UNIVERSITATEA DE MEDICINĂ ȘI FARMACIE
GRIGORE T. POPA IAȘI

BIOCHEMICAL INVESTIGATIONS – THE PREMISE IN BASIC INTERDISCIPLINARY AND CLINICAL MEDICAL STUDIES

Habilitation thesis

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ABBREVIATIONS

ABI – ankle-brachial index
AFI – amniotic fluid index
AGA – appropriate for gestational age
AHT – arterial hypertension
AIXao - aortic augmentation index
AIXbr – brachial augmentation index
AOS – antioxidant system AOS
BMI – body mass index
BMI – body mass index
BSA – bovine serum albumin
CAT – catalase
CD – cesarean deliveries
CHD – coronary heart disease
CK – creatinkinase
CMetS – cardiometabolic syndrome
CPAP – Continuous Positive Airways Pressure
CRL – crown rump length
CRP - C-reactive protein
CTG – cardio tocogrphy
CU – carotid ultrasound
DBP – diastolic blood pressure
DEG – diethylene glycol
DMF – N,N-dimethylformamide
DMF – N,N-dimethylformamide
EG – ethylene glycol
EHI – hypoxic ischemic encephalopathy
Fb – fibrinogen
FGR – fetal growth restriction
GPx – glutathione peroxidase
GR – glutathione reductase
GRF – glomerular filtration
HBP – high blood preassure
HCG – human chorionic gonadotropine
HDL – high density lipoprotein
HPC – hydroxypropylcellulose
HTN – hypertension normal
IMT – intima-media thickness
IPPV – Intermittent Positive Pressure Ventiltion
IUGR – intrauterine growth restriction
IVF – *in vitro* fertilization
LDH – lactate dehydrogenase
LPL – lipoprotein lipase
LVEF – ejection fraction
LVH – left ventricular hypertrophy
LVM – left ventricle mass
MCA – median cerebral artery

MDA – malonaldehyde
MDI – 4,4-diphenylmethane diisocyanate
NCEP ATP III - National Cholesterol Education Program, Adult Treatment Panel III
NEC – necrotizing enterocolitis
NICU – Neonatal Intensive Care Unit
PAPP – A1- pregnancy associated plasma protein A-1
PCR – polymerase chain reaction
PE – preeclampsia
PEA – polyethylene adipate
PEGA – poly(ethylene glycol)adipate
PI – pulsatility index
PIH – pregnancy induced hypertension
PlGF – placental growth factor
PMN – polymorphonuclear
PPao – pulse pressure
PTHF – polytetrahydrofuran
PU – polyurethanes
PWVao – aortic pulse wave velocity
ROS – reactive oxygen species
SBP – systolic blood pressure
SBPao – systolic blood pressure
sFlt-1 – soluble isoform of Flt-1 the transmembrane receptor for vascular endothelial growth factor
SGA – small for gestational age
sLDL – small LDL cholesterol
SOD – superoxide-dismutase
TAS – total antioxidant status of serum
TBA – tiobarbituric acid
TG – triglycerides
UtAPI – uterine artery mean pulsatility index
UTP – uterine apoplexy
VEGF – Vascular Endothelial Growth Factor
VLDL – very low densitity lipoprotein

REZUMAT

Elaborarea unei teze de abilitare în contextul etimologiei cuvântului *habilitare* "a se potrivi" impune prezentarea, dar mai ales demonstrarea capacității aspirantului de a dovedi prin descrierea cumulativă a muncii sale că aceasta are suficientă consistenț academică și științifică pentru a i se conferi *venia legendi.*

Conținutul acestei teze descrie preocupările și realizările mele științifice, profesionale și academice din perioada postdoctorală, dar și o parte din proiectele de viitor care au prins déjà contur și vor avea finalitate în anii ce vor urma. Structurarea acestei teze este realizată în conformitate cu recomandările Consiliul Naţional de Atestare a Titlurilor, Diplomelor și Certificatelor Universitare și metodologiei Școlii Doctorale a Universității "Grigore T. Popa" din Iași.

Prezentarea lucrării are ca început rezumatul conținutului, urmată de prezentarea cercetărilor, publicațiilor și a proiectelor de cercetare în care am fost implicată, fiind continuată cu descrierea proiectelor mele viitoare armonizând cei patru piloni ai formării mele profesionale: academic, ceretare științifică, activitate didatică și medicală.

Secțiunea I este structurată pe patru capitole și descrie cercetările realizate, rezultatele obținute și modalitatea de diseminare a rezultatelor.

Primul capitol prezintă rezultatele a cinci studii realizate împreună cu colectivele de cercetare de la Institutul de Chimie Macromoleculară "Petru Poni" din Iași și de la Departamentul de Științe Biomedicale al Facultății de Bioinginerie Medicală. În aceste studii au fost formulate și sintetizate noi membrane celulozice și de poliuretan pentru practica medicală.

După sinteza propriu-zisă, au fost efectuate o serie de teste pentru caracterizarea lor fizico-chimică, teste de biocompatibilitate, care au urmărit capacitatea de adsorbție a albuminei/proteinelor, teste de hemocompatibilitate, de citototoxicitate *in vitro* și *in vivo* și teste de potențial oxidativ.

În urma studiilor efectuate am apreciat că noii compușii sintetizați au o biocompatibilitate superioară și o adaptabilitate funcțională la contactul cu sângele datorită proprietăților lor cumulate, cum ar fi elasticitatea, hidrofilicitatea, neutralitatea suprafeței, adsorbția raportului albumină/fibrinogen mai mare, precum și datorită capacității oxidative reduse.

În capitolul al II-lea sunt abordate din perspectivă diagnostică, prognostică și terapeutică aspectele stresului oxidativ la nou-născuții cu asfixie. Studiile pe acest domeniu, de pionierat pentru România, au fost realizate în cadrul secției de anestezie și terapie intensivă din cadrul Spitalului de Obstetrică și Ginecologie "Cuza Vodă" din Iași, având acordul Comisiilor de Etică ale spitalului și ale Universității "Grigore T. Popa" din Iași.

Am abordat studierea impactului speciilor reactive de oxigen și modificările enzimelor implicate, în cazul asfixiei neonatale întrucât, conform datelor din literatur, asfixia perinatală, cu cea mai frecventă și severă complicaţie a sa, encefalopatia hipoxic ischemică (EHI), este principala problemă care poate apărea la naștere indiferent de modul de producere a acesteia (naturală sau prin cezariană).

Consecinţa majoră a EHI o constituie afectarea neurologică a nou-născuţilor cu răspunsuri clinice nefaste pe termen mediu și lung asupra calităţii vieţii acestor copii. Găsirea unui panel de teste, care, împreună cu examenul clinic și tehnicile de ventilaţie aplicate, să contribuie la direcționarea clinicianului către cea mai bună decizie terapeutică, au constituit principala direcție de studiu. În urma studiilor efectuate am standardizat și am validat metodele pentru dozarea activității principalelor enzime implicate în neutralizarea speciilor reactive de oxigen, precum și pentru activitatea enzimelor markeri în evaluarea neurologică.

Rezultatele obținute au fost folosite în monitorizarea activității enzimatice în cazul deciziilor terapeutice prin utilizare de fenobarbital și eritropoetină în cazul nou-născuților cu diverse grade de asfixie. Întrucât o parte din suferința nou-născutului este cauzată și de anumiți factori materni, am evaluat capacitatea de apărare antioxidantă a serului matern comparativ cu cea a nou-născutului în cazul retardului de creștere intrauterină acompaniat în cele mai multe cazuri de asfixie.

Capitolul următor prezintă suma cercetărilor privind evaluarea markerilor biochimici utili în monitorizarea și, mai ales, în predicția precoce a unei patogenii frecvente în obstetrică, respectiv preeclampsia. Deoarece subiectul prezintă numeroase valențe etio-patogenice, în lumina cărora s-au modificat și metodele de abordare terapeutică cu accent pe partea de prevenție și predicție, am analizat implicarea factorilor angiogenici în homeostazia endoteliului vascular, rolul determinării nivelului PlGF (factorul de creștere placentar), – sFlt-1 (izoforma solubila a Flt-1, receptorul transmembranar al factorului de creștere a endoteliului vascular), precum și raportul dintre aceștia.

O preocupare intensă a mea și a unor colegi clinicieni din specialitatea cardiologie a fost îndreptată asupra pacientului cu sindrom cardiometabolic, realizând trei studii pe care le-am prezentat în ultimul capitol.

Prin cercetările realizate am putut stabilii corelații între parametrii electrofiziologici, biochimici și manifestarile clinice la pacientul cu sindrom cardiometabolic, evidențiând importanța testării nivelului de sLDL (small LDL), particule cu potențial aterogen superior și marker de predicție pentru boala coronariană superior LDL-ului. Datorită implicațiilor majore asupra sănătății pacienților cu sindrom cardiometabolic am cercetat prezența unor polimorfisme la nivelul genei LPL (lipoprotein lipazei), primul realizat în România, cu scopul depistării și calculării frecvenței acestor polimorfisme în populația din nord-estul României.

Secțiunea a doua a tezei prezintă principalele mele preocupări de cercetare și dezvoltare acestea fiind structurate pe trei direcții strâns legate de activitatea mea educațională, pro-fesională și academică: studiile de biochimie moleculară, proteomică și genomică cu aplicații clinice directe la nou-născuți, gravide și la pacienți cu patologii oncologice.

Referințele bibliografice aferente studiilor prezentate și concluziile, care sumarizează temele, prezentate încheie lucrarea.

ABSTRACT

The elaboration of an habilitation thesis having in view the etymology of the word habilitation "to fit" requires the presentation, but especially the demonstration of the aspirant's ability to prove by the cumulative description of their work that they have enough academic and scientific consistency to confer them *venia legendi*. The contents of this thesis describes my scientific, professional and academic concerns and achievements in the postdoctoral period, but also some of the future projects that have already taken shape and will have finality in the years to come.

This thesis is structured in accordance with the recommendations of the National Council for Acknowledgement of University Degrees, Diplomas and Certificates and the methodology of the Doctoral School of "Grigore T. Popa" University Iași.

The presentation of the paper begins with a summary of the contents, followed by the presentation of the research, publications and research projects in which I was involved, then it continues with the description of my future projects harmonizing the four pillars of my training: academic, scientific research, teaching and medical.

Section I is structured in four chapters and describes the research carried out, the results obtained and the way in which the results are disseminated.

The first chapter presents the results of five studies conducted together with the research teams from "Petru Poni" Institute of Macromolecular Chemistry in Iași and from the Biomedical Sciences Department within the Faculty of Medical Bioengineering.

In these studies, new cellulosic and polyurethane membranes were formulated and synthesized for medical practice. After the actual synthesis, a series of tests were performed for their physical-chemical characterization, biocompatibility tests that monitored the absorption capacity of albumin/proteins, hemocompatibility tests, *in vitro* and *in vivo* cyto-totoxicity tests and potential oxidative tests.

Following the studies we found that the new synthesized com-pounds have superior bio-compatibility and functional adaptability to contact with blood due to their cumulative properties such as elasticity, hydrophilicity, surface neutrality, higher albumin/ fibrinogen absorption, and due to the reduced oxidative capacity.

The second chapter addresses from a diagnostic, prognostic and therapeutic perspective the aspects of oxidative stress in newborns with asphyxia. The studies on this field, pioneering for Romania, were carried out in the anesthesia and intensive care unit at "Cuza Vodă" Obstetrics and Gynecology Hospital of Iași, with the agreement of the Ethics Commissions of the hospital and of "Grigore T. Popa" University of Iași.

We approached the study of the impact of reactive oxygen species and changes in the enzymes involved in neonatal asphyxia because, according to literature, perinatal asphyxia, with its most common and severe complication, hypoxic ischemic encephalopathy (HIE), is the main problem that may occur at birth regardless of how it is produced (natural or by cesarean section).

The major consequence of HIE is the neurological impairment of newborns with adverse clinical responses in the medium and long term on the quality of life of these children. The main study directions were finding a panel of tests, which, applied together with the clinical examination and the ventilation techniques, could help directing the clinician to the best therapeutic decision.

Following the performed studies, we standardized and validated the methods for dosing the activity of the main enzymes involved in the neutralization of reactive oxygen species, as well as for the activity of marker enzymes in neurological evaluation.

The obtained results were used to monitor the enzymatic activity in the case of therapeutic decisions by using phenobarbital and erythropoietin in the case of newborns with various degrees of asphyxia. Since part of the newborn's suffering is also caused by certain maternal factors, we evaluated the antioxidant defense capacity of the maternal serum compared to the one of the newborn in the case of intrauterine growth retardation accompanied in most cases of asphyxia.

The following chapter presents the sum of research on the evaluation of biochemical markers useful in monitoring and, especially, in the early prediction of a common pathogenesis in obstetrics, respectively preeclampsia. As the subject has numerous etiopathogenic valences, in the light of which the methods of therapeutic approach with emphasis on prevention and prediction have changed, we analyzed the involvement of angiogenic factors in vascular endothelial homeostasis, the role of determining PGF (placental growth factor), – sFlt-1 (soluble isoform of Flt-1, the transmembrane receptor for vascular endothelial growth factor), as well as the ratio between them.

An intense concern of mine and of some clinician colleagues in the field of cardiology was directed to the patient with the cardiometabolic syndrome, conducting three studies that we presented in the third chapter.

Through research we were able to establish correlations between electrophysiological and biochemical parameters and clinical manifestations in patients with the cardiometabolic syndrome, pointing out the importance of testing the level of sLDL (small LDL), particles with higher atherogenic potential and predictive marker for coronary heart disease.

Due to the major implications on the health of patients with cardiometabolic syndrome, we investigated the presence of polymorphisms in the LPL gene (lipoprotein lipase), the first performed in Romania, in order to detect and calculate the frequency of these polymorphisms in the population of North-Eastern Romania.

The second section of the thesis presents my main research and development concerns as it is structured in three directions closely related to my educational, professional and academic activity: molecular, proteomic and genomic biochemistry studies with direct clinical applications in newborns, pregnant women and patients with oncological pathologies.

The bibliographic references related to the presented studies and the conclusions that summarize the presented topics conclude the paper.

SECTION I

ACADEMIC, PROFESSIONAL AND SCIENTIFIC ACHIEVEMENTS

Introduction

Learn from yesterday, live for today and hope for tomorrow. The most important thing is to never stop asking.

Professional activity

In more than 25 years of professional activity, I have always considered it essential to ensure and keep a high professional standard to complete and perfect my knowledge.

I graduated in 2001 from "*Grigore T. Popa" University of Medicine and Pharmacy of Iași,* Bachelor's Degree no. 641/9 April 2003, and "*Al. I. Cuza" University of Iaşi; Bachelor's Degree no. 291/21 June 1994*. I have acquired skills and I have learned new theoretical and practical notions in the new activity directions at the level of the medical analysis laboratory: clinical biochemistry, molecular biology, molecular biochemistry following various specialization programs:

- A training course within the ENDODIAB project – the training of specialists in the field of endocrinology and diabetes, Bucharest 2019-2020, "C. I. Parhon" National Endocrinology Institute;
- Course – Nutrigenetics – The use of nutrigenetics in defining individualized nutritional needs – 24-28 June 2019, Bucharest – GenetX and "Carol Davila" UMF of Bucharest;
- "PROTEOMICS – from Introduction to Clinical Applications" 9-14 July 2017, Iași, organized by UAIC Iași together with TRANSCEND – a research center of the Oncology Institute, Iași;
- A postgraduate course "Management of the modern health organization", 01- 15.03.2017, UMF Iași;
- A postgraduate course "Quality management in laboratory medicine", UMF Iași, 9-23.03.2013;
- Oncogenetics course Iasi, 24-26 October 2012, coordinator Prof. dr. Yves Jean Bignon;
- A training course – "Professional and organizational training of employees in immunology laboratories by the implementation of cutting-edge technologies and quality management" European project 2010-2013.

I apply the acquired professional expertise in the activity within the medical analysis laboratory of "Cuza Vodă" Obstetrics and Gynecology Hospital of Iași, where I have been working since 2013 and where I have been the head of the laboratory since 2018. I am responsible for the reconfiguration and involvement of laboratory activities in interdisciplinary collaborations with colleagues in the specialties of genetics, obstetrics and neonatology.

Scientific research activity

I started the scientific research activity in the bio-medical field since 1999 as a student at the second faculty and I was co-opted in the research team of 2 ANSTI grants:
ANSTI Code 3149, program director Gh. Iacob, topic "Research on the treatment of leukemia and malignant tumors by the magnetic carrier method", value of 30 million lei and Contract "Orizont 2000", program director Gh. Iacob, theme: "Research on the *in vitro*

concentration process of red blood cells by the separation method in high gradient of magnetic field. Magnetic sorter for immunomagnetic cells and microparticles", value of 40 million lei. I learn the biomedical role of the magnetic carrier and its multiple applications, a very approached topic by researchers at that time.

After having been admitted to doctoral studies in 2004 with a subject on the oxidative stress in certain human pathologies, I focused on the role of reactive oxygen species in newborns with asphyxia and who receive as the main therapy oxygen (administered in doses and different techniques).

The implications of reactive oxygen species in short and long-term neonatal pathology were new for neonatologists in those years, whereas the developed study was the first in Romania.

The conducted research conducted in the neonatal intensive care unit within "Cuza Vodă" Obstetrics and Gynecology Hospital Iași allowed me to determine the enzymes specific to oxidative stress, standardization of dosage methods, development of work protocols and introduction in the current practice in the files, monitoring of newborns with asphyxia of the determinations for the total antioxidant capacity of serum, CK and LDH (markers of stress and respectively neurological suffering).

The experience gained in the research of oxidative stress for blood parameters enabled the selection of 3 research grants in the teams.

IDEI Project, Contract no. 223/2007, "Interfering the pharmaco-biochemical mechanisms of homocysteine involvement in the atherogenic phenomenon", implemented in 2007-2010, the contractor "Grigore T. Popa" University of Medicine and Pharmacy of Iaşi; project director Associate Professor Albu Elena PhD.

Internal grant UMF Iaşi, Contract no. 28212/2011, "The role of quantifying the degree of subclinical atherosclerosis in patients with cardiometabolic syndrome, establishing the risk class and therapeutic behavior", implemented from 01.01.2012 to 20.12.2012, project director Professor Mitu Florin PhD.

Internal grant UMF Iaşi, Contract no. 30881/2014, "Optimal storage practices to preserve macronutrients, energy and total antioxidants in human milk from mothers of term and preterm newborns", implemented from 01.01.2015 to 30.12.2016, project director Associate Păduraru Luminiţa PhD.

My experience and professional training in both fundamental and biomedical sciences have been applied in the research activity carried out in the research teams of the following grants:

- Internal grant UMF Iaşi, Contract no. 17075/2009, *Study of the hemocompatibility of some polyurethane derivatives with potential biomedical applications*, implemented from 01.10.2009 to 30.09.2010, project director Associate Butnaru Maria PhD;
- PCCDI Project, Contract no. 39PCCDI/2018, *Intelligent materials for medical applications (INTELMAT)*; takes place from 01.03.2018 to 30.06.2021; coordinator of the Polytechnic University of Bucharest; partner "Grigore T. Popa" University of Medicine and Pharmacy of Iaşi; responsible for the project Professor Eng. Vereștiuc Liliana PhD.

The inclusion in the research team of the CNCSIS Project 2006-2008, contract no. 29/2006 with the title *Interdisciplinary platform of molecular medicine* 2006-2008, project director Professor Eugen Carasevici PhD allowed me access to modern and complex equipment and opened my perspective to learn new techniques of molecular biology, immunology and cell cultures, which helped me acquire sufficient skills and knowledge and win through competition UMF Internal Grant Iași, Contract no. 29232/2013 entitled "Study of the

prevalence of two polymorphisms of the lipoprotein lipase gene in patients with cardio-metabolic syndrome", implemented from 01.01.2014 to 30.06.2016; ***project manager***.

At present my research activity focuses mainly on clinical biochemistry and molecular biology, respectively, being concerned with genomic mutational changes in malignant pathologies of the pancreas. I can carry out this research by carrying out the *PCCDI Project, Contract no. 66PCCDI/ 2018 Pathogenic mechanisms and personalized treatment in pancreatic cancer using multi-homic technologies (PANCNGS), from 04.05.2018 to 30.06.2021; coordinator of Fundeni Clinical Institute, Bucharest; partner "Grigore T. Popa" University of Medicine and Pharmacy Iaşi; project manager Professor Scripcariu Viorel PhD,* a team I am also part of.

My participation in the mentioned grants and research projects and also the acquired skills have allowed me to write scientific papers as the lead author or co-author. The most important results are published ***in extenso*** in 25 articles in ISI Thomson Reuters, with a factor impact and a number of 9 papers in other journals from international databases. The number of citations accumulated according to the Web of Science is 131 and the Hirsh index (*h*-index=7).

I am the author of three specialty books and co-author of a chapter on an international treatise Polyurethane-10 *Biocompatibility and Biological Performance of the Improved Polyurethane Membranes for Medical Applications* as well as on two editions of the treatise of *Clinical Biochemistry* edition 2015 and 2020 edited by Minodora Dobreanu.

Academic activity

Since 1999, after being promoted by a contest as a university preparator, I have been working in the Biochemistry discipline at the Faculty of Medicine where I was involved in teaching students and residents from laboratory medicine, epidemiology, medical microbiology and diabetes. I coordinate bachelor's theses and I am part of the guidance commissions for doctoral theses from related disciplines.

In the same context I participated in educational programs for students and doctors:

- Training - "Career Strategies: Training in Medical Pedagogy", organized by CRU of "Grigore T. Popa" UMF Iași, 25-26 April 2017;
- CHRONEX-RD East-European Network of Excellence for Research and Development in Chronic Diseases October- November 2014;
- "Training Center for Specialists and Resources in Oral Rehabilitation", ID POSDRU/87/1.3/S/62208. Project co-financed from the European Social Fund by the Sectorial Operational Program of Human Resources Development 2007-2013. Priority Axis 1 – Education and vocational training in support of the economic growth and development of the knowledge-based society. Major field of intervention 1.3. – Development of human resources in education and training, Decision 23904// 29.10.2012.

