen-containing, loose and dense fibrous connective tissue that permeates the body and enables all body systems to operate in an integrated manner. Its injuries cause a significant loss of performance in recreational exercise as well as high performance sports, and could have a potential role in the development and perpetuation of musculoskeletal disorders, including lower back pain. Fascial tissues deserve more detailed attention in the field of sports medicine.

II. *Tissue engineering*

This represents another interest domain for immediate implementation which integrate my personal experience in bones (osteosynthesis) and pancreatic (stem cells) research.

II.1. Osseous synthesis - morphofunctional approach

The initial vacularization of the graft is extremely required for the graft to survive, integrate and function. The cortical type of graft gets slowly vascularised as compared to the cancellous grafts and requires an initial phase of bone resorption. They begin to unite at the host graft junction first and healing, gradually moves towards the shaft of the graft. The repair and remodelling of cortical bone occurs at a very slow rate, and any imbalance between resorption and bone formation can lead to bone loss and graft failure.

The fibrotic nonunions and late graft fractures are reported to be directly dependent upon the neovascularization of structural allografts with 25% clinical failure rate.

Recent studies shown that 1-2 years after surgical implantation, the fractures occur and are related to initiation of microfractures within the dead cortical bone. In contrast, cancellous bone graft is porous revascularize quickly, and can be rapidly incorporated and remodelled.

In this type of graft, osteoblastic bone formation occurs first on the surface of necrotic trabeculae, followed by osteoclastic bone remodelling and gradually resorbtion of the entrapped dead trabeculae and eventually replaces the entire graft with new living bone.

It is required that the biomechanical strength of the bone implant should be more due to the load bearing nature of the bone.

The scaffolds for bone should be ready to use specific materials so that there is minimum time paucity after the case is presented with complicated gap defects of bone. The bone bioengineering is an upcoming area which offers a viable solution for the problems like loss of bone and limb amputation.

The combination of regenerative cells and matrix not only provide a support at the site of loss but also provides the factors important for the bone regeneration and healing.

II.2. Genetic, epigenetic and laboratory studies on endocrine pancreas - stem cells therapy of diabetes mellitus type 2

The raising worldwide prevalence of Type 1 and Type 2 diabetes mellitus solicits the derivation of *in vitro* methods yielding mature and fully functional β-cells to be used in *Regenerative Medicine*.

The first morphological signs of pancreas development are detected at around embryonic day 9.5 (e9.5) in mice. At this stage, the endoderm evaginates into the overlying mesenchyme, forming the dorsal bud. This is followed by the budding of the ventral pancreas under the control of a different molecular cascade. The two buds eventually fuse at around e12–e13 to form a single organ, the pancreas.

Concomitantly, the process of endocrine specification is initiated, as outlined by the expression of several TFs, including Pancreas/duodenum homeobox protein 1 (Pdx1), Neurogenin 3 (Ngn3), Aristaless-related homeobox (Arx), and Paired box gene 4 (Pax4). While Pdx1 labels the early pancreatic progenitors, Ngn3 is a marker of pancreatic endocrine commitment. Arx expression restricts the endocrine-committed cells to a α-cell fate, while Pax4 expression guides cells towards a β- or δ-cell lineage.

Protocols to differentiate human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) into human pancreatic β-like cells have recently been developed. These methods, coupled with a bioengineering approach using biocompatible encapsulating devices, have recently led to experimental clinical trials showing great promises to ultimately end the battle of diabetic patients for managing hyperglycemia.

In vitro differentiation protocols face the challenge of achieving homogenous population of mono-hormonal insulin-secreting mature β-cells. Meanwhile, major epigenetic events such as DNA methylation, post-translational modification of histones and non-coding RNAs expression, orchestrate physiological endocrine pancreas specification into α-, β-, γ-, and δ-cells, both *in vivo* and *in vitro*.

They are associated to multiple pancreatic disorders including diabetes. Understanding the epigenomic and transcriptomic landscape underlying endocrine pancreas development could, therefore, improve *in vitro* differentiation methods.

Type 2 diabetes is caused either by a loss of insulin secretion or insulin resistance. Interestingly, recent in-depth analyses of the mouse and human genome by researchers (Lu et al., 2018) showed that in mice subjected to a high fat diet (HFD) and in humans with T2D, chromatin-state-defined transcriptome dysregulation led to a preferential loss of key β-cell TFs.

Equally important was the observation that such loss of identity was associated with a transition to a bivalent epigenetic state that is normally associated with stemness, suggestive of dedifferentiation. This is in accordance with the finding that humans with a high BMI display a decline in β-cell function. Interestingly, an embryonic ectoderm development protein (Eed)-containing PRC2 was shown to be required for the long-term maintenance of β-cell function in vivo. The Eed protein along with the Ezh2 histone methyltransferase and other proteins, form the PRC2.

The PRC2 catalyzes the trimethylation of H3K27, thus leading to target gene repression. The loss of the Eed-PRC2 complex was found to result in the expression of islet dedifferentiation factors, like GLI-Kruppel family member 2 (Gli2), and in the loss of the terminal β-cell identity.

Importantly, the simultaneous inhibition of a polycomb opposer, such as MII (a H3K4 methyltransferase), could rescue this loss of a β-cell phenotype, thereby raising hope in the context of T2D research. Equally noteworthy was the finding that multiple T2D risk loci are, in fact, α-cell-specific, as demonstrated while using ATAC-seq, further suggesting that T2D may also be associated with α-cell dysfunction and not just β-cell failure.

We focus on the main transcriptional and epigenetic events leading to pancreatic specification and on the applicative potential of novel epigenetic drugs for the establishment of innovative pharmacological therapeutic approaches. Although still controversial, most of the studies show strong evidence of the capacity of the adult endocrine pancreas to undergo regeneration. Several sources of islet cell neogenesis were proposed, and it appears that multiple mechanisms may act during this process.