I am concerned with the improvement and acquisition of good practices in accordance with the principles of ethics and university deontology, and I was involved in several projects:

- Member in the CEMED 2020 project *Quality in education through knowledge and respect for deontology and academic ethics*;
- Member in the CEMED 2018 project *Quality in education through knowledge and respect for deontology and academic ethics*;
- Member of the project POSDRU/18/1.2/G/40067 – *Quality standards and specific performance indicators in higher education in health* organized by "Iuliu Haţieganu" University of Medicine and Pharmacy of Cluj Napoca in partnership with the Romanian Agency for Quality Assurance in Education Superior of Health, September 2013.

I was concerned with establishing new collaborations between our university and other universities in the European space in the same context. I participated in cluster projects or ERASMUS teaching programs:

- AUF-ECO/2017 the project "Creation of a regional university network in the field of health, nutrition and food safety", implemented from 18.09.2017 to 31.12.2019; coordinator of Agence Universitaire de la Francophonie; partner "Grigore T. Popa" University of Medicine and Pharmacy of Iaşi; project manager associate professor Mihai Bogdan Mircea PhD;
- Project FP7, Contract no. 265435/2011, "Advanced cross disciplinary & integrated medical imaging for all Europeans through a network regional clusters and development strategies (AMI-4EUROPE)", implemented from 2010 to 2013, coordinator Asociacion Madrid Network Madrid, Spain; partner "Grigore T. Popa" University of Medicine and Pharmacy of Iaşi; responsible for the project Professor Pieptu Dragoş PhD/ Professor Ştefănescu Cipriana PhD;
- ERASMUS mobility – teaching internship September 3-10, 2019 University of Rennes;
- ERASMUS mobility – teaching internship, 3-8 September 2018, Lorraine Nancy University – France.

I strongly believe that the mission of the ones who have chosen this path of teaching career in medical higher education, must build it thoroughly, robustly and masterfully, by combining the three fundamental didactic levels, medical professional training and research.

CHAPTER 1

PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF MEMBRANES BASED ON POLYURETHANES AND CELLULOSE

1.1. Introduction

The need for appropriate biomaterials for medical applications is a widely discussed field. According to data of the last two decades it is clear that due to their wide structural availability, polyurethanes can satisfy the general requirements of "good materials" if biocompatibility is possible to ensue (Chen *et al*., 2008; Mendelson *et al*., 2006; Xinwen *et al*., 2007).

Polyurethanes – PU represent an important class of polymers permitting to obtain of the desired properties by a proper selection of different segments in their composition. The combination of polyols, diisocyanates and low molecular chain extenders gives a considerable position as useful biomaterials for implants or biomedical devices (Adhicari *et al*., 2003; Zdrahala *et al*., 1999).

Polyurethanes have been widely used for various commercial and experimental blood-contacting and tissue contacting applications, such as vascular prostheses, blood pumps, endotracheal tubes, mammary prostheses, heart valves, pacemaker lead wire insulations, intra-aortic balloons, catheters, artificial hearts, sensors and transducers, because of their generally favorable physical and mechanical properties, together with their fairly good biocompatibility and anti-thrombogenic characteristics (Ozdemir *et al*., 2002; Desai *et al*., 2000; Ramis *et al*., 2001).

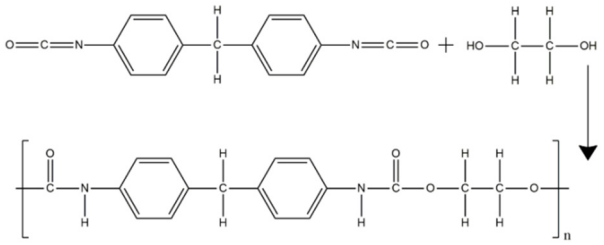


Fig. 1.1. Polyurethan synthesis.

Their blockpolymer structure could provide a large spectrum of physico-chemical properties and degradability, reasons for which the biological per-formances of PUs have been widely discussed in the last decade (Guelcher *et al*. 2007; Guelcher *et al.*, 2008; Christenson *et al.*, 2004; Fujimoto *et al.*, 2007).

However, it is well known that structural and mechanical adaptability of PUs is not always accompanied by cell and tissue biocompatibility. Therefore, numerous data in the literature are focused on biocompatibilization or functionalization of PUs (Yao, 2008; Sartori, 2008, Huang & Xu, 2010). Some promising methods for the improvement of biological response of PUs are conjugation, blending or coating with natural polymers

While the structural and mechanical adaptability of the PUs is indisputable, their bio-functionalization for a better biocompatibility is still a challenge. Most efforts are now focused on molecular conjugation, blending or coating as methods for functional improvement of the PU (Chen *et al*., 2010; Huang *et al*., 2010; Sartori *et al*., 2008; Yao *et al*., 2008).

From chemical point of view, the approach is directed to the molecules that provide a high level of molecular interactions, for example through hydroxyl radicals of polysaccharides including those of cellulose derivatives (Chen *et al.*, 2000; Raschip *et al.*, 2009).

Hydroxypropylcellulose (HPC) is an appropriate candidates for blending due to their demonstrate biocompatibility as well as to their possible hydrogen bonding that can enhance both mechanical and surface properties of the resulted materials, which will modify non-specific protein adsorption first (Doelker *et al.,* 1993; Janice *et al*., 2009).

An important overlooked tissue-material interfacing phenomenon is the oxidative stress production in the live tissue. In the last decade oxidative stress cell lesions induced by some degradable biomaterials previously considered biocompatible have been demonstrated (Jiang *et al.*, 2007).

Two mechanisms can be presumed. One deals with the excessive production of reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, hydrogen peroxide by the material constituents or by the tissues in response to the material actions.

The second mechanism considers direct affecting of the tissue antioxidant capacity produced by adsorption/inactivation of the tissue antioxidant compounds (Forstermann *et al.*, 2008). Therefore, free radical production and antioxidant capacity decrease may be considered predictable parameters for foreign body reaction and material time resistance.

Some diseases of the cardiovascular system are associated with increased production of ROS, and few treatments of the vascular tissue disorders involve antioxidant enzymes (Wattanapitayaku *et al*., 2001). The efficiency of enzyme-based treatment is dependent by the ability to achieve therapeutically adequate levels at the site of ROS-mediated injury (June *et al*., 2013).

Enzymes-biofunctionalized magnetic nanoparticles can increase the enzyme efficiency and limits adverse effects of thermal and proteolysis degradation (Chorny *et al*., 2010).

In terms of application, such magnetic nanoparticles have to be stable in the circulating system and to improve the halftime of the biological compounds, to reach the diseased tissue or to be involved in the in cellular mechanisms, to release the bioactive in the controlled manner, activated or not by external stimulus/stimuli. Also, they have to be biodegradable and eliminated from body without any toxic effects (Mahapatro *et al*., 2011). Moreover, in the aim to attach biomolecules on surface, the magnetic nanoparticles have to present reactive groups, like us: -OH and -NH_2, -COOH and -SH, most often obtained by coating with polymers.

Various polymers have been tested as coatings or networking structure in composites with magnetic materials (PLA, PLGA and copolymers, other synthetic polymers, chitosan, hyaluronic acid, gelatine) (Mahapatro *et al*., 2011). Catalase and superoxide dismutase (SOD) are two antioxidant enzymes containg active centers with coordinated metals, which decompose superoxide anion and hydrogen peroxide, respectively, the most important reactive oxygen species (Mora *et al*., 2014).

Haemocompatibility of biomaterials, in terms of thrombo-resistance is determined by surface characteristics such as: distribution of the electric charges, surface tension and hydro-phobe-hydrophile balance, which confer to the material more or less thrombo-resistant qualities.

It is well known that when a material is interacting with blood, first the adsorption of plasma proteins takes place. In function of the affinity of the material to certain proteins specific biological mechanisms occur, for example those of the coagulation cascade.

Proteins which decide the thromboresistance/thrombogenesis properties are albumin and fibrinogen, the first one is conferring thrombo-resistant qualities, and the second one is conferring thrombogene qualities, this latter protein being involved directly in the clot formation.

The adsorption of the fibrinogen is influenced on one hand by the chemical nature of the surface, in particular by the hydrophobe/hydrophile balance, and on the other hand can be the result of the pro-clot biological phenomena of the extrinsic pathway, such as the adhesion and

platelet activation. As to the second mechanism, fibrinogen adsorption accompanied by more accelerated prothrombin consumption, characteristic for a pro-clot situation with formation of the fibrin and finally the blood clot.

Personal contribution

"Study of the hemocompatibility of some polyurethane derivatives with potential biomedical applications" – Project UMF contract nr.17075/2009 – ***Internal project*** – ***Member***.

Maria Butnaru, Ovidiu Bredetean, Doina Macocinschi, **Cristina Daniela Dimitriu**, Laura Knieling and Valeria Harabagiu *Poyurethane*, Chapter 10 *Biocompatibility and Biological Performance of the Improved Polyurethane Membranes for Medical Applications*, InTech, 2012; 201-228.doi : 10.5772/34653

Published paper:

- Butnaru M, **Dimitriu DC**, Bredetean O, Macocinschi D, Harabagiu V. *In vitro Biocompatibility of HPC - modified Polyurethane Membranes*. Rev. Materiale Plastice, 2015, 52(3), 90-93, **Impact Factor – 0.842**;
- Butnaru M, Macocinschi D, **Dimitriu CD**, Vlad S, Filip D, Harabagiu V. *Proteine adsorbtion and oxidative properties of some cellulose-modified polyurethane membranes for medical applications*. Optoelectronics and Advanced Materials-Rapid Communications, 2011, 5(2), 172-176, **Impact Factor – 0.451**;
- Macocinschi D, Filip D, Butnaru M, **Dimitriu CD.** *Surface characterization of biopolyurethanes based on cellulose derivatives*. Journal of Materials Science: Materials in Medicine, 2009, 20, 775-783, **Impact Factor – 1.955**;
- Lupu M, Butnaru M, Macocinschi D, Oprean OZ, **Dimitriu C**, Bredetean O, Zagnat M, SI. *Surface properties of segmented poly(ester urethane)s and evaluation of in vitro blood compatibility and in vivo biocompatibility*. Journal of Optoelectronics and Advanced Materials, 2007, 9(11), 3474-3478, **Impact Factor – 0.827**;

1.2. Aim of the studies

Study 1 and study 2 – These two articles aimed to study the effects of the chemical structure of segmented polyurethanes (study 1) and polyurethanes-cellulose (study 2) on their surface properties such as blood protein adsorption, the haemocompatibility and the tissue biocompatibility.

Investigations are based on the geometric mean approach of Owens and Wendt, Rabel, Kälble (Owens *et al*., 1969; Rabel *et al*., 1977; Kälble *et al*., 1969) on the Lifshitz-van der Waals acid/base approach of van Oss, Good and Chaudhury (van Oss *et al*., 1988; van Oss *et al*., 1994), as well as on the theoretical methods involving quantitative structure-property rela-tionships (Bicerno *et al*., 1996).

Study 3 – The aim of this study was to highlight the functional integration possibility of HPC-modified polyurethanes previously demonstrated for their good mechanical properties (Lupu *et al*., 2007, Maccocinschi *et al*., 2009).

Study 4 – Based on the results of the 3 studied mentioned above, where we demonstrated that small amounts of HPC in some PU structures can enhance surface hydrophilicity, neutralize surface charge, decrease surface roughness and improve bulk porosity of the resulted membranes, in this study we have showed the hydrolytic modifications of the material structures, *in vitro* biocompatibility as well as polymorphonuclear (PMN) leucocytes activation.

Study 5 – The aim of this study was measurement of total Antioxidant Status (TAS) in plasma. The TAS measurement was made by standard protocol provided by Randox TAS kit.

1.3. Materials and methods

1.3.1. Materials and reagents used in the studies

Reagents used for membranes preparation:

- poly(ethylene adipate)diol (PEA, Mn=2000 g/mol) from Fibrex SA Săvineşti, România;
- polytetrahydrofuran (PTHF, Mn=2000 g/mol) from BASF;
- poly(propylene)glycol (PPG, Mn=2,000 g/mol) from BASF;
- ethylene glycol (EG), diethylene glycol (DEG) as chain extender;
- poly(ethylene glycol)adipate (PEGA);
- N,N-dimethylformamide (DMF) from Merck;
- 4,4-diphenylmethane diisocyanate (MDI, distilled under reduced pressure prior to utilization) and 2,4-tolylene diisocyanate (TDI) from Merck;
- hydroxypropylcellulose LF (HPC with average weight molecular weight Mw=95 000 g/mol) from Klucel.

Reagents used for membranes characterization:

- Thiazolyl Blue Tetrazolium Bromide (MTT), suitable for cell culture;
- Dulbecco's Phosphate Buffered Saline (DPBS), modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered, suitable for cell culture;
- Hank's Balanced Salt Solution (HBSS) based on HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), modified, without calcium chloride and magnesium chloride, liquid, sterile filtered, suitable for cell culture;
- Dulbecco's Modified Eagle Medium (DMEM), high glucose with L-glutamate and Pyruvate;
- Penicillin-Streptomycin-Neomycin (PSM) solution (5,000 units penicillin, 5 mg streptomycin and 10 mg neomycin/mL) stabilized, sterile-filtered, suitable for cell culture;
- Fetal Bovine Serum (FBS) heat inactivated, non-USA origin, sterile-filtered, suitable for cell culture;
- dextran T500; bovine serum albumin (BSA); 0.4% Tripan Blue solution.

All these materials and reagents were purchased from Sigma-Aldrich.

1.3.2. Preparation of the samples

1.3.2.1. Preparation of the materials based on PU

The samples were prepared by the reaction of aromatic diisocyanates such as 4,4'-methylene diphenylene diisocyanate (MDI) or 2,4-tolylene diisocyanate (TDI) with poly(ethyleneglycol)adipate (PEGA), and diethylene glycol (DEG) as chain extender, by using a two steps polyaddition process in N,N-dimethyl formamide (DMF) (Grigoriu *et al.*, 2001).

The obtained samples were noted as follows:

- PEGA/MDI-DEG;
- PEGA/TDI-DEG.

1.3.2.2. Preparation of PU casting films

Each PU was dissolved in DMF, to reach a concentration of 1 g/dl. The solutions were cast on a glass plate and initially solidified by slow drying in DMF saturated atmosphere, for 7

days and finally by drying at 50°C under vacuum (48h). The polyurethane films thus prepared were subjected to surface analysis.

Also, the same types of samples were plasma-treated. The low pressure plasma treatment was performed on an installation with the following characteristics: intensity – 3000 V cm^{-1}, frequency – 1.3 MHz, pressure – 58 Pa, duration – 10 min. Uniform drops of the test liquids with a volume of 2 μL were deposited on the film surface and the contact angles were measured after 30 s, with a video based optical contact angle measuring device equipped with a Hamilton syringe in a temperature-controlled environmental chamber.

All measurements were performed in air, at a temperature of 25°C. Repeated measurements of a given contact angle were all within ±3°C. As probe liquids, double-distilled water, ethylene glycol and glycerol were used, as purchased at maximum obtainable purity.

1.3.2.3. Preparation of PU/HPC membranes

Briefly, isocyanate terminated urethane prepolymers were first synthesized by the polyaddition reactions between MDI and macrodiols in DMF as solvent. PEA, PTHF or PPG were used as macrodiols. The urethane pre-polymers were treated in a subsequent step with EG as chain extender. Finally, HPC was added to PU solutions to obtain the following compositions for all PU/HPC samples: macrodiol/MDI/EG/HPC=52.24/36.57/7.27/3.92 (weight ratios).

As the molar ratio between isocyanate groups in MDI and the sum of hydroxylic groups in macrodiol and EG was 1.02, the excess of isocyanate groups linked to PU prepolymers were available to bind a part of HPC chains. Membranes with about 1 mm thickness were prepared by pouring PU/HPC DMF solutions in distilled water, at 40°C.

The formed films were dried under vacuum for several days and kept in distilled water for solvent removing. PU/HPC functionalized samples based on PEA, PTHF and PPG macrodiols were named as follows:

- PEA-HPC;
- PTHF-HPC;
- PPG-HPC.

1.3.2.4. Preparation of magnetic nanoparticles

Biofunctionalized magnetic nanoparticles with antioxidant enzymes have been obtained by Catalase/SOD attaching on particles through carbodiimide chemistry method.

Briefly, 5 mg enzyme was dissolved in 20 mL phosphate buffer saline (PBS, 0.05 M, pH=8.00) and then mixed with a suspension of nanoparticles (100 mg in 30 mL of 0.05 M PBS, pH=8.00) and slowly mechanically stirred for 24 h. The particles have been collected from suspension through magnetic separation, purified by repeated washes with PBS (2x) and deionised water (3x) and finally freeze-dried.

1.3.2.5 Total Antioxidant Status (TAS)

The TAS measurement was made by standard protocol provided by Randox TAS kit.

1.3.3. Characterization techniques

1.3.3.1. Contact angle measurement

PU films were dissolved in DMF, to reach concentration of 1 g/dl. The solutions were cast on a glass plate and initially solidified by slow drying in DMF saturated atmosphere for 7 days and finally by drying at 50°C under vacuum (48 h). The PU films thus prepared were subjected to surface analysis. Also, the same types of samples were plasma treated. The low-pressure plasma treatment was performed using an installation, done in our laboratories, with the following characteristics: intensity: 100 V/cm; frequency: 1.2 MHz; pressure: 0.24 mbar; duration: 10 min.

Uniform drops of the test liquids with a volume of 2 ml were deposited on the film surface and the contact angles were measured after 30 s, with a video-based optical contact angle measuring device, a modular instrument for goniometry, done in our laboratories, equipped with a Hamilton syringe in a temperature controlled environmental chamber.

Repeated measurements of a given contact angle were all within ±3°C. As probe liquids, double distilled water; ethylene glycol and methylene iodide were used.

1.3.3.2. Surface tension parameters

For the calculation of the surface tension parameters, the geometric mean method (Eqs. 1 and 2), the acid/base method (LW/AB) (Eqs. 3-5), and theoretical method based on the structure-property relationship considering the group contribution techniques (Eq. 6), were used.

$$\frac{1+\cos\theta}{2}\frac{\gamma_{lv}}{\sqrt{\gamma_{lv}^{d}}} = \sqrt{\gamma_{sv}^{p}}\cdot\sqrt{\frac{\gamma_{lv}^{p}}{\gamma_{lv}^{d}}} + \sqrt{\gamma_{sv}^{d}} \tag{1}$$

$$\gamma_{sv} = \gamma_{sv}^{d} + \gamma_{sv}^{p} \tag{2}$$

$$1+\cos\theta = \frac{2}{\gamma_{lv}}\left(\sqrt{\gamma_{sv}^{LW}\cdot\gamma_{lv}^{LW}} + \sqrt{\gamma_{sv}^{+}\cdot\gamma_{lv}^{-}} + \sqrt{\gamma_{sv}^{-}\cdot\gamma_{lv}^{+}}\right) \tag{3}$$

$$\gamma_{sv}^{LW/AB} = \gamma_{sv}^{LW} + \gamma_{sv}^{AB} \tag{4}$$

$$\gamma_{sv}^{AB} = 2\sqrt{\gamma_{sv}^{+}\cdot\gamma_{sv}^{-}} \tag{5}$$

$$\gamma(298\,\mathrm{K}) \approx 0.75\cdot[E_{coh}/V(298\,\mathrm{K})]^{2/3} \tag{6}$$

θ is the contact angle determine for water, ethylene glycol and CH_2I_2, subscripts "lv" and "sv" denote the interfacial liquid-vapour and surface-vapour tensions, respectively, while superscripts "p" and "d" denote the polar and disperse components, respectively, of total surface tension, γ_{sv}. Superscripts "LW" and "AB" indicate the disperse and the polar component obtained from the γ_{sv}^{-} electron donor and the γ_{sv}^{+} electron acceptor interactions, while superscript "LW/AB" indicates the total surface tension is the total surface tension, E_{coh} the cohesive energy and V the molar volume.

Water sorption was evaluated by weighing of the polymeric film samples (5×5 mm) under dry and wet state. The maximum hydration was considered at the moment of passing from floating to immersed state. Distilled water was employed. Water sorption of the films was calculated from the following relation (El-Rehim *et al*., 2004):

$$\text{Water uptake (\%)} = (Ww - Wd)\times 100/Wd \tag{7}$$

where Ww and Wd represent the weights of wet and dry films, respectively. The water uptake is the average value of six samples prepared under the same conditions.

For protein adsorption experiment were used:

- bovine serum albumin – BSA solution (1 mg/ml and 3 mg/ml) in phosphate buffered

saline – PBS, pH 7.4 (study 1);
- 3 mg/ml bovine serum fibrinogen (95% clotable) in solution 9% NaCl and human blood plasma (obtaining from human blood on 3.8% sodium citrate 9:1 v/v) (study 2);
- individual protein solutions of fibrinogen (FB 95 % clotable; 3 mg/ml) and BSA (45 mg/ml) of normal physiological concentration (study 3);
- FB-BSA mix physiological solution, 3 mg/ml for BSA and 45 mg/ml for FB (study 3);
- complex protein solution: platelet poor blood plasma – PPP (study 3).

The solutions were always freshly prepared before every adsorption experiment. Prior to adsorption experiment, the samples were equilibrated with PBS for 24 h (study 1)/ 72 h (study 3) or 9% NaCl for 72 h (study 2). In order to perform adsorption experiments, PU films/ PU-HPC membranes with known surface area were introduced into tubes with containing:
- 5 ml BSA solution at 37°C for 15 min (study 1);
- 0.5 ml fibrinogen solution or sanguine human plasma at 37°C for 1 h (study 2);
- 0.25 ml FB/ BSA/ FB-BSA/ PPP at 37°C for 30 min (study 3).

For study 1 the adsorption was carried out by gently shaking the tubes. Any air bubbles were removed by allowing the samples to cross the buffer surface several times. After 15 min of incubation, PU films were removed and the amount of adsorbed protein was determined by assaying the remaining concentration of BSA by a UV-spectrophotometer.

For studies 2 and 3, after incubation, the films of PU/PU-HPC were removed and the amount of remaining protein in solution or plasma was determined by using Na_2SO_4 reaction and spectrophotometrical assaying with Piccos UV–VIS.The adsorbed amount of protein was calculated by following equation:

$$\text{Adsorbed BSA } (mg/cm^2) = (Co - Ce)\ V/S \qquad (8)$$

Where Co and Ce are the initial and equilibrium concentrations of BSA solution (mg/ml), V is the volume of protein solution (ml) and S is the surface of the PU/ PU-HPC sample.

Total antioxidant capacity (TAS) in blood plasma was determined after 24 h incubation of 0.25 ml freshly heparinized human plasma with $0.5\times0.5 cm^2$ well hydrated PU/HPC samples. The TAS measurements were made by standard Randox TAS kit protocol. Control serum was used for data validation.

1.3.3.3. *In vitro* haemocompatibility tests of PU and PU/HPC samples

The thrombogenic potential of the surface film was judged by the blood clot formation test, as described in the literature (El-Rehim *et al*., 2004). The PU film (2×2 cm^2) was incubated in water saline (0.9 % w/v NaCl, pH=7.4) for 24 h, 37°C.

To this swollen sample 0.2 ml human blood from a healthy donor was added on 3.8 % sodium citrate (9:1 v/v), followed by the addition of 0.2 ml $CaCl_2$ solution (0.1 mol/L) to start the thrombus formation. After 8 min, 2 ml deionized water was added to stop the reaction. After another 5 min, the water was removed from the sample and the formed thrombus was taken out with a spatula.

The separated thrombus was soaked in 1 ml 37% formaldehyde solution for 5 min, at room temperature, and then soaked in water for another 5 min. The fixed thrombus was blotted with pieces of filter paper, entirely collected and weighed. The thrombus weight percentage on the PU film was calculated on the basis of the equilibrated thrombus on the glass, which under the same conditions, is assumed to be 100%.

Haemolysis experiments were performed on the film surface, as described in the literature (Bajpai *et al*., 2005). In a typical experiment, a dry polyurethane film (2×2 cm^2) was equilibrated

in physiologic serum for 24 h at 37°C, human blood from a healthy donor on sodium citrate (0.25 ml) being added on the polyurethane film.

After 20 min, 2 ml physiologic serum was added on the film to stop haemolysis, and the sample was incubated for 60 min at 37°C. Positive ($A_{(+)control}$) controls of absorbance ($A_{test\ sample}$) were obtained by adding 0.25 ml human blood from a healthy donor on sodium citrate to 2.0 ml bidistilled water; negative ($A_{(-)control}$) controls of absorbance were obtained by adding 0.25 ml physiologic serum, again to 2.0 ml bidistilled water.

The incubated sample was centrifuged at 1000 g for 20 min. The supernatant was taken out and on a spectrophotometer the absorbance was recorded at 545 nm.
The percent of haemolysis was calculated with the following relationship:

$$\%\ Hemolysis = \frac{A_{test\ sample} - A_{(-)control}}{A_{(+)control} - A_{(-)control}} \quad (9)$$

1.3.3.4. *In vitro* haemocompatibility tests of magnetic nanoparticles

The blood was collected by venous puncture from healthy volunteers and was incubated with an anticoagulant (sodium citrate solution, 3.3%; ratio 1/9 v/v). In 3 ml of blood was added a suspension of nanoparticles (0.5 ml; 0.01%) and incubated at 37°C for 1 h and 12 h (under slight agitation). Finally, the particles were separated by centrifugation (1000 rpm, 10 min) and the blood was biochemical analyzed. The study was approved by the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy Iași.

1.3.3.5. The polymorphonuclear (PMN) leucocytes migration test

The polymorphonuclear (PMN) leucocytes migration experiment was performed on PMNs isolated from human blood of healthy volunteer donor, using gradient separation method described in literature (Meshki *et al*., 2006). The PMN cells were counted using improved Neubauer hemacytometer. Cell viability was determined by widely used 0.2% Tripan Blue solution in DPBS (Freshney *et al*., 2005).

Only the cell suspensions with over 90% of cell viability were considered appropriate for the experiments. PMN migration experiment was performed using Falcon HTS Transwell-24 plates with 8μm pore size membrane and $1\times10^5/cm^2$ pore density. The lower compartments of the plates were filled with 0.1 ml of PU extract, while the upper compartments were filled with 0.5 ml of PMN suspension in HBSS, using 1×10^6 cells/ well.

The PMNs were activated by adding to the cell suspension 10 μL of 1 mM calcium chloride. The plates were kept for 30 min at 37°C and 95% humidified atmosphere followed by 10 min incubation at +4°C. After incubation the upper insert compartment was removed and the PMN cells migrated in the lower compartment were counted using inverted phase-contrast optical microscope. The migration experiment was performed in triplicate for each PU extract. As negative control HBSS was used.

1.3.3.6. *In vitro* cytotoxicity test

In vitro cytotoxicity was performed on primary rat fibroblasts isolated from rat skin, using the explant method (Freshney *et al*., 2005). Briefly, $1cm^2$ of skin was decontaminated using 3-step washing procedure in DMEM with decreasing PSN concentration (4%, 2% and 10%). After the washing procedure, the hypodermic part of the skin was removed and dermis was cut in small (1-2 mm) pieces and plated on the bottom of a petri dish, covered with a thin layer of FBS. The skin tissue pieces were covered with 1.5 ml of DMEM supplied with 15% FBS and 1% PSN and incubated at 37°C, 5% CO_2 and 95% humidity.

Culture media was changed every 3 days, for about 2-3 weeks, until a confluent cell radiated corona was formed around the plated tissue pieces. The migrated cells were passed on 75cm^2 culture area flask, according to standard subculture technique (Freshney *et al*., 2005), until a confluence monolayer was reached. Cells from 2 and 3 passages were used for biocompatibility studies.

For the aimed experiments the PU/HPC membranes were cut in 6 mm discs and decontaminated by their immersion in 70% sterile ethylic alcohol for 20 min.

In order to perform in vitro cytotoxicity tests the sterilized membranes were washed 3 times in sterile DPBS and then incubated overnight in DMEM culture media supplemented with 1% PSN at 37°C, 5% CO_2 and 95% humidity.

Each pre-equilibrated polyurethane disc, was placed over preplated fibroblasts at 2.5×10^4 well cell density, in 24-well plate. The cytotoxicity assay was performed using standard MTT technique. The obtained results were normalized to negative control (cultures kept in the same conditions, but not incubated with materials).

The cytotoxicity experiment was performed in parallel with the morphological assessment of the cells. Briefly, the cell cultures were washed with DPBS, fixed for 20 min in 4% glutaraldehyde solution then stained using hematoxylin-eosin (HE) staining protocol. The stained cells were analyzed using Leica DMIL inverted microscope at 10× magnification objective.

1.3.3.7. ***In vivo*** **biocompatibility test**

In vivo biocompatibility was performed on male rats 200 g weight. Testing protocol was performed with respect of European Convention, Romanian Association for Laboratory Animals Sciences and "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi regulations regarding the protection of vertebrate animals used for experimental and other scientific purposes.

In vivo biocompatibility was assessed by inserting small polyurethane film disks (10 mm diameter size), under dorsal skin; the disks were removed after 16 days together with the adherent tissue. All surgical procedures were done under thiopental anesthesia. After prelevation the disks and the tissue were prepared for light microscopy observation. The fragments were fixed in 10% formaldehyde water solution, embedded in paraffin wax, sliced in 15 μm pieces and stained with hematoxylin – eosin – methyl blue and acid periodic – Schiff (PAS). The samples were analyzed with a Nikon E600 light microscope (Nikon, Japan).

1.3.3.8. **TAS in blood plasma**

The plasma was obtained from human blood centrifugation at 1000 G for 20 min. PU samples were incubated in blood plasma for 1, 2 and 3 days at 37 oC and mild orbital shacking. The TAS measurement was made by standard protocol provided by Randox TAS kit. Thus, 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS)® was incubated with a peroxidase (metmyoglobine) and H2O2 to produce the ABTS®+ radical cations having a stable blue-green colour that was measured at 600 nm on a Biocompatibility and Biological Performance of the Improved Polyurethane Membranes for Medical Applications 205 spectrophotometer mentioned in the previous section.

By adding blood plasma containing antioxidants a suppression of this colour to a degree which is proportional to their concentration is observed. Control serum ("standard" provided by the determination kit) was used for data validation. TAS values were calculated based on the measured absorbance in the standard, blood plasma sample and blank (buffer provided by the kit) before and after H2O2 adding.

The absorbance differences (ΔA) between measurement before and after H2O2 adding for standard, sample or blank solutions were used for calculation of TAS concentration

according to relations/ TASmMol/L=Factor·(ΔAblank-ΔAsample), for which Factor (f) = standard concentration/(Ainert reactive -A standard).

1.4. Results

The methods used for the determination of surface tension (van Oss *et al*., 1989) are based on contact angle measurements between the liquid meniscus and the polyurethane surface. A contact angle below 90° indicates that the substrate is readily wetted by the test liquid, while an angle over 90° shows that the substrate will resist wetting.

Table 1.I lists the contact angles between double distilled water, ethylene glycol or glycerol and PU samples, before and after plasma treatment. A decrease of the contact angle after plasma treatment indicates a higher oxygenation of the surface, leading to an increase in hydrophilicity. On the other hand, Table 1.II lists the contact angles between double distilled water, ethylene glycol, or CH_2I_2 and PU/HPC samples, before and after plasma treatment.

Table 1.I. Compositional parameters of soft and hard segments, number average molecular weights, polydispersity indices and contact angle of different liquids – PU samples, before[a)] and after[b)] plasma-treatment.

Samples	**Weight ratio (%) P_1: ($D_{1\ or\ 2}$: C_1)**	**M_n**	**M_w/M_n**	**Water**	**Ethylene glycol**
PEGA/MDI-DEG	75.43: (16.36 : 8.21)	20800	1.77	62[a)]	45[a)]
				25[b)]	31[b)]
PEGA/TDI-DEG	80.11 (13.24 : 6.65)	20100	1.67	53[a)]	41[a)]
				17[b)]	22[b)]

Table 1.II. Contact angle degrees of different liquids and PU samples before and after plasma treatment.

Sample code	**Untreated samples/plasma-treated samples**		
	Water	Ethylene glycol	CH_2I_2
PEA-HPC	45/60	40/35	40/29
PTHF-HPC	45/52	36/29	33/29
PPG-HPC	60/50	45/30	32/27

Using Eq. 1-6, surface tension parameters for untreated and plasma treated PU films have been determinated. The obtained results from Table 1.III and 1.IV shown that the sample with MDI in hard segment has a higher hydrofilicity, which increases through plasma treatment.

From this reason, sample with MDI in hard segment was selected for biomedical analysis *in vitro* by water uptake tests (Wang *et al*., 2004), adsorption experiment, clot for-mation tests, hemolysis assay (Bajpai *et al*., 2005) and *in vivo* biocompatibility experiments (Xu *et al*., 2005).

Table 1.III. Surface tension parameters for untreated [a)] and plasma treated [b)] PU films according to Eqs. (1), (2) and (6).

Sample	Eqs. (1), (2)			Eq. (6)
	γ_{sv}^{d}	γ_{sv}^{p}	γ_{sv}	γ
PEGA/MDI-DEG	0.62 [a)]	67.65 [a)]	68.03 [a)]	47.30
	0.73 [b)]	89.02 [b)]	89.76 [b)]	-
PEGA/TDI-DEG	5.44 [a)]	43.83 [a)]	49.28 [a)]	47.66
	0.61 [b)]	89.06 [b)]	89.67 [b)]	-

Table 1.IV. Surface tension parameters for untreated [a)] and plasma treated [b)] PU films according to Eqs. (3) and (4).

Sample	Eq. (3), (4)			
	γ_{sv}^{LW}	γ_{sv}^{-}	γ_{sv}^{+}	$\gamma_{sv}^{LW/AB}$
PEGA/MDI-DEG	4.24[a)]	59.01[a)]	7.87[a)]	47.35[a)]
	2.93[b)]	79.66[b)]	13.42[b)]	68.31[b)]
PEGA/TDI-DEG	9.89[a)]	38.72[a)]	5.83[a)]	39.94[a)]
	1.39[b)]	81.20[b)]	15.99[b)]	73.46[b)]

Following the plasma treatment the disperse component of surface tension, γ^{d}_{sv}, increases in absolute value, while the polar component surface tension γ^{p}_{sv}, decreases except PPG-HPC sample for which these dependences varies in a less extent (γ^{p}_{sv} increases from 32.2 to 38.9 mN/m, and γ^{d}_{sv} increases from 9.1 to 10.7 mN/m).

Before and after plasma treatment all samples exhibits predominant electron donor properties and shows the contribution of the polar component to the total surface tension obtained from the geometric mean method GM for untreated and plasma treated polyurethanes.

The polar term γ^{p}_{sv} generally gives a large contribution to γ_{sv}, due to the large electron donor γ_{sv} – interactions. The polar component decreases after plasma treatment, except the same PPG-HPC sample. The total, disperse and polar surface tension parameters are influenced by the matrix structure of polyurethanes possessing various soft segments.

Generally, all samples possess high polar surface tension parameters, which decrease after low-pressure plasma treatment, except the PPG-HPC sample. In comparison, the surface tension of PU/HPC (PEA-HPC, PTHF-HPC, PPG-HPC) samples are shown in tables 1.V and 1.VI. For a better correlation of these results, in table 1.VII are presented the compositional parameters of PU/HPC samples, number-average molecular weights and polydispersity indices of the samples and in tabel 1.VI are shown some phyico-chemical properties of PU/HPC samples.

Table 1.V. Compositional parameters, number-average molecular weights and polydispersity indices of the samples.

Sample code	**Soft segment**	**Hard segment**	**Composition macrodiol/MDI/EG/HPC, wt%**	**M_n**	**M_w/M_n**
PEA-HPC	PEA	MDI-EG;HPC	52.24/36.57/7.27/3.92	134522	1.865
PPG-HPC	PGA	MDI-EG;HPC	52.24/36.57/7.27/3.92	72951	1.669
PTHF-HPC	PTHF	MDI-EG;HPC	52.24/36.57/7.27/3.92	70291	1.590

Table 1.VI. Physico-chemical properties of the studied PU/HPC samples.

Characteristics/Samples	**PEA-HPC**	**PTHF-HPC**	**PPG-HPC**
Mechanical testing (Dry/Conditioning in saline water 0.9% for 24 h)			
Young modulus, M Pa	90/113	70/30	75/39
Elongation at break, %	71/84	72/159	53/56

Table 1.VII. Physico-chemical properties of the studied PU/HPC samples (continued).

Characteristics/Samples	**PEA-HPC**	**PTHF-HPC**	**PPG-HPC**
Tensile strength at break, MPa	19/22	14/10	15/9
Toughness, MJ/m^3	9.3/13.1	7.7/11.8	5.6/3.5
Dynamic Contact angle/Water uptake			
θ_{adv} (advanced)	84.9 ± 1.1	77.4 ± 1.1	85.6 ± 1.1
θ_{rec} (receding)	44.2 ± 0.5	42.9 ± 0.5	44.8 ± 0.5
Hysteresis, %	47.9	44.6	47.7
Water uptake	140 ± 4	167 ± 3	92 ± 6

Table 1.VIII. Surface tension parameters (mN/m) for untreated and plasma treated PU/HPC samples according to the geometric mean method and to the acid/base method.

Polymer code	Untreated samples/plasma-treated samples				
	γ_{sv}^{p}	γ_{sv}^{d}	γ_{sv}^{-}	γ_{sv}^{+}	γ_{sv}
PEA-HPC	57.0/24.8	2.1/16.6	55.2/23.6	12.5/4.7	59.1/41.4
PTHF-HPC	53.1/34.7	4.7/12.9	50.7/33.2	10.2/6.6	57.8/47.6
PPG-HPC	32.2/38.9	9.1/10.7	30.7/37.2	6.2/7.4	41.3/49.6

Table 1.IX. Contribution of the polar component to the total surface tension obtained from the geometric mean method for untreated and plasma treated PU.

Polymer code	Untreated samples $\gamma_{sv}^{p}/\gamma_{sv} \cdot 100$ (%)	Plasma-treated samples $\gamma_{sv}^{p}/\gamma_{sv} \cdot 100$ (%)
PEA-HPC	96.5	60.0
PTHF-HPC	91.9	73.0
PPG-HPC	78.0	78.5

1.4.1. Water sorption, plasma protein absorption and *in vitro* haemocompatibilty tests of PU and PU/HPC samples

The biocompatibility of materials depends on their ability to swell in aqueous media. A high water level on the surface of the biomaterial provides a low interfacial tension with blood, thus reducing fibrinogen adsorption, cell adhesion and clot formation (Grigoriu *et al*., 2001, Imay *et al*., 1972).

Thus, the water uptake is a precursory test for hemocompatibility analysis. The water uptake correlated with the value of adsorbed BSA, the amount of blood clot and the degree of hemolysis of PU samples is presented in Table 1.X.

Table 1.X. Water uptake, amount of blood clot, hemolysis degree and absorbed BSA on PU samples.

	Water uptake (%)	**Clot weight (mg)**	**Hemolysis (%)**	**Absorbed BSA (mg/cm^2)**	
				1 mg/ml BSA solution	3 mg/ml BSA solution
PEGA/MDI-DEG	22.14 ± 8.6	13.10 ± 0.63 (30%)	6.67 ± 0.21	0.063 ± 0.02	0.3 ± 6.4
glass	-	45.15 ± 0.61 (100%)	12.94 ± 0.20	-	-

In figure 1.2 A are shown the weight of polymer sample in dry and maximum hydrated state and the amount of absorbed fibrinogen from physiological solution and blood plasma.

It is observed that the water uptake is given by the PEA-PU sample (reference PU sample without HPC, PEA/MDI/EG, Mn=109.613, Mw/Mn=1.3), PEA-HPC and PTHF-HPC samples (151%, 140% and 167%, respectively) and in a less extent by the PPG-HPC (92%) due to its less polar soft segment having the lateral $-CH_3$ substituents which confer a different geometry to the poly-urethane internal microporous structure, unfavourable for water uptake.

The experimental data related to the amount of adsorbed fibrinogen before and after incubating of polymers with a physiological solution of fibrinogen (3.00 mg/ml) and blood plasma (2.98 mg/ml) are presented in figure 1.2 (B).

The postincubation fibrinogen concentration for the incubated materials (1h at 37°C), preincubation and postincubation fibrinogen concentration for reference sample in comparison with the physiological normal limits are given in table 1.XI. Incubation of the samples with blood plasma realized in the same conditions of the incubation in solution and leaded to the results from figure 1.2 (B).

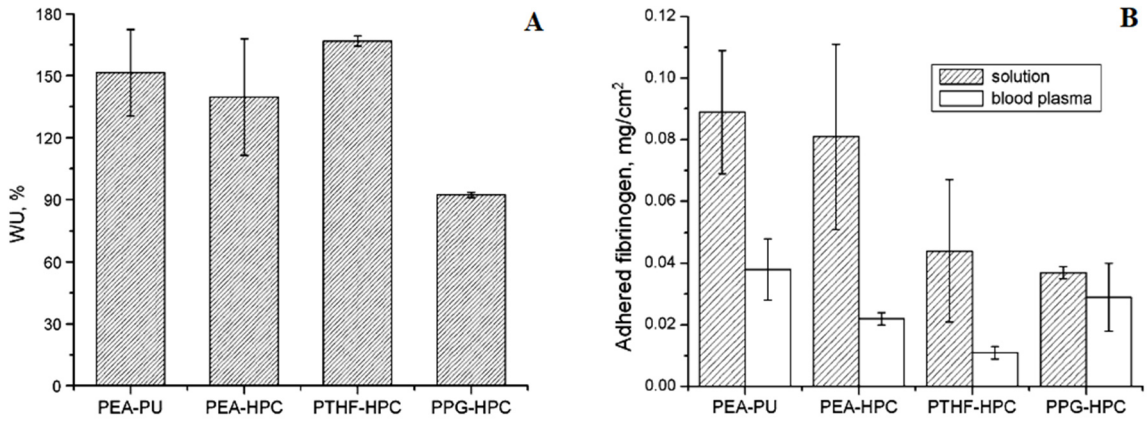


Fig. 1.2. A. Weight of polymer sample in dry and maximum hydrated state; **B.** Amount of absorbed fibrinogen from physiological solution and blood plasma.

Determination of the adhered fibrinogen from blood plasma was coupled with the determination of the prothrombin time, *i.e.* the time of transformation of the prothrombin in thrombin, followed by transformation of the fibrinogen in fibrin and clot formation.

It is observed that the amount of fibrinogen adsorbed from blood plasma is less in comparison with that in solution, for all the materials except PPG-HPC, for which the differences are not significant. Also, it is remarked that prothrombin time stand in physiological normal limits (Table 1.XI) so studied PU samples did not affect the clot formation mechanisms.

Table 1.XI. Prothrombin time and fibrinogen concentration after the contact blood plasma with PU samples.

Parameter	Physiological normal limits, mg/ml	Reference sample (blood plasma)	PEA-PU	PEA-HPC	PTHF-HPC	PPG-HPC
Prothrombin time (s)	8.3-11.3	10.43 ± 0.04	11.06 ± 0.4	10.9 ± 0.09	10.9 ± 0.09	10.9 ± 0.07
Fibrinogen concentration (mg/ml)	2.84-3.69	Preincubation 2.98 ± 0.04				
		Postincubation				
		2.96 ± 0.05	2.79 ± 0.04	2.87 ± 0.04	2.9 ± 0.01	2.77 ± 0.07

The results on TAS modification of blood plasma are shown in table 1.XII. It can be observed that the hydrophilicity of PEA-HPC and PHTF-HPC samples do not affect the antioxidant capacity of blood plasma after 24h of incubation, compared to the relatively more hydrophobic PPG-HPC sample which decreases the blood TAS by 20%.

These preliminary results do not allow the access to the involved mechanisms. However, as blood plasma without cells was used (PPP), we can suppose that the mechanism involves the antioxidant enzymes adsorption and/or inactivation on the material surface.

Table 1.XII. Antioxidant status of blood plasma incubated with PU-HPC samples.

Samples	24 h (Mmol/L)	72 h (Mmol/L)	Decrease	Physiological value
Plasma control	1.3 ± 0.17	0.84 ± 0.05	0 %	1.3 – 1.77
PEA-HPC	1.23 ± 0.06	0.9 ± 0.08	5.4 %	
PTHF-HPC	1.2 ± 0.11	0.84 ± 0.06	7.7 %	
PPG-HPC	1.03 ± 0.09	0.75 ± 0.03	20.5 %	

As functional test for biointegration capacity of PU/HPC samples, the amount of blood clot formed in contact with a material was determined. The results for clot weight are shown in the table 1.XIII.

As shown by the data in table 1.XIII, PTHF-HPC film characterized by less adsorbed fibrinogen, no charge on surface and greater water uptake has little contribution to clot formation, while the other two materials with a roughly similar charge but different hydrophilic properties contributed to blood clotting proportional to their hydrophilicity.