For us, of special interest is to check the capacity of glucagon cells to regenerate, making alpha-cells as a possible progenitor cell that is prone to transdifferentiation into functional beta-cells.

III. *Multiscale modelling of vascular tumour growth in anatomy*

The multiscale modelling of vascular tumour growth is an emblematic and primordial research plan of ours.

Solid tumours progress through two separable phases: the avascular and the subsequent vascular one. In the initial phase that a tumour goes through, nutrients and oxygen are delivered to the tumour cells via diffusion processes alone from surrounding host capillaries. It could remain at this critical size, where cell proliferation is balanced by cell death, for a period of several months or even years without causing any serious damage to the

host, which is exploitable by in vitro experimentation, where tumour cells are cultivated in the laboratory as three-dimensional spheroids.

The mass of the tumour reaches the critical point of transition from avascular to vascular phase and develops an intrinsic blood supply network which allows it to grow further. At this point, tumour cells secrete angiogenic factors such as vascular endothelial growth factor (VEGF) in response to hypoxia and thus, the angiogenic activation process termed as the angiogenic switch, occurs. The endothelial cells will proliferate and eventually, the tumour will be penetrated by vessels. Finally, maturation of several vessels will occur as a result of molecular competition between activators and inhibitors regulating angiogenesis.

Experimental and clinical evidence suggest that tumour angiogenesis, the tumour-induced growth of new capillary blood vessels in the body from already existing vasculature, is a key process in tumour development and cancer invasion. In particular, once angiogenesis has obtained its goal, new vessels provide the tumour mass with nutrients and oxygen, which are clearly of vital importance for its survival and further growth. Hence, the tumour may soon reach a cancer cell population of the order of magnitude, give rise to the first symptoms and eventually form metastases in distant organs. Angiogenesis occurring in a physiological context, e.g. during early embryogenesis as well as in the female reproductive system is completely different in numerous aspects comparing to tumour – induced angiogenesis. The latter, does not tend to create mature and stable vessels able to provide normal blood supply. Instead, tumour vasculature is characterized by a complex and dysfunctional structure resulting in higher fractal dimension.

The concept of antiangiogenic treatment is based upon the idea that the growth of a tumour and it is strongly dependent on the amount of blood vessels that it induces to grow. However, it was not until the nineties that initial experimental results suggesting that angiogenesis blockage could result in tumour regression came up. Hence, the original target of antiangiogenic treatment was to block the transfer of nutrients and oxygen to the tumour by destroying the tumour vasculature until all cancer cells starve to death. That is why it was believed that the specific kind of treatment would be able to cure the disease. Even though there is now enough evidence that this is not the case, several mechanisms of action of angiogenic inhibitors have been elucidated in order to explain their anticancer effect. These findings could be used in early tumour diagnosis radiologic protocols by tracking an antiangiogenesis compound.

It remains controversial whether targeting tumour vasculature can improve radiotherapeutic efficacy. Anti-angiogenic or vascular-destructive agents potentially enhance tumour responses to radiotherapy. Several anti-angiogenics have been clinically evaluated in combination with radiotherapy but their benefits are controversial.

Appling the datas from our future study into the simulation domain is expected to play a significant role in tumour response to therapy.

IV. *Educational Pathern in Anatomy*

Against a backdrop of ever-changing diagnostic and treatment modalities, stakeholder perceptions such as undergraduate, clinicians and anatomy educators are crucial for the design of an anatomy curriculum which fulfils the criteria required for safe medical practice.

As the nature of medical training and academic clinical training pathways continue to evolve, it is important that the learning requirements and educational preferences of graduate entrants are also evaluated.

This is why one of my plan for future research is to conceive, apply and customize survey studies in areas which envolve the association of anatomy with medical education and clinical practice, mode of instruction, and curriculum duration.

The collection and analysis of data related to current trends in the content and mode of delivery of anatomy curricula is expected to provide an evidence base for assessing whether existing curricula meet the needs of the modern medical workforce. To that end, a survey was designed and tailored for medical undergraduate, clinicians, and academic anatomy educators to: investigate perceptions of the relevance of anatomy curricula to the modern medical curriculum as well as current medical practice based on the following survey domains: level of agreement, or otherwise, with statements concerning the role of anatomy in the medical curriculum and its relevance to clinical practice; level of satisfaction with time allocated for anatomy within curriculum; ranking, in terms of perceived importance, of various modes of instruction in anatomy education.

The anatomy course initiatives including the introduction of inter-professional activities at the preclinical level (including anatomy) may help to foster a sense of professional identity, and increase competencies in areas such as teamwork, communication, and awareness of ethics.

Previous work has shown that junior medical doctors in particular use anatomical knowledge in all phases of the consultation, especially during physical examination.

Most of the specialist in medical education field recognized that the viewpoint of clinicians should be taken into account if the aim is to develop an anatomy curriculum relevant to daily clinical practice. Previous work has shown that while there may be areas of clinical care which rely to a greater (e.g. imaging and diagnostics) or lesser (e.g. doctor-patient communication) extent on anatomical knowledge, all groups in the current study (echoing previously published results) rated clinical relevance as a crucially important element of the anatomy curriculum.

The expected results regard to achieve a necessary inhumanity expected to undergraduates to gain from the dissection course they went through as a “rite of passage” at the beginning of their university careers. Learning human body structure by performing hands-on dissections in the anatomical theatre has become a fundamental element of modern medical education, almost everywhere on the globe. I propose “Living and Virtual Anatomy” as one possible alternative approach to undergraduate’ first encounter with the human body.

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