These results suggest that the oxidative stability and haemocompatibility properties are depending more on surface neutrality and hydrophilic properties in good correlation with serum albumin (SA)/FB adsorption ratio.

Table 1.XIII. Amount of blood clot formed in contact with PU/HPC samples.

Incubated surface	Clot weigh at 240 sec (mg)
Clot without material	24.8 ± 2.0
Collagen membrane	42.5 ± 3.5
PEA-HPC	31.9 ± 2.6
PTHF-HPC	28.4 ± 2.9
PPG-HPC	46.8 ± 2.2

1.4.2. *In vitro* haemocompatibility tests of magnetic nanoparticles

The hemocompatibility tests showed normal values for the concentration in haemoglobin, hematocrit, red blood cells, leukocytes and platelets. Also, the values obtained for the prothrombin time and fibrinogen were within normal limits after 1 h of incubation of the nanoparticles with blood.

1.4.3. *In vitro* cytotoxicity of the PU/HPC samples

The results of cytotoxicity test on PU/HPC samples are presented in figure 1.3. According to this figure, the least cytotoxic and most biocompatible PU sample was PU/ HPC-PTHF that assures about 87% of cell viability after 48 hours of cells-material incubation.

The most cytotoxic PU sample was PU/HPC-PPG. After 48 hours this material decreased cell viability to 30% compared to the control. The PU/HPC-PEGA sample expresses a low cytotoxic effect in the first 24 h of culture.

After 48h the cell viability in the presence of this sample has grown and became more comparable with those expressed by PU/HPC-PTHF.

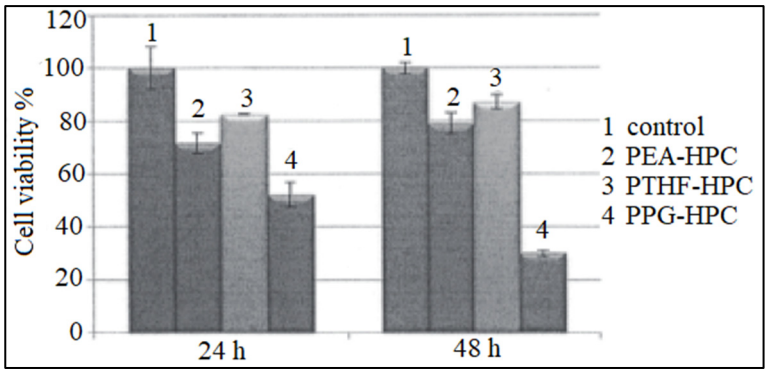


Fig. 1.3. Cell viability of the primary fibroblasts after 24 and 48 h of incubation with PU/HPC samples.

1.4.4. The polymorphonuclear (PMN) leucocytes migration test

PMN leucocytes are white blood cells that are involved in nonspecific body defense. These are the cells that will first react to a microbial invasion and tissue injuries including those associated with implantable devices.

The mechanism by which PMN are attracted to the affected area is chemotaxis, the phenomenon by which the cells move toward the highest concentration of some molecules named chemoattractants.

From figure 1.4, one can resume that all materials have some PMN calling power, significantly higher than blank control. The highest chemotactic property is expressed by PU/HPC-PPG while the lowest one by PU/HPC-PTHF. These results are well correlated with the cytotoxicity data.

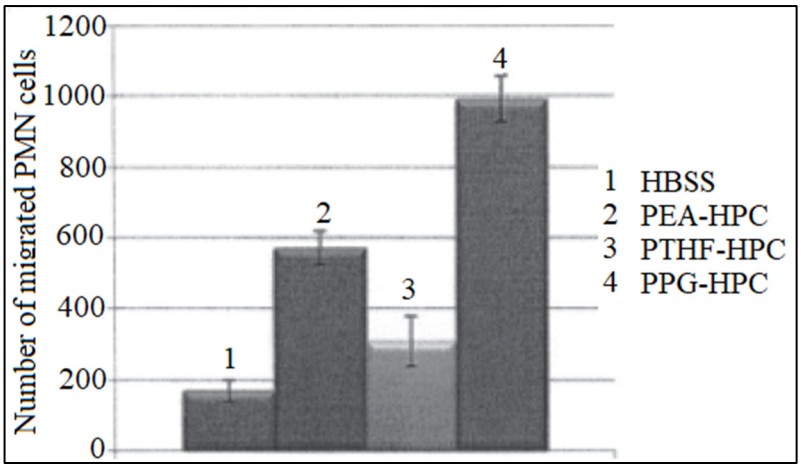


Fig. 1.4. Number of migrated leucocytes in the lower culture compartment.

1.4.5. *In vivo* biocompatibility test

Subcutaneous implantation is an important *in vivo* step to test various polymers for their biocompatibility and degradation properties. Rats are suggested to be of choice for soft tissue degradation studies because of their low cost and the rich background data for the animal.

In our studies, subcutaneous implantation of PU samples showed a good tolerance for the tested material. None of the animals died and the histomorphologic analyses demonstrated light fibrous collagen structures around the implants as well as the absence of cellular inflammatory reactions (Fig. 1.5 A, B; Fig. 1.5 A). In implant area it can be seen debris of PU with pronounced porous structure (Fig. 1.5 A).

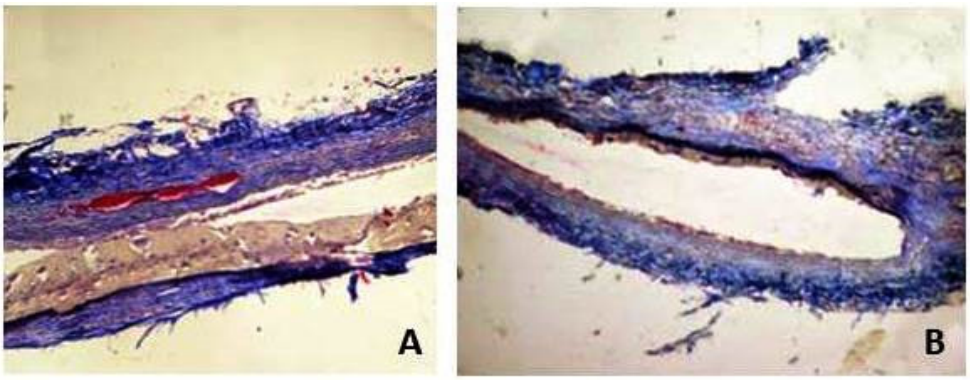


Fig. 1.5. Rat skin: **A**. PU sample, collagenised and well vascularised derm, hypoderm's remainder. HEA stain, ×40; **B**. Gap in the area of implant insertion with PU wreckage, surrounded by conjunctive fibrilar tissue, ×100.

Although on some areas of histologyc samples it was seen vascular hyperemia, moderate lymphocytes, macrophages and fibroblasts infiltration, these had a regressive postsurgical reparatory character (Fig. 1.6. A, B).

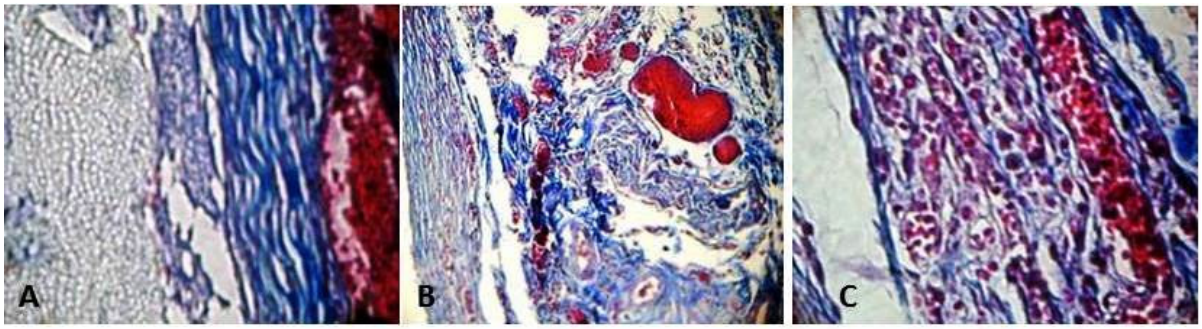


Fig. 1.6. Rat skin, HEA stain: **A**. PU implant with porous structure; fascicular conjunctive tissue, ×100; **B**. Hyaline venous crom. Periferic fibrocellular reaction, capillary neoangiogenesis, ×400; **C**. Polymorfic cellular hyperplasy with lymphocytes, histocytes and fibrocytes. Vennular hyperemy,× 400.

1.4.6. Values for TAS in blood plasma

Assigning to SA molecules the main role in protective effect, we analysed the interaction of PU/HPC membranes with blood plasma, following the plasma antioxidant status. To define the importance of SA adsorption on material surface, the membranes were incubated at 37°C in blood plasma and TAS was measured periodically. The results are shown in Fig. 1.7. Two PU samples (PU-PEGA and the more hydrophobic PU-PPG/HPC) had significant tendency to quickly decrease TAS activity in the first 48 hours. Due to the complexity of TAS, it is difficult to speculate on the mechanism by which the decreasing phenomenon arises and certainly more examinations are needed. However, one can suppose that PU PEGA alter the TAS activity as a result of plasma pH modification that leads to sustained free radical generation in the presence of the material.

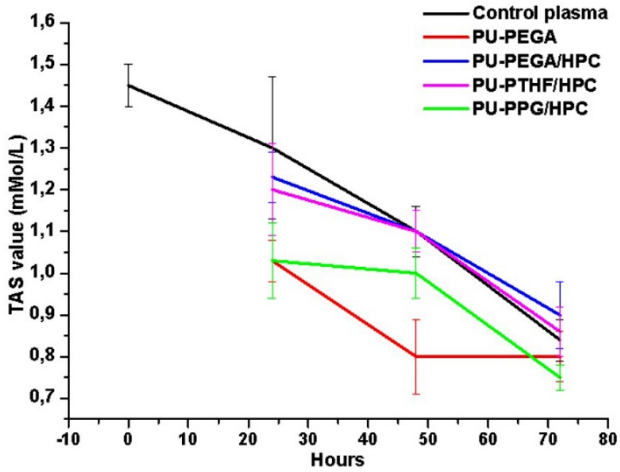


Fig. 1.7. TAS evolution after PU/HPC incubation in blood plasma at 37°C.

1.5. Discussions

The segmented poly(ester urethane)s and poly(etherurethane)s prepared in a two-step polyaddition process were investigated to obtain information on their surface tension parameters and hydrophilicity, both before and after plasma treatment.

Calculations of the surface tension parameters are based on the geometric mean method, on the acid/base method, and on theoretical methods involving structure-property relationships.

Surface hydrophilicity, evaluated by the free energy of hydration between compounds and water, confirms the influence of different hard segments and, on the other hand, the influence of the plasma treatment. This treatment creates a desirable increase in the polar surface free energy of the samples for potential applications.

Future studies on the design and characterization on hydrophilic/hydrophobic character of these segmented PU surfaces would benefit from considering the surface topology in potential biological and coating applications. The balance between hydrophilicity and hydrophobicity, blood protein adsorption proprieties, as well as the thrombogenic and the hemolytic properties of PEGA/MDI-DEG sample represents the first favorable results for biomedical applications.

The significant differences between adsorptions of the fibrinogen solution and blood plasma, suggest that the fibrinogen adsorption properties of the polyurethane samples, under physiological condition are affected by the concurrent affinities for other plasma proteins, which do not disturb the haemostatic mechanisms.

Probably among the plasma proteins that can concur with fibrinogen is albumin, which was previously investigated (Lupu *et al*., 2007), and it was found that the adsorption value of a sub-physiological solution of serum albumin, (3 mg/ml), to PEA-PU materials was 0.3±0.06 mg/cm^2.In the literature many research studies are dealing with PU regarding contact angle measurements, water sorption and protein adsorption. Water plays an important role in determining biocompatibility characteristics of the synthetic material. It is very well known that high water levels on the surface of the biomaterial providing a low interfacial tension with blood consequently reduce fibrinogen adsorption and cell adhesion on the surface similarly to biological tissues. The water contact angle for polyurethane type Biospan^{TM} is 70° (Abraham *et al*., 2001).

For phospholipids-grafted segmented PU high water contact angles were between 99-105° (Korematsu *et al*., 1999) while for cross-linked multiblock pellethene polyurethane water contact angle is 73° (Yoo *et al*., 2004). As it was observed in our studies water contact angles for studied polyurethanes are placed at lower values comparing with those above mentioned examples, due to the introduction of the hydrophilic cellulose derivative (HPC) which is very well known also to form hydrogels in water.

The best functional biointegration oxidative capacity was shown by PTHF-HPC and PEA-HPC materials. This means that these materials do not affect the functionality of the tissue antioxidant defense mechanisms and consequently they are more oxidative stable.

The thrombogenicity testing highlighted that only PTHF-HPC sample possesses high haemocompatibility properties. In contact with blood, biomaterials are limited in their usefulness primarily in thrombus formation at the bloodmaterial interface, which is triggered by the preferential adsorption of some plasma proteins. Fibrinogen plays a central role in haemostasis participating not only in the coagulation cascade, but it also promotes adhesion of platelets and activates then when adsorbed onto certain solid surfaces.

The protein adsorption is selective relative to albumin and fibrinogen and this selectivity depend on the modified PU surfaces and in the adsorbed fibrinogen on polyurethane surface depend on the migration of the amphiphilic character to the surface (Abraham *et al*., 2001). In our studies it is observed that the fibrinogen adsorbed from blood plasma is less in comparison with that in solution except PPG-HPC sample.

Reaching the destination, the leucocytes induce a complex acute inflammatory reaction that is continued with a chronic phase and finally with rejection or isolation of the affected area in a thick fibrous capsule (Anderson, 2001). The solvents, additives, synthesis process contaminants, residues and degradation products are the most frequent PMN calling signals issued by the materials. Thus, an *in vitro* method for PMN calling power could have a predictive value for *in vivo* behavior. The found PMN calling effect and cytotoxicity of the PPC-HPG sample might be due to the internal porous structure, consisting in small, less interconnected pores as well as due to higher hydrophobicity of this sample compared to the other two, data reported in our previous paper (Butnaru *et al*., 2012).

The mentioned PPG-HPC characteristics most probably disrupt the release of the residual compounds from the material structure. The mentioned PU/HPC-PPG caracteristics most probably disrupts the release of the residual compounds from the material structure. The results obtained in this section provide a prediction for a proinflammatory behaviour of the PU samples *in vivo*.

Furthermore, the results from *in vivo* experiments brought out more argues regarding the biocompatibility of PEGA/MDI-DEG polymer. The implantation of PEGA/MDI-DEG polymer didn't produce any kind of chronic inflammatory reactions so we can consider this material to be biocompatible. Oxidative in vitro behavior Oxidative degradation of PUs caused by hydrolytic or enzymatic mechanism was intensively discussed (Christenson *et*.al., 2004).

First of all, PUs designed for tissue-contact devices undergo hydrolytic degradation as a result of watering with physiological solutions. This process has an impact especially on poly(ester-urethane)s that can generate hydroxy-acids, being susceptible to induce reactive oxygen species (ROS) production following the materialtissues interaction. By means of this mechanism, PUs can be implied in the sustained oxidative degradation and a wide range of pathological states.

As it is well known, ROS can trigger subtle mechanisms responsible for diseases generation through the peroxidation of cell membrane lipids and DNA damage (Marnett, 2002). The most susceptible organs to oxidative aggression are the heart, vessels, lung, gut, liver, brain and nerves (Förstermann, 2008; Rahman *et*. *al*., 2002).

In a normal body state, ROS appear constantly as a result of some biological errors or as a consequence of some short living reactive intermediate products generated by the cell aerobic metabolism. Endogenous enzymatic and nonenzymatic pathways are responsible for the formation of free radicals.

1.6. Conclusions

Cellulose-based polyurethanes have been studied taking into consideration that the functional groups given by cellulose chains constitute preferential sites for bioactive interactions for biocompatible devices.

Considering all the results and discussions we can conclude that nature of the PU soft segments strongly influence the functional capacity integration due to its implication in the hydrophilic/hydrophobic balance, while HPC adjusts the surface biocompatibility through the beneficial positivation of the surface potential.

Between the studied samples (PEA-HPC, PTHF-HPC, PPG-HPC), PTHF-HPC sample is characterized by enhanced biocompatibility and functional blood-contact adaptability due to its cumulative properties such as elasticity, hydrophilicity, surface neutrality, higher albumin/fibrinogen ratio adsorption as well as due to its low oxidative capacity.

The stability of the pH value of biological media and the ratio of adsorbed albumin and fibrinogen from blood plasma were found to be the most valuable screening criteria to evaluate the blood-interface functionality, but not only.

These criteria could provide information on material capacity to keep stability of the main body balances (oxidant/antioxidant, haemostasis/haemolysis) that are responsible for material acceptance in the early phase, followed by structural and functional integration in the later stages. These characteristics together with other important material properties as surface neutral charge and desired porous structure are keys points for good results expectance as was demonstrated.

Mechanism of blood clot formation, positively influenced by increasing the concentration of HPC, and low amount of fibrinogen adsorbed confirm that polyurethanes, from this category, could be used in biomedical applications.

CHAPTER 2

REACTIVE OXYGEN SPECIES AND THEIR ROLE IN THE DIAGNOSIS, MONITORING AND TREATMENT OF PREMATURE AND TERM BABIES WITH ASPHYXIA

2.1. The role of antioxidant enzymatic systems in oxidative damage after the asphyxiating insult at premature infants and newborns

2.1.1. Introduction

Starting from the positioning of medical biochemistry as a link between paraclinical and clinical between the fundamental part of medicine and the clinical part, I developed my research activity based on this reality.

The title of the doctoral thesis "BIOCHIMIC ASPECTS OF OXIDATIVE STRESS IN ETHYOLOGY DIVERSION TRAUMATISMS" allowed the focus of research on a group of subjects little studied in Romania up to that time, *i.e.* newborns at term and premature in the neonatal intensive care unit. The reason for studying the aspects of oxidative stress in these has gone from the question of how long is the optimal time of exposure to their oxygen therapy and especially what is the most effective method of those that a neonatologist can use to revive and then support a newborn that requires this type of intervention.

The problem of oxidative stress occurs for any newborn right from the moment of birth, as a result of the fact that the transition from birth from fetal to newborn life involves complex physiological changes, and the speed of their production is extremely fast and sometimes not successfully completed.

Statistics show, of course different from country to country, that in about 6%-10% of births due to fetal and/or maternal factors, the newborn's ability to adapt adequately to ectopic life is affected. Perinatal asphyxia is a predominant cause of neonatal mortality and neurological morbidity. Newborns with severe perinatal hypoxia will experience long-term neurological sequelae, neonatal encephalopathy and the suffering of organs and systems in the first hours of life.

Perinatal asphyxia with the most common and severe complication of its hypoxic ischemic encephalopathy (EHI) are the main problems that can occur at birth regardless of how it is produced (natural or by caesarean section). The "trigger" factors in triggering EHI are considered: hypoxia, ischemia along with decreased certral blood flow.

The major con-sequence of EHI is the neurological impairment of newborns with adverse clinical responses in the medium and long term to the quality of life of these children. The installed asphyxiating event, the sequence of physiopathological changes at the molecular, cellular, organic and systemic levels can have multiple consequences that can lead to a negative prognosis of newborns. More than 80% of newborns who survive Stage III EHI have severe complications, 60–70% have neurological disorders (spastic diplegia or tetraplegia, mental retardation), 10-20% minor disabilities and 10% are normal.

Even in the absence of neurological manifestations – of varying degrees – in the immediate neonatal period, in the long term, complications may occur. Approximately 15-20% of school-age children who have had average EHI experience learning difficulties, so these children should be monitored for a long period of time. In severe forms, mortality can reach 50%, with half of these deaths occurring in the first month of life.

It is important to note that once the asphyxiated insult is installed, a period of cascading events follow during which brain damage occurs. After postnatal resuscitation, oxygenation and brain reperfusion are restored. During this recovery phase, concentrations of inorganic phosphates, cellular metabolites and pH values will return to normal.the emerging energy failure generates the secondary phase of the event 6-72 hours after the asphyxiating insult.

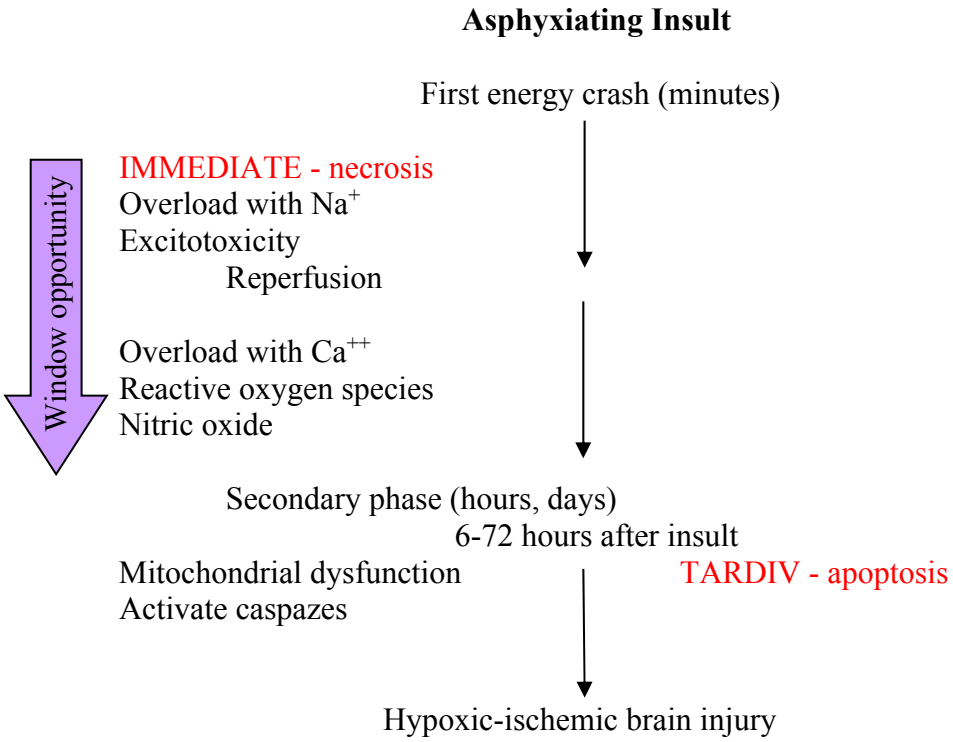


Fig. 2.8. Installation of hypoxic-ischemic brain injury (after Ferriero., 2004).

The administration of oxygen therapy required by asphyxia generates additional amounts of free radicals that will amplify the severity of the lesions. The involvement of free radicals in the development of diseases of the newborn with long-term consequences is the keystone of lesions caused by hypoxia and ischemia and amplified by prolonged oxygen administrations.

In the premature newborn, due to its immaturity, asphyxia and implicitly reactive oxygen species are involved in conditions such as: periventricular leukomalacia, pulmonary bronchodysplasia, retinopathy of prematurity, ulceronecrotic enterocolitis etc.

The pathologies mentioned represent the motivation of the concern of the literature in the therapeutic approach to the limitation of free radical harmfulness. The choice of the technique of performing oxygen therapy, as well as the time of administration of oxygen, should be judiciously evaluated in order to prevent further complications.

Once installed ischemic lesions are irreversible, any therapeutic way of limiting them can bring real benefits in minimizing neurological consequences.

As the literature shows the incidence of perinatal asphyxia is inversely proportional to the gestational age: 6% in the term newborn and much higher in the premature newborn under 36 weeks of gestation. The frequency of production of a hypoxic-ischemic event is different from country to country between 9-15%. At the time of the start of our study for Romania the incidence was 14-15% and there was no study of appreciation of the role of reactive oxygen species in newborns exposed to oxygen therapy.

Considering that there is no etiological therapy to combat the side effects of oxygen therapy in the newborn with asphyxia, we considered it necessary to deepen the study effort on the choice of therapy, an antioxidant protective behavior against reactive oxygen species,

but also to find the most useful biochemical, specific and sensitive parameters for monitoring the evolution of newborns.

2.1.2. The role of antioxidant defense systems in annihilating reactive oxygen species

Direct measurement of free radicals in biological products (serum, plasma, homogeneous from tissues, etc.) is difficult due to their short half-life.

Some forms of stress benefit from specific markers, for example in oxidative stress a number of biochemical parameters are measured that show either the body's ability to adapt to increase the concentration of reactive oxygen species, (enzymatic or non-enzymatic biochemical markers), or changes produced by the action of free radicals (malondialdehyde, total antioxidant capacity, etc.).

The mechanism of production of ROS (reactive oxygen species) being complex and at various molecular levels is controlled by enzymes whose activity changes by increase or decrease and at the same time the activity of these enzymes is mutually conditioned (Fig. 2.9).

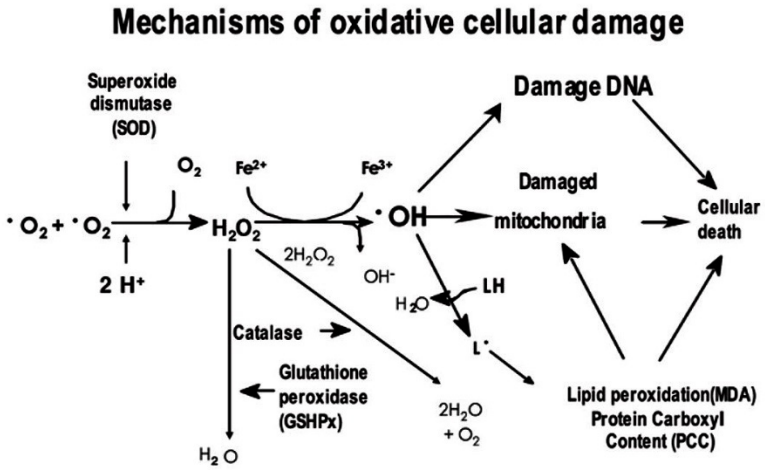


Fig. 2.9. Oxidative stress in the celle (Úrsula Muñoz Morón, 2012).

The activity of the following enzymes was dosed: superoxide-dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation levels by determining malonaldehyde activity (MDA).

The antioxidant status of serum (TAS) was evaluated by measurements of antioxidant activity, which reflects its ability to inhibit free oxygen radicals in any substrate. At the same time, it is obvious that the antioxidant system of blood plasma (serum) is not only the sum of the contributions of individual antioxidant enzymes, but depends on the interaction between them, but also on the presence of other oxidizing or prooxidant compounds present in serum or plasma.

In assessing the effect of ROS in newborns requiring the administration of oxygen-therapy the design of studies together with the activity of enzymes involved in the complex process of generating molecules with oxidative activity, the activity of two enzymes respectively lactate dehydrogenase (LDH) and creatinkinase (CK), which have proven to be useful markers in the monitoring of the newborn during oxygen therapy to measure the level of neurological impairment.

2.1.3. Aim of the studies

The study was conducted between 2004-2008 at the Clinical Hospital of Obstetrics and Gynecology "Cuza Vodă" Iași, Neonatal Intensive Care Unit. We measured the activity of the enzymes superoxide dismutase, glutathione peroxidase and the antioxidant capacity of the serum 48 hours after the asphyxiation event and 72 hours when we also measured the level of malondialdehyde as a marker of lipid peroxidation in full-term and premature infants with perinatal asphyxia who received oxygen therapy. We established the reference values for each parameter analyzed according to the chosen control groups.

We chose 48 and 72 hours, respectively, because as we observed in the doses of creatine kinase and lactate dehydrogenase enzymes as the first indicators of asphyxia, the prognostic period in terms of neurological evolution falls within this time frame.

The lots taken into consideration have been distributed as follows:

- Healthy full-term newborns – (gestational age 37.8±1.7 weeks deduced from serial ultrasound evaluations, birth weight 3340±800 grams) batch NNS - 20 subjects;
- Healthy premature newborns – (gestational age 28.3±2.3 weeks deduced from serial ultrasound evaluations, birth weight 1188±356 grams) PMS batch - 20 subjects;
- Full-term newborns with asphyxia – (gestational age 38±2 weeks deduced from serial ultrasound evaluations, birth weight 3580±1070 grams) NNTA batch with O_2-therapy –22 subjects.

Premature infants with asphyxia – (gestational age 28±5.83 weeks deduced from serial ultrasound evaluations, birth weight 1232.35±717.65 grams) PMA group with O_2-therapy – 30 subjects. Newborns with sepsis or severe birth defects were excluded from the study. It should be noted that the number of subjects initially investigated was higher (216), but due to the inhomogeneity of the data required for processing as well as the lack of values of certain parameters, those presented above were selected. Newborns with asphyxia have shown severe forms of perinatal asphyxia.

Oxygen therapy is administered temporarily in acute hypoxia, but also prolonged in case of advanced or chronic diseases. Oxygen administration is performed on the mask, with a balloon or after intubation by IPPV (Intermittent Positive Pressure Ventiltion) or with the help of a CPAP (Continuous Positive Airways Pressure) nasal cannula. The goal is to obtain a normal oxygenation-normoxia or in other words a fraction of oxygen inspired enough to have a pink child (**International Liaison Committee on Resuscitation**, 2005).

The researches has been conducted with the approval of the Ethics Committee of the University of Medicine and Pharmacy "Grigore T. Popa" Iași and in accorance to the European Communities Council Directive 86/609/EEC, with the informed consent of the patients.

2.1.4. Materials and methodes

Biochemical determinations made to investigate antioxidant status

Determination of antioxidant status was achieved by determining superoxide-dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH), lipid peroxidation (MDA), catalase activity (CAT) and measuring total antioxidant capacity of serum (TAS) methods that have been used in all studies targeting the effect of reactive oxygen species in newborns.

Determination of antioxidant status was achieved by determining superoxide-dismutase activity (SOD) – Metoda RANDOX (RANSOD)

Principle of the method:

The first line of defense against superoxide radical anion is superoxide dismutase, which catalyzes the dismutation of superoxide radical anion to hydrogen peroxide and oxygen and consequently to the limited diffusion of this radical anion.

Results:

A_2-A_1/3=ΔA/min.

Calculate the percentage of inhibition according to the standard curve. Express the results in SOD/ml units. SOD/ml=SOD unit/ml on the curve x dilution factor.

Conversion into units of hemoglobin:

SOD unit/ml/g hemoglobin=SOD unit/g Hb.

Determination of antioxidant status was achieved by determining glutathione peroxidase activity (GPx) – method (RANSEL)

Principle of the method:

The dosing of glutathione peroxidase (GPx) with RANSEL reagents is based on the Paglia and Valentine method (Paglia and Valentine., 1967).

Glutation peroxidase catalyzes glutathione oxidation (GSH) by cumen hydroperoxide (ROOH) according to the equation below.

In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) is immediately converted to reduced form (GSH) with NADPH oxidation to $NADP^+$.

The activity of glutathione peroxidase GPx is evaluated by decreasing absorption at 340nm due to NADPH oxidation at $NADP^+$ (Kraus. *et al*., 1980).

Results:

A2-A1/2= ΔA/min.

U/l hemolyzed=8412xΔA/min.

Determination of malondialdehyde (MDA) activity

Principle of the method:

The dosing method is based on the measurement of the concentration of malonin dialdehyde (MDA) the main biodegradation product of lipid peroxides.

The biological material containing lipid peroxides is subjected to acidic hydrolysis at high temperature, when malonil dialdehyde (MDA) is formed alongside other products of decomposition of lipid peroxides. MDA reacts with tiobarbituric acid (TBA) with the formation of an aduct that can be dosed spectrophotometrically at 532 nm (Ohkawa *et al*., 1979).

Calculate results:

For the calculation of the results, account shall be taken of the value of the molar extinction coefficient of the MDA-tiobarbituric acid bring α532=1,56x105 $M^{-1}cm^{-1}$.

Determination of total antioxidant capacity of serum – total antioxidant status (TAS) – RANDOX method

Principle of the method:

2,2'-Azino-di-[3-ethylbenztiazole sulfonate] (ABTS®) is incubated with peroxidase (metmioglobin) and H_2O_2 to produce an ABTS®+ cationic radical. It has a relatively stable blue-green color, which is measured at 600nm. Antioxidants in the sample to be analysed (ser) added cause the production of this colour to be suppressed up to a level that is proportional to their concentration.

Results:

Calcul – Total antioxidant status

Factor = standard concentration/(inert reactive aa -A standard)

mmol/L=Factor x (ΔAinert reactive - ΔA sample).

Determination of the activity of enzymes involved in the evaluation of oxidative stress produced in the case of perinatal asphyxia was measured on a wet chemistry analyzer of type Imola, using calibrator and control serums for each parameter analyzed.

Determination of creatine-kinase activity

Principle of the method:

Creatine-kinase (CK) catalyzes the ADP transphosphorylation reaction to ATP. $NADH+H^+$ is produced by a sequence of enzymatic coupling reactions. The amount of $NADH+H^+$ produced is directly proportional to the activity of the enzyme creatine kinase.

This method determines the amount of $NADH+H^+$ produced per minute (rate). The final product has a maximum absorption at 340 nm. The method of determination is kinetic and the activity of the enzyme is expressed in U/L.

Performance: linearity up to 1500 U/L. For samples above the limit of detection, dilution was performed with a 7% BTS dilution reagent specific to the VITROS 950 system. The validity of the results is confirmed by the use of certified ED-T50 control sers (NCCLS 1992).

Determination of lactatedehydrogenase activity

Principle of the method:

Lactatedehydrogenase (LDH) specifically catalyzes the oxidation of lactate to pyruvate, with subsequent reduction of NAD^+ to $NADH+H^+$. The rate at which $NADH+H^+$ occurs is directly proportional to the activity of lactatedehydrogenase.

The amount of $NADH+H^+$ is measured by the rate method (absorption per minute) at a wavelength of 340 nm. The method of determination is kinetic, and the activity of the enzyme is expressed in U/L.

Performance: linearity up to 2200 U/L (Nicholson., 2004).

For samples above the limit of detection, dilution was performed with a 7% BTS dilution reagent specific to the VITROS 950 system. The validity of the results is confirmed by the use of certified ED-T50 control sers (NCCLS 1992).

The criteria for establishing the diagnosis of perinatal asphyxia were those of the American Association of Perinatology which proposes five elements:

- hypoxic event occurring before, during or after birth;
- sudden fetal bradycardia or late decelerations in fetal heart monitoring;
- Apgar score between 0-3 to 5 minutes;
- multi-organic dysfunction with onset within the first 72 hours of life;
- early cerebral ultrasound images that objectify asphyxia.

Personal contribution

Published paper:

- Stamatin M, **Dimitriu C**, Iliescu R, Dimitriu AG. *Le stress oxydatife, comme facteur limitant de l'oxygenotherapie chez le nouveau-ne premature*. Archives de pediatrie, Juin 2006; vol.13-N 6, 944.
- Stamatin M, **Dimitriu DC**, Filip C, Paduraru L, Iliescu R, Petrariu A. *Perinatal hypoxia – generatrix cause of oxidative stress*. Journal of Perinatal Medicine, 35, suppl. II, ISSN 0936-174X, 2007, 245.

2.1.5. Results

The activity of the creatine kinase (CK) parameter in full-term infants with asphyxia is recorded at a maximum within 24-48 hours (2647.11±2325.234 U/L and 2533.60±1923.937 U/L, respectively) followed by a decrease at 72 hours after birth 1102.75±617.16 U/L) but much higher than the reference value 65-600U/L) (Fig. 2.10).

For asphyxiated preterm infants, creatine kinase (CK) activity is maximal at 4 hours (1093.75±1370,634) and decreases at 72 hours after birth. An increased value of creatine kinase activity in the first 4 hours after birth is not sufficient to consider it a true parameter of asphyxia, but rather a marker of stress due to birth.

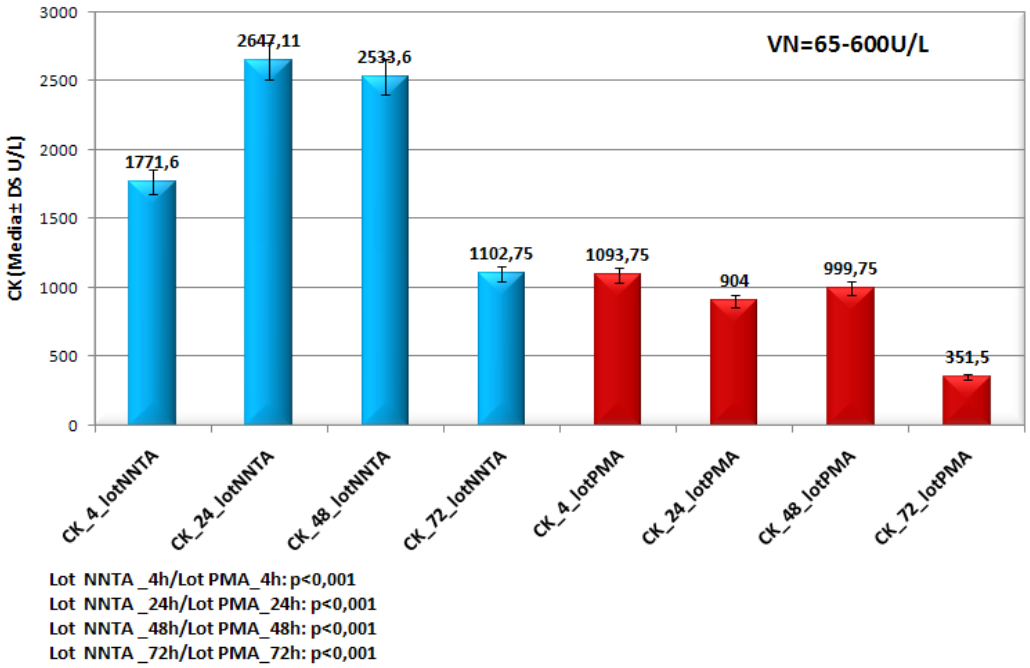


Fig. 2.10. Variation in creatine kinase activity at various times in term infants and premature babies undergoing oxygen therapy.

The reference values for the CK creatine kinase parameter analyzed in the case of full-term or premature healthy newborns are higher than the accepted values for adults (26-140 U/l for women and 38-174 U/l for men) because in the first 24 hours after birth due to the effort made by the newborn during labor, the muscular activity increases intensely due to the pressure exerted upon the muscles, being accompanied by an increase in the activity of the creatine kinase, whereas in the case of cesarean births the values are higher, probably due to the anesthetics administered to the mother that reach the fetus, but also as a result of lesions caused by the surgery underwent by the mother that will determine the increase in the values of the creatine kinase that will be found in the newborn (Sanath. *et al*., 2008).

An increased value of the creatine kinase in the first 4 hours after birth is not enough to consider it a true parameter of asphyxia because even in newborns without asphyxia the values of this parameter may increase due to the stress caused by the birth (natural or cesarean section) and they show fluctuating values in the first 12 hours of life by returning to normal limits in the interdependence with the individual reactivity of the newborn.

Maintaining increased values of the creatine kinase's activity with a tendency to triple or exceed the normal upper limit draws attention to severe neurological impairment. Thus, the activity of the creatine kinase can be a stress marker when values are increased up to 24 hours of life, and it will be analyzed after this hourly interval in order to assess asphyxia after this period only together with the activity of lactate dehydrogenase (LDH).

The lactate dehydrogenase activity has a maximum value at 4 hours (3024.67±1609.913 U/L) after which it gradually decreases, reaching half of the maximum value at 72 hours in the case of the group of full-term newborns with asphyxia (1590.75±212.315 U/L), but higher than the reference range (160-450U/L) (Fig. 2.11).

The values for lactate dehydrogenase (LDH) peak within 24-48 hours (3750.00±2215.647 U/L and 3672.00±2275.442 U/L) in the case of premature babies with asphyxia and remain significantly higher than the reference values and at 72 hours (Fig. 2.11).

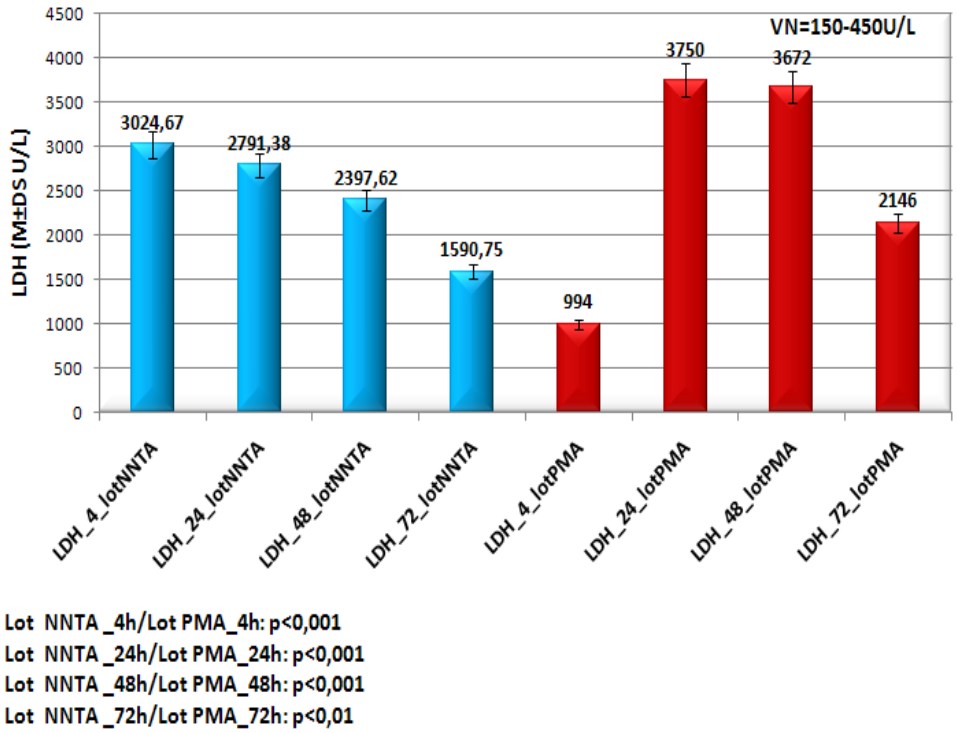


Fig. 2.11. Variation of lactate dehydrogenase activity at various time intervals in full-term and premature infants undergoing oxygen therapy.

The progressive decrease of the LDH parameter values after 72 hours is associated to a favorable evolution if it is accompanied by a decrease of the CK values.

We evaluated the level of oxidative stress for newborns with asphyxia by measuring the activity of the superoxide dismutase enzymes, glutathione peroxidase enzymes as well as the antioxidant capacity of the serum 48 hours after the asphyxiation and after 72 hours we measured the level of malondialdehyde as a marker of the lipid peroxidation. The reference values for each parameter as well as the values obtained after making the determinations are presented in Tables 2.XIV and 2.XV.

Table 2.XIV. Values of superoxide dismutase SOD enzyme activity, GPx peroxidase glutathione, MDA malondialdehyde concentration and antioxidant activity of TAS serum for term infants with asphyxia.

Statsitic description of the studied lots				
Studied lot	Evaluated parameter	Media (M)	Standard deviation (SD)	Standard error. (SE)
Lot NNTA with O_2^- therapy.	SOD (V=263±47 U/L)	768.75	73.200	21.131
	GPx (4171-10881 U/L)	17979.50	445.670	128.654
	MDA (1.173-1.324 µmol/L)	2.5375	0.52393	0.13098
	TAS (VN=1.33-1.77 mmmol/L)	1.3017	0.26059	0.07523

Table 2.XV. Values of superoxide dismutase SOD enzyme activity, GPx peroxidase glutathione, MDA malondialdehyde concentration and TAS serum antioxidant activity for premature babies with asphyxia.

Statsitic description of the studied lots				
Studied lot	Evaluated parameter	Media (M)	Standard deviation (SD)	Standard error (SE)
LOT PMA with O_2^- therapy	SOD (VN=263 ±47 U/L)	622.86	200.430	53.567
	GPx (4171-10881 U/L)	15703.50	2561.267	640.317
	MDA 1.173-1.324 µmol/L)	2.8850	1.09135	0.31505
	TAS (VN=1.33-1.77 mmmol/L)	1.1433	0.28500	0.08227

Also for the LDH parameter, the values at 4 hours are uneven, higher in the NNTA group. The evolution of LDH after 24 hours shows a behavior similar to the one for CK at the same hourly interval, there are correlations between the LDH values after 48 hours for the NNTA group and the PMA group ($p<0.001$).

The values above 3500 U/l after 24 hours showed 4 premature newborns in whom the transient tricuspid regurgitation with normal ETF was also identified. The values above 4000 U/l and that remain increased after 48 hours for both full-term and premature newborns were associated to an unfavorable prognosis and death.

We chose 48 hours and 72 hours respectively because, as we observed in the doses of the creatine kinase enzymes and lactate dehydrogenase enzymes as the first indicators of asphyxia, the prognostic period related to the neurological evolution falls within this time frame.

Assessment of the activity of superoxide enzymes dismutase SOD, glutathione peroxidase GPx in the serum of newborns with asphyxia

For both groups analyzed the value of the superoxide dismutase activity is far above the reference value, with an average of 622.86 U/L compared to an accepted maximum of 310 U/L. There are decreases in values in the case of the batch of premature babies versus newborns at term and with statistical differences between the two groups ($p<0.01$) justified by the immaturity of the enzyme system of the premature, but also by an individual variability for all subjects analyzed (Fig. 2.12).

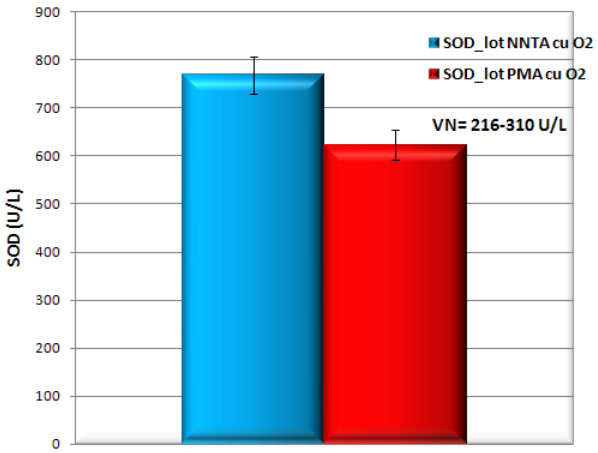


Fig. 2.12. Variation of SOD superoxide dismuta activity for groups of term and premature babies with asphyxia.

The increase in the SOD activity in the NNTA reflects an antioxidant reaction to neutralize the toxicity of the superoxide radical. A lower rate of the SOD activity in the LDCs reflects the fact that for a premature infant it is more difficult to cope with the continuous generation of free radicals, whereas the SOD activity differs from one tissue to another one, therefore it is difficult to make an accurate assessment and thus lower values can be explained in erythrocytes in certain premature infants compared to others to which we must add the particular features given by the difference in the gestational age.

The oxidative stress due to asphyxia generates reactive oxygen species that will increase the activity of the superoxide dismutase in the serum of the newborns, a value that has individual variations depending on the severity of the asphyxia.

The premature with asphyxia responds slowly to the asphyxiation attack aspect illustrated by the diminished antioxidant defense. The level of activity of the enzyme glutathione peroxidase is significantly increased in the group of term newborns with asphyxia compared to premature babies with asphyxia, but above the maximum value accepted as the maximum reference value (10881U/L).

Between the two strings of values is established a correlation between them there is a statistical difference ($p<0.01$), but also a positive Pearson correlation. After the administration of oxygen, the mean value of G-Px increased significantly in the NNTA group and PMA group) in comparison to the normal reference values (Fig. 2.13).

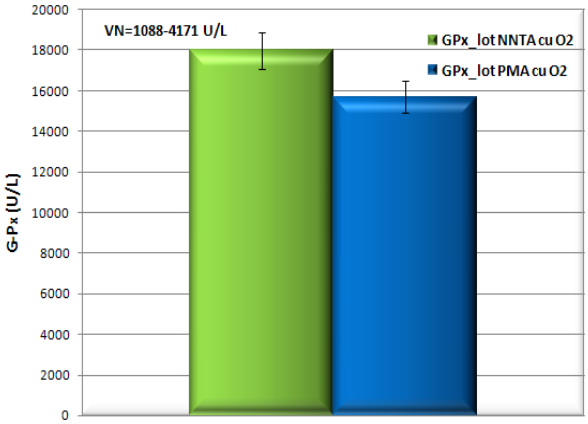


Fig. 2.13. Variation of glutathione peroxidase activity for groups of term and premature infants with asphyxia.

The statistical differences between the groups in which oxygen was administered in comparison to the ones that did not need oxygen therapy were strongly statistically significant ($p<0.001$). There are also statistically significant differences between the group of full-term newborns with oxygen therapy in comparison to premature newborns with oxygen therapy ($p<0.001$). No correlations were found between the values of the compared strings ($p> 0.05$).

There is an increase in SOD and GPx activity in subjects with asphyxia compared to non-asphyxiated subjects, with significant differences between SOD and GPx activity in term infants compared to premature infants.

The increase in SOD and GPx activity reflects the fact that these antioxidant enzymes have not been able to cope with the continuous generation of free radicals. Furthermore, the induction of these enzyme activities could have been a response to oxidative stress caused by resuscitation with pure oxygen administered as a result of asphyxia (Kim *et al.*, 2002).

Antioxidant defense at the cellular level involves the cooperative action of superoxide dismutase with glutathione peroxidase. The major defense is provided by superoxide dismutase

which neutralizes the toxic effect of the superoxide radical, followed by the action of glutathione peroxidase that neutralizes hydrogen peroxide. Increased levels of hydrogen peroxide may inhibit the activity of glutathione peroxidase, the value of which may be low in severe forms of ischemic hypoxic encephalopathy (Blum., 1985).

Assessment of serum anti-oxdante capacity in newborns with asphyxia

At present, as we have shown in the previous chapter for assessing the impact of free radicals on antioxidant enzyme systems, measurements of their activity are made at the erythrocytic level useful process, but difficult to achieve in dynamics and with sufficient costs requiring not only a high-performance laboratory, but also a staff specialized in this problem.

Assessing the antioxidant status of the serum by measuring antioxidant activity, an activity that reflects its ability to inhibit free oxygen radicals in any substrate proves its usefulness. However, most papers emphasize the role of individual enzymes in antioxidant defense.

At the same time, it is obvious that the antioxidant system of the blood plasma (serum) is not only the sum of the contributions of individual antioxidant enzymes, but depends on the interaction between them, but also on the presence of other oxidizing or prooxidant compounds present in serum or plasma (Fig. 2.14).

Most research focuses on assessing the total antioxidant activity of serum (TAS), which is a complex parameter that characterizes the state of the entire antioxidant system (AOS).

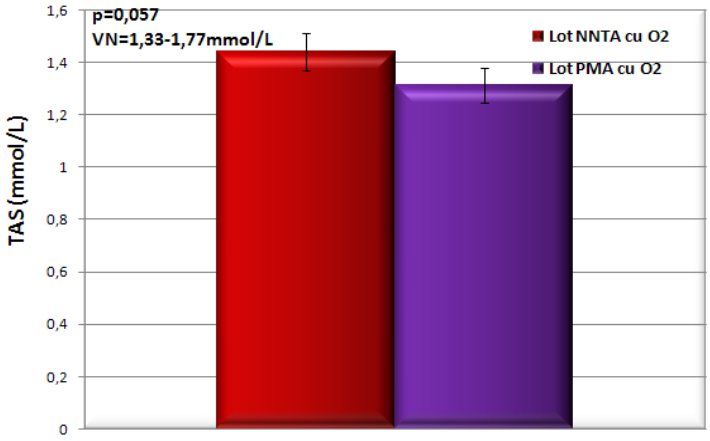


Fig. 2.14. Variation in the antioxidant capacity values of the TAS serum for the groups of term and premature babies with asphyxia.

Changes in the antioxidant capacity of serum show a decrease in this value in newborns with asphyxia, reflecting the effort of enzyme antioxidant systems to counteract at the cellular level the destructive action of free radicals.

A significant decrease was observed more evident in the preterm group (average 1.14 mmol/L) but with values of 0.9 mmol/L in premature babies with severe hypoxic ischemic encephalopathy. The average value for term newborns was around 1.30 mmol/L with individual variations and significantly lower for those with chronic asphyxia.

The administration of oxygen after asphyxia worsens the lipid peroxidation, whereas the generated free radicals trigger an inflammatory process responsible for the long-term effects at the level of the various tissues in which they are generated. The concentration of lipid peroxides changes whereas the concentration of their decomposition product is malon dialdehyde (MDA) (Fig. 2.15).

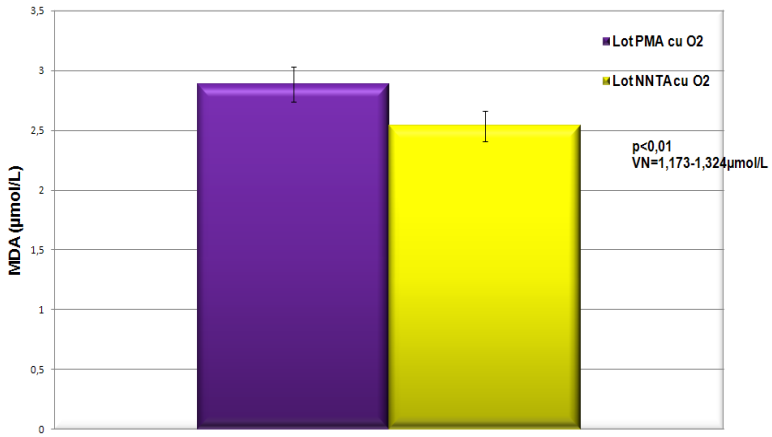


Fig. 2.15. Variation of MDA malonaldehyde values for groups of term and premature babies with asphyxia.

The degree of lipid peroxidation was evaluated by measuring the level of malonalhide in the serum of newborns with asphyxia and we considered that 72 hours after the occurrence of the ischemic attack is better evaluated the oxidative membrane process which as we have shown in other studies of ours continues over a longer period of time being directly proportional to the intensity of the attack and the degree of encephalopathy developed.

There is an increase in malonaldehyde values for both preterm and term infants. (0.95 mmol/L for healthy newborns versus 1.17 mmol/L compared to preterm infants at 24 hours) and for premature babies continue to increase to 1.32 µmol/L at 72 hours.

There are statistical differences between the value of the malondialdehyde concentration in the group of full-term infants with asphyxia in comparison to the group of premature infants with asphyxia ($p < 0.01$).

Oxygen therapy will amplify the oxidative stress and implicitly the lipid peroxidation, as it can be seen from the obtained data, an average value of 2.88 µmol/l in the group of premature infants in comparison to an average of 2.53 µmol/l for the group of full-term newborns.

We can notice an emphasized growth rate of the lipid peroxidation in premature infants with asphyxia (272%), in comparison to (216.2%) for full-term newborns with asphyxia. When the dynamics is measured, there is a faster recovery after the asphyxiation attack at the reference values for the full-term newborn in comparison to the premature infant.

For the cases of premature infants with more than 5 days of intermittent oxygen therapy and by using various techniques (free flow, intubation, IPPV, CPAP), an increase in the lipid peroxidation was recorded. The determinations performed 14 days after the birth of premature infants who received oxygen therapy for 7 to 10 days showed MDA values above 2 µmol/mL, which suggests a continuous oxidative process.

2.1.6. Discussions

The values increased for LDH at 48 hours have a more accurate diagnostic significance of asphyxia especially when they are correlated with increased CK activity values in the same time frame and can be a marker of late asphyxia. Increased values of creatine kinase activity after 24 hours of life together with increased lactate dehydrogenase activity may be predictive factors in perinatal asphyxia.

The activity of the antioxidant system which includes two antioxidant mechanisms – a group of antioxidant enzymes (superoxide dismutase, catalyse, and glutathione peroxidase) and the so-called anti-radical chain represented by a sequential chain of reversible states

oxidation-reduction reactions (ascorbate-glutation and tocopherol oxidation) – provide protection for tissues and organs against the aggressive action of free radicals.

The harmful effect of reactive oxygen species is manifested only when active oxygen species are formed and antioxidant defense is insufficient.

In this context, newborns are considered to be particularly prone to the toxic effects of active oxygen species due to the fact that the fetus develops under conditions of relative hypoxia. The additional suffering scans that occur as a result of hypoxia at birth increase the risks and increase the severity of the lesions in newborns either premature or at term which must receive additional oxygen therapy.

Superoxide dismutase and glutathione peroxidase are antioxidant enzymes important in the defense of biological structures from the harmful action of free radicals their activity being different from one tissue territory to another even in the same time frame (Yoshida *et al*., 1994).

The study of total antioxidant activity of serum in healthy term newborns in our group revealed values close to adults 1.33-1.77 mmol/l and in accordance with Kosov's study (Kosov *et al*., 2001). We did not separate the study groups of babies born naturally or caesarean section, but according to the data in the literature there are no significant changes in the values for the antioxidant capacity of the serum in the case of the two types of births for healthy term infants.

All this demonstrates that newborns need high levels of antioxidants and a complicated cellular redox balance to counteract the toxic action of increased amounts of free radicals, and the behavior of antioxidant defense systems is specific to each tissue territory according to its peculiarities.

Serum antioxidant capacity values in healthy premature infants were within the normal range, but a dispersion of the variable total antioxidant capacity in the left side of the range (with an average of 1.35 mmol/l) acceptable in the context of prematurity was observed.

For both term and healthy premature infants, significant individual fluctuations were recorded even though the values were close. It seems that the mode of birth in the case of a healthy baby is not influenced by the type of birth because the natural antioxidant system is intentionally created to be a system resistant to extreme effects (Kosov *et al*., 2001).

Following the determinations made, there is a decrease in antioxidant activity in the serum of premature babies compared to that of newborns at term after oxygen therapy, confirming once again that prematurity increases oxidative stress, and antioxidant defense mechanisms have been shown to be less active in premature babies than in babies born at term. Our data is consistent with those of Geeta Gathwala and Seema Sharma (Gathwala, 2000).

We must consider in both study groups that one of the factors favoring asphyxia is placental insufficiency which can cause hypoxia, but it seems that there is a high degree of compensatory-adaptation reactions in all cases of placental insufficiency.

Their consequence is an increase in placental blood flow and normalization of the fetus' oxygen supply. It appears that placental compensatory reactions are a stabilizing factor that contributes to the normal formation of fetal antioxidant activity, even in conditions of chronic hypoxia.

However, excessive side effects may favor the depletion of placental compensatory reactions followed by the occurrence of decompensated placental insufficiency, leading to a deficiency in the antioxidant defense system and increased lipid peroxidation (Kosov *et al*., 2001).

The neonatal erythrocytes are more vulnerable to lipid peroxidation and malondialdehyde formation. Newborn erythrocytes have a smaller number of sulfhydryl groups in the membrane and contain more residual hemoglobin, neonatal hemoglobin. They consume more oxygen and produce more hydrogen peroxide in comparison to adult erythrocytes.

The higher hydrogen peroxide production may be derived from hemoglobin self-oxidation due to the fact that the globular mean of hemoglobin is significantly higher in newborn erythrocytes in comparison to adults.

The increased sensitivity may be related to the deficiency of antioxidants that occurs in neonatal erythrocytes with obvious differences between premature and full-term newborn. The oygen therapy will increase the vulnerability of the erythrocyte membranes and it will increase the lipid peroxidation.

Table 2.XVI. Criteria for determining the degree of ischemic hypoxic encephalopathy (after modified Sarnet).

Weak I	**Hypertension, minor changes in tone, reduced feeding difficulty and recovery in 48 hours**
Moderated II	Lethargy, more pronounced abnormalities of tone, low appetite and convulsions with recovery within 7 days
Severe III	Coma, inability to maintain adequate ventilation, deep hypotonia, convulsions

The evaluation of the degree of lipid peroxidation according to the degree of encephalopathy according to Sarnet's criteria and Sarnet appreciates that the peroxidation process is intensified and remains high as the time of administration of oxygen therapy increases, and the neurological lesions are more pronounced.

Pregnancy due to the increased need for oxygen as well as due to the large number of mitochondria in the placenta predisposes to oxidative stress. This is highlighted in studies in which markers of oxidative stress have been identified even at normal pregnancies (Toescu *et al*., 2002).

The behavior at the ischemic hypoxic attack of full-term newborns is different from that of premature babies, and in the group of premature babies the gestational age together with the birth weight influences each premature individual.

2.1.7. Conclusions

We validated the dosing methods for the evaluation parameters of oxidative stress in the age category of premature newborns (in healthy groups of newborns with neonatal asphyxia).

We have shown that a useful parameter in the short and medium term monitoring of the evolution of newborns is the measurement of the antioxidant activity of the serum (TAS). For this we established our own reference values by age groups, respectively newborns at term and respectively healthy premature ones. These values constituted and constitute the reference criteria used for studies and research in other doctoral theses.

Validation of a method for rapid assessment of the total antioxidant capacity of serum is extremely useful because it allows the determination of both the antioxidant status of the mother and the newborn regardless of gestational age.

Starting from the obtained results, we realized that it is necessary:

Assessment of the total antioxidant defense capacity of serum (TAS) and malondi-aldehyde in all newborns subjected to oxygen therapy due to perinatal asphyxia and the intro-duction of these biochemical parameters in the monitoring sheets of the long-term follow-up program of newborns who presented perinatal asphyxia.

Newborns need a high level of antioxidants and a complicated cellular redox balance in order to counteract the toxic action of the increased amounts of free radicals, whereas the behavior of the antioxidant defence systems is specific to each tissue territory depending on its particular features.

The behavior during the ischemic hypoxic attack of full-term newborns is different from the one of premature babies, whereas in the group of premature infants the gestational age together with the birth weight individually influences each premature infant.

Elaboration of feasible tests to highlight the role of free radicals in post-asphyxiated cerebral distress in the newborn in order to design therapeutic schemes to reduce the aggression of free radicals generated by the administration of oxygen therapy to newborns with asphyxia at birth.

The antioxidant status of the newborn is closely related to that of the mother (maternal-fetal system). The correlations between the level of antioxidant saturated serum in mothers and newborns highlighted in the literature indicate the use of determining the antioxidant capacity of serum in mothers and newborns in order to assess impairments that may occur during and after birth as well as for monitoring possible antioxidant therapy.

Starting from the data from the literature and in agreement with the analysis of cases of premature birth or not, but having the intrauterine growth restriction (IUGR) as a triggering factor, we conducted a study is to identify correlations between total antioxidant status values of mothers and their infants.

2.2. Oxidative stress for blood (plasma serum) in newborns with premature asphyxia and at term with and without growth restriction

Published paper:

- Scripcariu SI, Avasiloaiei A, Socolov D, Mihălceanu E, **Dimitriu DC**, Moscalu M, Stamatin M. *Total antioxidant status as marker of oxidative stress in infants with intrauterine growth restriction*. Revista Română de Medicină de Laborator Vol. 28, Nr. 2:2020. **Impact factor – 0.945**.

2.2.1. Aim of the studies

In this study was identify correlations between TAS values of mothers and their infants and compare these values in accordance to the presence or absence of *intrauterine growth restriction* (IUGR).

The identification of IUGR or restriction as a separate entity was discussed in the mid-20th century, when the age at birth and the birth weight were overlapping concepts. The World Health Organization then recommended that a weight of less than 2500 g at birth means prematurity. The scrrening for IUGR represents a central interest point for obstetricians and neo-natologists because this condition is associated to maternal and fetal mortality and morbidity.

Even if there is a set of risk factors for IUGR, their prediction capacity is still small. Some of the risk factors are: smoking, the mother's weight and low height, preexisting cardio-vascular disorders. All these are generators of reactive oxygen species through the generated pathophysiological mechanisms.

The endoltelial dysfunction and the local inflammation are considered by certain central elements in the pathogenesis of placental ischemic disease. The presence of the vascular cell adhesion molecules VCAM, the soluble intracellular adhesion molecule-1 (sICAM-1) and E-selectin, in this process is also known in the case of these pathologies. The increased levels in the circulating blood in pregnant women may indicate the presence of endothelial dysfunction, but there are still difficulties in the dosing process.

The prediction of placental ischemic disease is a challenge for any clinician in the context in which there is a special clinical polymorphism, whereas the etiopathogenic events are disparate in time and space. For the diagnosis of PE the markers have been standardized to measure the severity of the disease as well as to assess the placental imbalance, but for IUGR,

the placental abruption and especially the early identification of these pathologies, many studies are still ongoing.

2.2.2. Materials and methods

The study over three years (2016-2018) in "Cuza Vodă" – Iași maternity hospital on a group of 52 infants. Thirty-six infants had IUGR, diagnosed over the second or third trimester of pregnancy and were also small-for-gestational-age (SGA) – below the 10th percentile on the intrauterine growth curves (study group). Sixteen appropriate-for-gestational-age (AGA) infants, from healthy pregnancies were used as controls. We excluded from our study infants with congenital malformations of any kind.

The samples, which were taken immediately prior to delivery from peripheral blood in mothers and from the cord blood in infants, were preserved up to 36 hours in the refrigerator (+2 – +8°C) or in the freezer up to 14 days at -20°C and analysis was performed on an RX Imola®.

As reference for TAS, we used values between 1.23 and 1.77 mmol/L. Hemolyzed samples were considered inadequate and discarded. The study was approved by the Hospital's ethics committee by decision number 1124/01.02.2017. Informed consent was taken from the infant's parents prior to any harvest of biologic material being done. The data were analyzed using SPSS 24. (SPSS, Chicago, IL, USA).

Numerical variables of continuous type were expressed as means and standard deviations, and for comparison, the t-Student test or Mann-Whitney U test were applied. To evaluate the correlation among variables, the correlation coefficient was calculated, and statistical significance was assessed through the Pearson test. Statistical significance was defined as $p<0.05$.

2.2.3. Results

The infants we included had a mean gestational age of 36.6 weeks, a mean birth weight of 2456 grams, a mean length of 47.1 cm and a mean ponderal index of 2.25, with significant statistical differences between the two groups (Table 2.XVII).

Serum TAS values in the mothers were between 0.89 and 1.98 mmol/L, with statistically significant differences ($p=0.018$) between the mean values in the IUGR group (1.32 mmol/L) and the non-IUGR group (1.46 mmol/L) (Table 2.XVIII, Fig. 2.16).

TAS values determined in the serum of neonates were between 0.9 and 2.08 mmol/L and also showed significant differences ($p<0.001$) between the means we obtained in the IUGR group (1.34 mmol/L) and the ones in the non-IUGR group (1.61 mmol/L) (Table 2.XVIII, Fig. 2.16). In both groups, we found a significant direct correlation between maternal and neonatal values of serum TAS ($p<0.001$ for both groups) (Fig. 2.16).

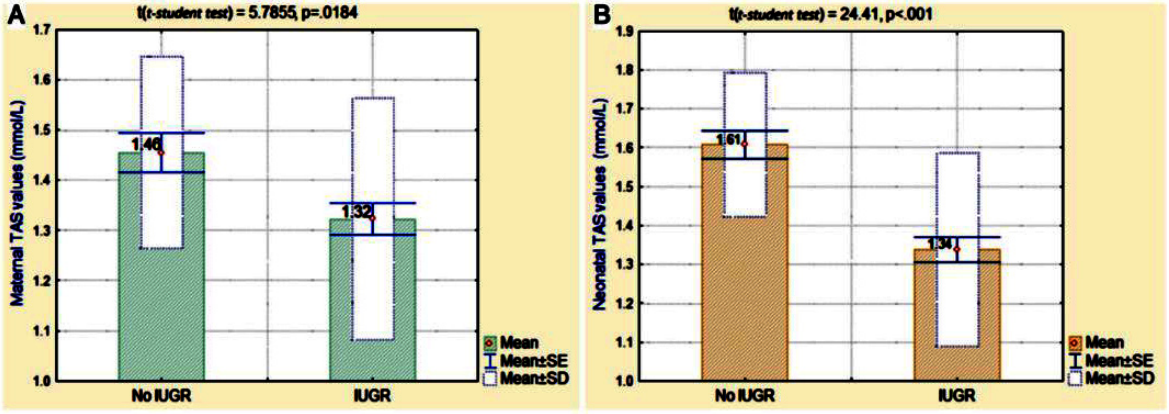
Table 2.XVII. Characteristics of the studied groups.

Characteristic (†)	**Total group**	**Study group**	**Control group**	**P-value (‡)**
Gestational age (weeks)	36.6±2.8	35.8±2.9	38.3±1.4	<0.001*
Birth weight (g)	2456.1±830.9	2033.8±620.7	3406.9±252.7	<0.001*
Birth length (cm)	47.1±4.8	45.2±4.7	51.2±1.2	<0.001*
Ponderal index (kg/cm^3)	2.25±0.3	2.12±0.3	2.48±0.13	<0.001*

† mean± standard deviation; ‡ t-Student test or Mann-Whitney U Test; (*) Marked effects are significant at $p<0.05$.

Table 2.XVIII. TAS values in studied groups.

TAS values (†)	Total group	Study group	Control group	P-value (‡)
Mothers (mmol/L)	1.36±0.23	1.32±0.24	1.46±0.19	0.018*
Infants (mmol/L)	1.42±0.26	1.34±0.25	1.61±0.18	<0.001*



mean± standard deviation; † t-Student test; ‡ Mann-Whitney U Test; (*) Marked effects are significant at $p<0.05$.

Fig. 2.16. Mean values of (A) maternal and (B) neonatal serum TAS (mmol/L).

2.2.4. Discussions

Antioxidants are responsible for the protection of cells from peroxidation reactions, the limitation of cellular damage, as well as the maintenance of cellular membrane integrity (Bharadwaj *et al*., 2018).

TAS represents an overall measure of the antioxidant activity of biological fluids and it provides an integrated parameter, rather than the simple sum of measurable antioxidants, by taking into account their synergistic interaction (Ghiselli *et al*., 2000) thus providing an integrated parameter rather than the simple sum of measurable anti-oxidants. The capacity of known and unknown antioxidants and their synergistic interaction is therefore assessed, thus giving an insight into the delicate balance *in vivo* between oxidants and antioxidants.

Although it is known that IUGR is a condition marked by limited antioxidant defense, some studies investigating antioxidant activity in IUGR show conflicting results. Toy *et al*. showed that serum TAS levels were significantly lower in women with IUGR pregnancies, compared to the control group, whereas TAS levels and the oxidative stress index were significantly higher in the IUGR group compared to controls.

Our study showed, in a similar fashion, that both mothers and infants from IUGR pregnancies have significantly lower values of serum TAS, compared to those from normal pregnancies. Moreover, there is a significant direct correlation of maternal and neonatal TAS values, both in IUGR and non-IUGR pregnancies. One of the strong points of our study is that it demonstrates that the antioxidant status of the IUGR infant is closely linked to that of its mother and, thus, can be influenced by it.

Oxidative stress in IUGR pregnancies can be reduced to a point by antioxidant supplementation, such as vitamins and trace elements. Not only is this difficult to quantify objectively but also, IUGR pregnancies are more often than not, high-risk pregnancies, due to possible malnourishment or other conditions of the mother, such as pre-eclampsia or hypertension.

Maternal malnourishment ultimately leads to intrauterine malnutrition, which is linked to a rise in oxidative stress. Gupta *et al*. 2001 ascertained this in their study, by the increase of

malondialdehyde activity and decrease of antioxidant enzymes, such as superoxide dismutase, catalase and reduced glutathione, catalase, reduced glutathione, and serum malondialdehyde.

Obesity and its link to oxidative stress may be another risk factor for the appearance of IUGR. Thus, the study of comorbidities that obesity can be associated with and the link between them through oxidative stress has been an interesting subject in literature.

An interesting fact is that, while ischemia-modified albumin has not been demonstrated to be associated with obesity as a result of oxidative stress in this pathology (Higdon *et al*., 2003, Yigitbasi *et al*., 2017), studies in literature link ischemia-modified albumin to oxidative stress in pathologies such as stroke and chronic heart failure (Jena *et al*., 2017). So, oxidative stress has been demonstrated as a pathophysiologic factor in multiple diseases, but the mechanisms through which it functions are still incompletely investigated, making this subject an interesting and promising one.

Pre-eclampsia is an independent risk factor for oxidant-antioxidant imbalance (Sharma *et al*., 2016). Infants from mothers with pre-eclampsia have high lipidic peroxidation markers and low TAS, as demonstrated by Namdev *et al*., 2014. Moreover, in their study, it was proved that the level of oxidative stress was linked to poor neonatal outcome, such as necrotizing enterocolitis, sepsis or respiratory distress. However, while oxidative stress may be different in preterm and term infants, TAS is not influenced by gestational age, as it was demonstrated by Ferencz *et al*., 2015 in their study on IUGR infants.

2.2.5. Conclusions

TAS values are significantly lower in mothers and infants with IUGR, as compared to mothers with normal pregnancies, resulting neonates without IUGR. In addition, we have shown that there is a direct correlation between TAS values of infants and their mothers, regardless of the presence or absence of IUGR. The TAS values, as an important marker of the oxidative status of patients, are correlated with the presence of IUGR and values recorded from blood samples of the mother may be predictive for the oxidative status of the infant, thus of IUGR.

2.3. Oxidative stress for blood (plasma serum) after treatment of perinatal asphyxia in term newborns

Personal contribution

We conducted a study aimed at modifying some parameters of oxidative stress in newborns with perinatal asphyxia who received treatment with phenobarbital and erythropoetine.

Published paper:

- Avasiloaiei A, Dimitriu C, Moscalu M, Păduraru L, Stamatin M. *High-dose phenobarbital or erythropoietin for the treatment of perinatal asphyxia in term newborns*. Pediatrics International, 55(5):2013, 589-593. **Impact Factor – 0.985**.

2.3.1. Aim of the studies

Systemic hypoxia and cerebral ischemia lead to a decrease in oxygen and glucose. The consequence is anaerobic glycolysis, followed by decrease of ATP and acidosis, all of these contributing to impairment of brain function. Various intertwined mechanisms (excessive excitatory aminoacids receptors synthesis, intracellular calcium accumulation, free radical generation) influence the outcome of hypoxic-ischemic injury. Reactive oxygen species can promote the expression of adhesion molecules, leading to activated granulocyte accumulation, which amplifies cellular destruction.

Phenobarbital and erythropoietin are two additional therapies that might be used in addition to or instead of therapeutic hypothermia. Phenobarbital acts by suppressing the oxidative cerebral metabolism. Aim of the studies randomized controlled trial to examine the potential beneficial effects of phenobarbital and erythropoietin for infants with perinatal asphyxia who had signs of HIE. Evaluation of the effectiveness of therapy was carried out by measuring the values of enzymes involved in the evaluation of oxidative stress and total antioxidant status: superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase), malondialdehyde (MDA), total serum antioxidant status (TAS).

2.3.2. Materials and methods

We conducted a prospective randomized study of 67 term neonates with perinatal asphyxia, admitted from 1 January 2010 to 30 September 2011 to the "Cuza Vodă" Clinical Hospital of Obstetrics and Gynecology Neonatal Intensive Care Unit (NICU). Of 9302 term infants admitted to the "Cuza Vodă" Clinical Hospital of Obstetrics and Gynecology during the 21 months of the study, 67 (0.72%) were diagnosed with perinatal asphyxia.

The following data were collected and analyzed: birthweight, gestational age, Apgar scores at 1, 5 and 10 min, cord blood pH, red blood cell activity of antioxidant enzymes (SOD, GPx), TASand MDA. TAS was analyzed using the ABTS® technique (Boehringer-Mannhein, Germany) and RANDOX reactants (Randox Laboratories, Crumlin, County Antrim, Northern Ireland). SOD was measured by the degree of formazan inhibition using RANSOD kits (Randox Laboratories) with control serum. GPx was measured using RANSEL reactants through the Paglia and Valentine method. MDA was analyzed using thiobarbituric acid reaction.

The samples were collected at 4, 24, 48, and 72 h and at 7 days of life, and measured using a Beckmann spectrophotometer. Neurologic clinical examination was performed as soon as possible after birth and periodically thereafter, noting the presence or absence of neurologic abnormalities (of muscle tone and inborn reflexes) and their duration.

The infants were randomly assigned to supportive treatment (oxygen, volume expanders, inotropes, diuretics, antibiotics), a single dose of i.v. phenobarbital, 40 mg/kg, during the first 4h after birth plus supportive treatment, or s.c. rythropoietin, 1000 IU/kg per day, for the first 3 days plus supportive treatment. Infants were allocated to treatment groups by unblinded, randomdraw, numerical assignment.

The data were analyzed using SPSS V.19.0. (SPSS, Chicago, IL, USA). Descriptive statistics were used to express characteristics and tendencies of studied parameters. The independent variables – treatment-derived differences among parameters – were analyzed using ANOVA test, for normal frequency distribution. In other cases, the non-parametric Kruskal–Wallis test was used, based on the analysis of attributed ranks. Statistical significance was defined as $p<0.05$.

2.3.3. Results

The enrolled newborns had a mean gestational age of 40.4 weeks and a mean birthweight of 3278 g. The newborns admited in NICU diagnosed with perinatal asphyxia is presented in figure 2.17. The treatment groups were homogenous. Apgar scores at 1 min were between 3 and 4.1 and had a mean of 3.6. As resuscitation continued, Apgar scores at 5 min rose slightly to a mean of 5.3, and at 10 min – 6.6. Low cord blood pH confirms the diagnosis of perinatal asphyxia (7.00 – 7.09).

Antioxidant enzymes (SOD and GPx) were lower for infants treated with phenobarbital or erythropoietin compared to control infants (Fig. 2.18). When compared to reference values for healthy term newborns (SOD, 216–310 U/L; GPx, 4171–9881 U/L), the high values in the control infants suggested the existence of oxidative stress following perinatal asphyxia.

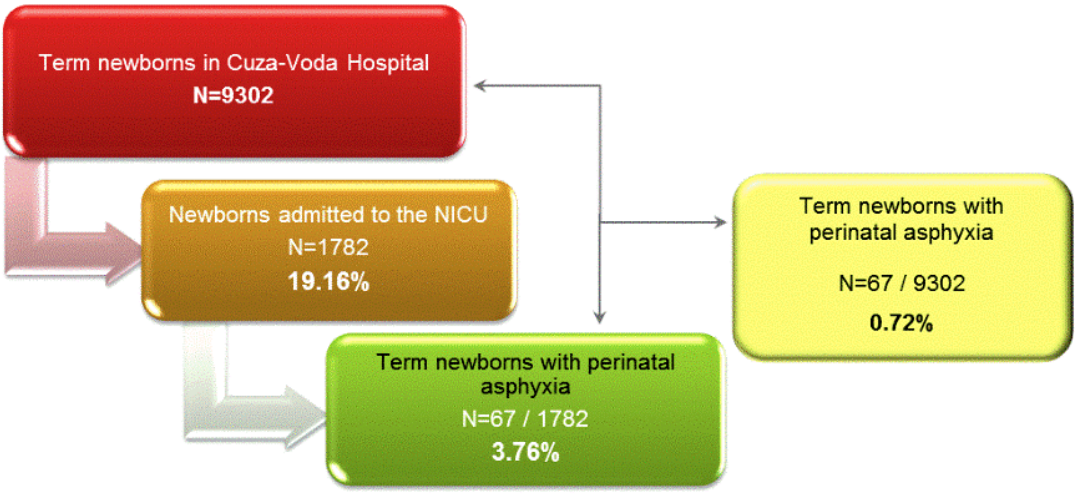


Fig. 2.17. Incidence of perinatal asphyxia NICU, neonatal intensive care unit.

The TAS was higher, although not significantly ($p<0.5$), in the phenobarbital and erythropoietin groups, compared to the control group (Fig. 2.18). The mean reference values for TAS in healthy term newborns are 1.20-1.30 mmol/L. Lipid peroxidation measured by MDA in plasma 7 days after the initial hypoxic insult was high in all of the patient groups compared to the normal range for healthy term newborns (1.17-1.32 mmol/L) (Fig. 2.18).

At birth all of the infants had clinical neurologic abnormalities, and this aspect persisted at 6 and 12 h; after 72 h neurologic disorders were found in only 53.7% of the newborns. Four deaths occurred in the control group (17%); one (5%) in the phenobarbital group; and one (5%) in the erythropoietin group.

Treatment correlation with long-term neurologic outcome took into account the presence of various items during the clinical follow-up examination: motor disabilities, and disorders of receptive language, expressive language, and cognitive development (Fig. 2.19).

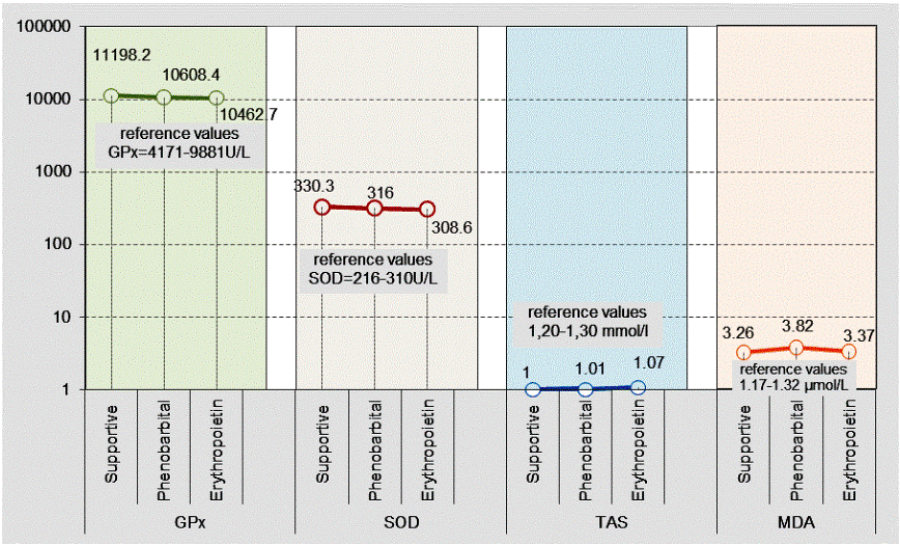


Fig. 2.18. Assement of antioxidant enzyme GPx, glutathione peroxidase; MDA, malon-dialdehyde; SOD, superoxide dismutase; TAS, total serum antioxidant status. Reference values: GPx, 4171-988 U/L; MDA, 1.17-1.32 µmol/L; SOD, 216-310 U/L; TAS, 1.20-1.30 mmol/L.

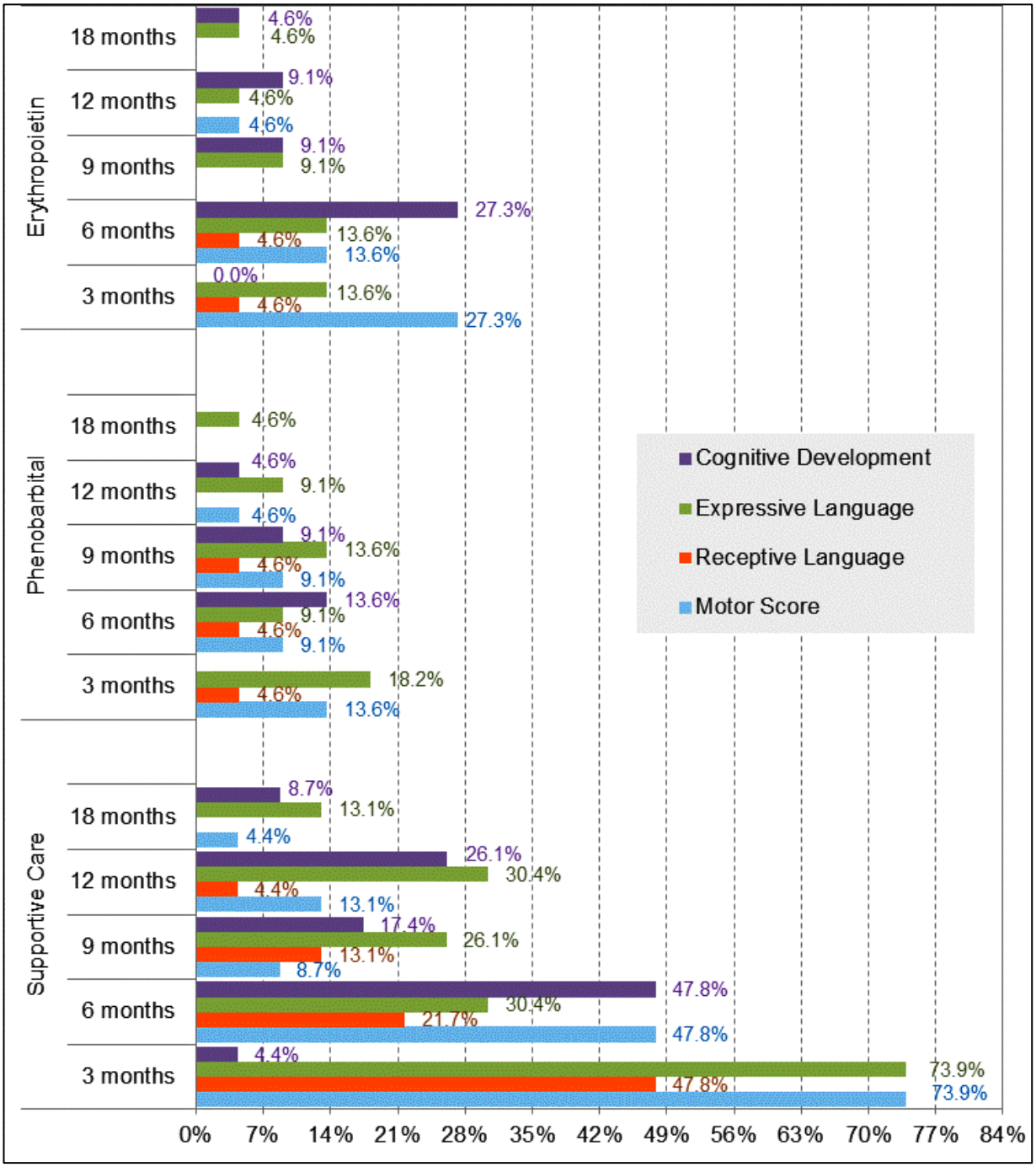


Fig. 2.19. Results of Bayley II assessement: purple – cognitive development; green – expressive language; orange – receptive language; cyan – motor score.

2.3.4. Discussions

Decrease of plasma activity of antioxidant enzymes among the infants treated with erythropoietin is due to the known role of erythropoietin in preventing oxidative stress and decreasing lipid peroxidation (Villa *et al*., 2003, Juul, Felderhoff-Mueser, 2007).

Phenobarbital decreases cellular metabolic rate and oxidative stress, favoring the decrease of antioxidant enzyme activity TAS was higher in the erythropoietin group than in the other groups (Gathwala *et al*., 2010). This could represent the effort of antioxidant systems to counterbalance free radical injury, generated by hypoxia-ischemia. Also, TAS may be greatly influenced by other medications used during the first 72 h after perinatal asphyxia.

Lipid peroxidation is as low process, and it is a good reflection of oxidative stress generated by both perinatal asphyxia and oxygen therapy. Because lipids are key components of cellular membranes, their peroxidation leads to severe disruptions of membrane structure and function.

The degree of lipid peroxidation can be measured by the levels of MDA in plasma and cerebrospinal fluid (Gathwala *et al*., 2010), because MDA is very persistent in plasma. It results from the peroxidation of fatty acids with three or more double links and it is the cause of cross-linking and polymerization of membrane components.

Malondialdehyde was lower in the erythropoietin group compared to the other groups, although the difference was not statistically significant ($p<0.05$). This could be explained by the various models of neonatal hypoxia, which showed that in doses ranging from 1000 IU/kg to 30 000 IU/kg, erythropoietin has anti-apoptotic and anti-inflammatory effects in the acute postinjury period, with neurogenic and vasculogenic effects in the recovery period (Chang, *et al*., 2005).

The descending trend in neurologic abnormalities of muscle tone and reflexes, as well as seizures suggests the recovery of cerebral metabolism following the early transient failure of cerebral blood flow. The mortality rate was lower in the phenobarbital and erythropoietin groups (both 4.6%) than in the control group (17.4%; c2=7.26, P=0.0087, 95% confidence interval). This may be explained by the facilitation of antioxidant defense mechanisms through the use of phenobarbital or erythropoietin.

The dynamics of the parameters involved in antioxidant defense were more pronounced in the erythropoietin group than in the other groups, but the differences between the phenobarbital and erythropoietin groups were not statistically significant. Follow-up results were better in the phenobarbital group than in the erythropoietin group in the fields of motor and cognitive function at 3 and 6 months and worse for expressive language.

At 18 months, however, the differences between these two groups were not significant. The present results regarding the efficacy of high-dose phenobarbital during the immediate recovery period are consistent with the work of (Gathwala *et al*., 2010), who noted decreased oxidative stress after phenobarbital. The present study shows that high-dose pheno-barbital improves long-time neurologic outcome, as shown by Hall.

2.3.5. Conclusions

High-dose phenobarbital or erythropoietin along with supportive treatment has a positive influence on the outcome of newborns with perinatal asphyxia. Phenobarbital also has the advantage of low cost and simplicity. Unlike therapeutic cooling, phenobarbital can easily be given in any NICU (Neonatal Intensive Care Unit), even in low – or modest – resource countries.

Therefore, newer and more effective neuroprotective therapies are urgently needed. Phenobarbital and erythropoietin are two additional therapies that might be used in addition to or instead of therapeutic hypothermia. Phenobarbital acts by suppressing the oxidative cerebral metabolism and diminishing the neuronal response to glutamate High-dose i.v. phenobarbital, used early after the neurologic insult, lowers the cerebral metabolic rate and lipid peroxidation in plasma and cerebrospinal fluid, decreases the incidence of seizures and that of long-time complications, is well-tolerated and does not influence mortality. Erythropoietin has been shown to have potential for ameliorating the neurological sequelae of HIE

2.4. Oxidative stress for other biological fluids (breast milk)

Personal contributions

Internal Grant Member

"Optimal storge practices to preserve macronutrients, energy and total anti-oxidants in human milk from mothers of term and preterm newborns", project manager Luminița Păduraru – 2015-2016.

Published paper:

- Păduraru L, **Dimitriu DC**, Avasiloaiei A L, Moscalu M, Zonda GI, Stamatin M. *Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates*. Pediatrics and Neonatology 59(6): 2018: 600-605. **Impact Factor – 1.232**.
- Păduraru L, Zonda GI, Avasiloaiei AL, Moscalu M, **Dimitriu DC**, Stamatin M. *Influence of refrigeration or freezing on human milk macronutrients and energy content in early lactation: Results from a tertiary centre survey*. Paediatrics & Child Health 24(4): 2019, 250-257. **Impact Factor – 2.348**.

2.4.1. Aim of the studies

The objectives of *the first study* were to determine the total antioxidant status (TAS) of human milk and to evaluate the differences between premature milk and term milk at different moments of lactation, while the aim of *the second study* was to identify the time during lactation when the macronutrients provide maximum energy. For both studies were evaluated the effects of refrigeration and freezing.

2.4.2. Materials and methods

These two studies involved 90 lactating mothers with infants born between July 2015 and October 2016 in a level III maternity hospital from eastern Romania, an area with the highest natality rate, but with low income and poor economic status.

The mothers were assigned to two predefined groups: one group – enrolling 30 mothers who delivered at term (gestational age ≥37 weeks) and the other group – enrolling 60 mothers who delivered prematurely (gestational age <37 weeks).

The inclusion criteria were: mothers of premature or term infants whose lactation started in the first 3±1 days postpartum, aged 16-40 years, with normal recovery after delivery. Otherwise the exclusion criteria were: acute illness, treatment with drugs potentially excreted in human milk, chronic treatment for epilepsy, cancer, thyroid conditions, or arterial hypertension, and localized breast infection.

Human milk from days 3±1 (*colostrum*), 7±1 (transitional milk), and 30±1 (mature milk) was pumped under the same conditions, between 8:00 and 11:30 a.m. and divided in five aliquots/subject (2 ml sample in Eppendorf plastic tubes). Two samples were refrigerated at +4°C, two were frozen at -20°C and one, with fresh milk, was analyzed in the following 2h.

For *the first study*, TAS was measured from the following milk types: fresh (labeled FM), refrigerated for 24 h (labeled R24) and 72 h (R72), and frozen for 1 week (F1) and 12 weeks (F12), respectively. For each patient a total of 15 aliquots were obtained and analyzed. Results were expressed as mmol/L. Samples where a concentration higher than 2.5 mmol/L was detected were diluted with a solution of double distilled water and remeasured.

For *the second study*, that aimed to determine the energy and macronutrient content, fresh milk was analyzed the same day, within 2 hours after expression. Refrigerated milk was analyzed the next 24, 48, and 72 hours (R24, R48, R72), after rewarming to room temperature. Samples of frozen milk were thawed at room temperature 1, 2, 4, 8, and 12 weeks after

sampling (F1, F2, F4, F8, F12) the same way milk is thawed for feeding infants in our neonatal intensive care unit. For each patient, 54 aliquots were analyzed.

The method used ABTS spectrophotometric technique (Boeringer-Mannheim, Germany), Rx-IMOLA analyzer (Randox Laboratories, Crumlin, County Antrim, Northern Ireland), RANDOX reagents and calibrators. Results were expressed as mmol/L. Samples where a concentration higher than 2.5 mmol/L was detected were diluted with a solution of double distilled water and remeasured. In the present study few samples were retested, as there were very few values of less than 2.5 mmol/L.

Statistical analysis were performed by SPSS v.24.1 (IBM) software, in case of the first study and by SPSS V.20.1 (SPSS, Chicago, IL, USA) and MATLAB (MathWorks, Natick, MA, USA) software programs, in case of the second study. Data were expressed as means (95% confidence interval [CI]). A paired-samples t test was used for comparison of means at different times. Statistical comparisons between all groups were made by parametric (t-Student, analysis of variance) and nonparametric (Kruskal-Wallis or Mann-Whitney U) tests for the first study and by analysis of variance (ANOVA), for the second study. Statistical significance was defined as $p<0.05$.

Informed consent was obtained from each subject and the study was approved by the "Grigore T. Popa" University's Ethics Committee.

2.4.3. Results

The mean values of TAS in the two groups increased from day 3 to day 30. This pattern was constant regarding fresh term milk (1.39 *vs*. 2.1 *vs*. 2.55 mmol/L), whereas in preterm milk TAS values were slightly higher in transitional FM compared to mature FM, although the difference was not significant (2.03 *vs*. 1.95 mmol/L) (Fig. 2.20).

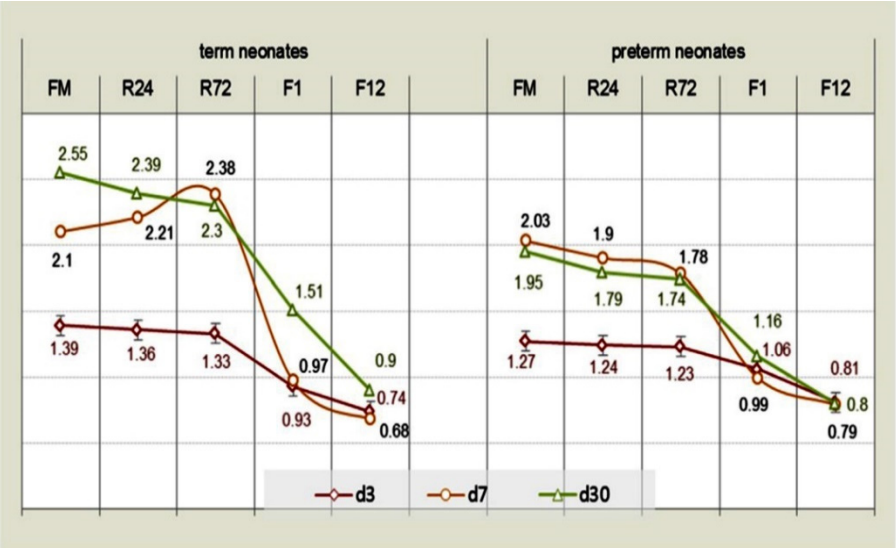


Fig. 2.20. TAS dynamics depending on storage method of different types of human milk (mmol/L). FM; R24, R72 refrigerated milk for 24, and 72 h, respectively; F1, F12 frozen milk, for 1 and 12 weeks, respectively; d3, d7, d30 days 3,7, 30 of lactation.

Term (group 2) FM had a higher TAS mean value than preterm (group 1) FM at each moment of lactation (1.39 *vs*.1.27, 2.1 *vs*. 2.03, 2.55 *vs*. 1.95), with a statistically significant difference only for mature FM (p Z 0.038978).

A similar pattern was maintained if the milk was refrigerated for 24 h (p Z 0.033047) or frozen for 1 week (p Z 0.5407). For R72 the differences were maintained, but they were not statistically significant (Table 2.XIX). The multivariate analysis showed that TAS was

influenced by mother's age and parity: mothers >25 years had a significantly higher TAS than those <25 years (b Z 0.517, p Z 0.013, OR Z 2.3) and multipara higher than primipara (b Z _0.226, p < 0.00001, OR Z 2.2) (Table 2.XX).

These findings needs to be confirmed by further studies in order to be better explained and understood. Neither social, nor financial status had any influence on TAS.

In preterm milk, the TAS increase stops after the first week at similar values with transitional term milk. In each phase of lactation refrigeration generated a slight decrease in TAS and there were no significant differences whether it was 24 or 72 h refrigeration time, excepting transitional term milk which showed a trend of preserving and even increasing the TAS content by refrigeration, although this finding was not significant. These data suggest that 24 h refrigeration is better than 72 h, but 72 h of refrigeration for human milk remains a reasonable time in order to avoid severe decreases in TAS concentration.

Table 2.XIX. Level of signifiance (*p*) in comparison of TAS values in human milk from term and preterm mothers.

Day of lactation	p[a] (fresh milk)	p[a] (R 24 h)	p[a] (R 72 h)	p[a] (F1)	p[a] (F12)
	TAS: preterm vs. term				
Day 3	0.079330	0.081682	0.180936	0.055115	0.201615
Day 7	0.860016	0.285988	0.078413	0.771669	0.095554
Day 30	0.038978[a]	0.033047[a]	0.056289	0.005407[a]	0.235300

TAS—total antioxidant status.
[a] p-value <0.05 was considered to be statistically significant.

Table 2.XX. Multivariate analysis concerning factors that influence TAS values.

TAS Model predictors	Sig. p-value[a]	Adjusted Odd Ratio	95%CI for adjusted OR	
			Lower	Upper
Mother's age (≥25 y)	.017	2.311	1.878	2.936
Marital status (Couple/married)	.967	1.023	.358	2.923
Financial level (Medium, good)	.462	.861	.577	1.284
Parity (Multipara)	.023	2.263	1.928	3.718

95% confidence intervals, OR- Adjusted odds ratio.
[a] p-value <0.05 was considered to be statistically significant.

Analyzing the frozen samples for 1 week (F1), the decrease of antioxidant capacity is constant, for every type of milk, in both groups, more dramatically in day 3 and day 7 term milk, and with similar values for both groups, term and preterm (0.93 and 0.97 mmol/L, *vs* 1.06 and 0.99 mmol/L, respectively).

These values are inferior to any value of TAS in fresh milk, even from D3. Compared with R24 and R72, F1 samples have significantly lower TAS content, suggesting that freezing, even for 1 week is worse than refrigeration for 3 days in terms of global antioxidant properties. After 3 months of freezing, almost all antioxidant status is limited to around 0.8 mmol/L (with an interval of 0.79-0.81 mmol/L for group 1 *vs* 0.68-0.9 mmol/L for group 2).

Proteins

In fresh milk, the protein level decreased constantly during lactation, with a significant difference between the two groups starting after 3 weeks of lactation. Preterm milk of day 21 and day 30 has significantly lower protein than term milk (1.27 versus 1.43 g/dL, P=0.015 and 1.13 versus 1.28 g/dL, P=0.001).

After day 60, protein continued to decrease, but with no significant difference between term and preterm samples (1.04 versus 1.10 g/dL, P=0.129). Refrigeration for 72 hours of term milk (group 1) decreased protein content less than freezing, although not significantly so. Preterm colostrum (day 3) and preterm mature milk from day 60 lose protein by refrigerating

more than 24 hours. Freezing more than 1 week affects protein from preterm milk significantly more than from term milk.

Carbohydrates

Carbohydrates in fresh milk increased constantly over the first 2 months of lactation. They were significantly higher in preterm than term milk, especially in day 21 (7.11 versus 6.82 g/dL, P=0.014) and day 30 (7.16 versus 6.91 g/dL, P=0.003) samples. The carbohydrate content of term milk did not drop significantly regardless of storage method. Only preterm milk from day 60 lost carbohydrates if refrigerated 72 hours or frozen for 2 months.

Lipids

In fresh milk from term mothers, the lipid content increased constantly during the first month, followed by a slight decrease over the next 30 days to a value similar to that on day 21 of lactation. For preterm milk, the lipid content significantly increased during the first 2 to 3 weeks, followed by a slight decrease at the end of the first month and an increase during the second month. Also, it has a constantly higher fat content than term milk; the maximum concentration was reached on day 60 for preterm and on day 30 for term samples. Mature fresh milk from day 30 shows significant differences in terms of lipid content between term and preterm (3.89 versus 3.36 g/dL, P=0.029).

2.4.4. Discussions

Oxidative stress is one of the major problems for ill neonates, especially because of the high rate of prematurity (over 14% in our population), so energy intake and antioxidant defense can be crucial for such neonates. They are frequently exposed to oxidative stress due to infection, oxygen therapy and mechanical ventilation, so oxygen is a required treatment but also a potential dangerous "drug".

Parenteral nutrition, blood transfusions, necrotizing enterocolitis, intraventricular/periventricular hemorrhage, and retinopathy of prematurity are all thought to be conse-quences of this imbalance between antioxidant capacity and oxidative stress (Zarban *et al*., 2009), added to an already demonstrated reduced antioxidant capacity. Also, for severely asphyxiated infants, either preterm or term, TAS is reduced, together with different fractions of radical scavenging activity in plasma.

Zarban *et al*.'s extensive study on milk samples (*colostrum*, transitional and mature milk) collected from 115 healthy women only with full-term neonates demonstrated that TAS was obviously higher in *colostrum* than transitional and mature milk. These data suggest that using *colostrum*, during the first days of life is vital, due to its high antioxidant potential.

Quiles *et al.* studied coenzyme Q10 as a marker of total antioxidant capacity and found higher concentrations for *colostrum* and transition milk in the full-term *vs* the preterm group, but values decreased through lactation in mothers delivering full-term infants.

Ezaki *et al*.'s study, on 56 cases of premature delivering Japanese mothers found that TAS tended to decrease in time when studying milk's composition up to 150 days, with TAS values homogenously distributed during the first month of lactation. Fidanza *et al*., 2002 reported a high, but not significant antioxidant capacity in *colostrum*, using ORAC (oxygen radical absorbent capacity) assay on only 30 samples (Ezaki *et al*., 2008).

Abuhandan *et al*., 2015, found that the oxidants and antioxidants in the milk of mothers of premature infants were significantly higher than those of full-term birth mothers. Although the study groups enrolled similar number of subjects, the prematurity focused on 30-32 weeks' gestation, and the method used differed in terms of technique (Erel method) and units, so absolute values cannot be compared with ours. Moreover, the samples were collected on day 5

and preserved for an unspecified interval of time at -80°C, so comparison with our data would be inappropriate.

In a recent study on 15 subjects in different stages of lactation, Mehta showed the identified enhancement of the antioxidant capacity of human milk by bioactive proteins that are lacking in commercial formula, and this study supports consideration of breast milk as the ideal nutrition for preterm-born neonates (Mehta, 2014, Petrova, 2014). Endogenous antioxidants in breast milk such as catalase, glutathione peroxydase, and superoxide dismutase were thought to increase with the passage of days after birth (L'Abbe, Friel, 2000). This explains our results, as TAS in fresh breast milk increased gradually during the first postnatal month.

Our data show that preterm milk has lower TAS levels than term milk at every moment of lactation we studied, but differences are not significant until 30 days. The discrepancy with other studies may be due to ethnic particularities, nutritional habits and tradition. As the antioxidants generally accumulate better during the last trimester of pregnancy as demonstrated by blood determinations, it is probable that a woman who has given birth prematurely would not synthetize a higher TAS concentrated milk as she would when delivering at term. In preterm milk TAS does not increase after 7 days, suggesting that premature infants benefit most from the antioxidant capacity of *colostrum* and transitional milk.

In terms of the storage method, our data were consistent with other studies in demonstrating that refrigeration for 3 days was better than freezing human milk at -20°C. In a study by Xavier *et al*. performed on 20 term and preterm delivering mothers, the highest TAS was found in *colostrum* and decreased over time, more through freezing (-8°C) than refrigeration, with no difference between term and preterm milk, which emphasizes the need of awareness and curtailment of the practice of storing and later use of human milk in medical practice and home care (Xavier *et al.*, 2011).

According to the Spanish Pediatric Association (Hernandez-Aguilar, 2013) the study of different ways to preserve the antioxidant capacity of breastmilk throughout lactation and of factors to improve mother and infant antioxidant status are important fields of research.

Although some early studies considered human milk composition as relatively homogenous (Hall, 1979), more recently, several studies revealed large variations in macro-nutrient composition within the same feeding, with duration of lactation, diurnally, between different populations (Jensen, 1993, Prentice, 1995, Wojcik. *et al*., 2009) and maternal factors like age, weight, parity, diet (Zachariassen *et al*., 2013, Lev *et al*., 2014), but also with expression method (Beker *et al*., 2015, pasteurization, storage container, freezing temperature, and thawing method. Macronutrient composition in particular differs between preterm and term milk, with preterm milk tending to be higher in protein and fat early in lactation (Ballard, Morrow, 2014).

We studied a period of refrigeration of 72 hours and found that macronutrient and energy levels were preserved with minimal changes. This finding agrees with the results of Slutzah *et al*., 2010 who studied milk composition over a 96 hour period and found little change in the composition of fresh milk refrigerated for 4 days at 4°C.

In practice, human term milk can be used after being refrigerated for 3 days without significant protein loss. Term *colostrum* may be kept frozen for 3 months, whereas milk from D14 to D21 for only 1 month; mature milk expressed after 30 days of lactation can only be kept for only 2 months.

Preterm milk of day 21 and day 30 has a significantly lower protein level than term milk. This finding is not consistent with other data (Bauer, Gerss, 2011) and may have been influenced by specific demographic aspects, ethnic differences, nutritional intake, or nutritional status. Our data showed a significant influence of the infant's gender on protein content in their mother's milk, as also shown by Powe (Powe *et al*., 2010).

Results on breast milk fatty acid composition are not consistent (Kovács *et al*., 2005, Aydin *et al*., 2014) found increased alpha-linolenic acid in milk of mothers who deliver prematurely while (Kumbhat *et al*., 1985) reported no significant difference in fat concentration between preterm and term milk.

Moreover, Gross *et al*. 1981 reported no variation in fat concentration with either duration of lactation or gestational age and Paul *et al*., 1997 observed a significant increase in lipid concentration with the progression of lactation but no significant difference between term and preterm milk. We found an increased content of lipids in fresh milk during the first month of lactation, providing greater energy intake for the infants during this period.

Population type, geographical area, and nutritional habits generate wide differences between studies. The method used for determinations, type of containers (plastic or glass), and thawing process contribute also.

Other measurable conditions like maternal BMI, age, financial status, and parity may also influence milk macronutrient composition. Sauer *et al*., 2017 observed high variability in macronutrient composition of expressed human milk, even when standardized samples that were not exclusively foremilk or hind milk were used.

The practical implications are real and of large interest in neonatal practice and home care and nursing of either term or preterm infants. We emphasize the large variability of human milk composition related to different population and storage methods. Access to milk banks is rare or even impossible for large number of centers, so our results will be of real help in deciding the best strategy.

Due to the fact that the number of participants to this study was relatively small, it was impossible to perform a multivariate analysis to assess the potential factors that could explain why the storage method has a different impact on the macronutrient content of milk from different time periods.

2.4.5. Conclusions

Breastfeeding remains an essential tool to help in the protection against free radicals, oxygen reactive species and oxidative stress. Fresh human milk has the highest antioxidant capacity. Protein content in fresh milk varies between term and preterm mothers in an inconsistent manner, influenced by multiple factors and conditions, mainly the mother's BMI, age, financial status, and the infant's gender.

As a final remark, when fresh milk is not available, preserving milk in the refrigerator for a short time up to 72h is a better option than freezing.

Human milk to have the most appropriate composition for the growth and development of an infant. A better understanding of human milk composition and the potential impact of storage and pasteurization on milk components is important for the nutritional management of fragile, highrisk infants.

CHAPTER 3

THE STUDY OF MATERNAL CONDITIONS WITH CONSEQUENCES ON THE NEWBORN

3.1. Introduction

Collaboration with obstetricians allowed the development of these studies aimed at establishing biochemical parameters other than those established in the assessment of prognosis, as well as monitoring of pregnant women with pre-eclampsia.

The disorders associated with placental ischemia are the major problems of pregnancy. Understanding the detailed pathophysiological features of implantation and vascular remodeling would have a major impact to predict and prevent these problems.

It is evident that although there is extensive knowledge about these important topics, large and vital knowledge gaps need to be filled. The mechanisms of normal and abnormal trophoblast invasion and how they relate to spiral artery vascular remodeling are important topics for resolution. The control of spiral artery remodeling that is likely due to more than normal trophoblast invasion must be resolved.

Despite the value of "lumping" the placental ischemic disorders, we must determine what differentiates IUGR, preterm delivery, recurrent pregnancy loss, and preeclampsia. Especially pertinent is the elucidation of the components responsible for maternal systemic pathophysiology being related largely to preeclampsia. Where possible examinations of markers and pathological changes should be done both before and during full disease and with rigid accurate diagnoses (Roberts, 2014).

Early studies have targeted the association of the level of an inflammatory marker of C-reactive protein with early onset in pre-eclampsia compared to specific enzymes or proteinuria. We established the protein correlations between blood pressure values, established biochemical and haematological gestatinal age and choleline protein dynamics and C-reactive protein dynamics in pre-eclampsia pregnant women, compared to pregnant women without other pathologies.

At the same time, a mathematical model was created to assess the values of two protein molecules the anti-angiogenic factors, such as sFlt-1(soluble fms-like tyrosine kinase-1 receptor), play an important role during the first part of the pregnancy, being related to the physiological vascular neoformation he is important during the second part of the pregnancy and to the physiological vascular remodeling.

Antiangiogenic factors, such as sFLT-1 (soluble tyrosine kinase receptor fms-like) and sEng (soluble endoglin) play animportant role in the first part of pregnancy. They are linked to physiological vascular neoformation, and, in the second part of the pregnancy, grant the endothelial functionality and physiological vascular remodeling. Soluble FLT-1 is a circulating anti-angiogenic protein that binds to the receptor of the PIGF and VEGF, thus preventing interaction with endothelial receptors, causing endothelial dysfunction. Endoglin is a surface co-receptor protein of TGF (transformig growth factor) β1 and β3.

The sEng factor is its soluble form, a novel anti-angiogenic factor that acts in synergy with sFLT-1. In normal pregnancy, a proangiogenic status appears, with low levels of sFLT-1 and increased levels of PIGF, by the end of the second trimester.

Towards the end of the pregnancy these levels return to normal. In pregnant women with toxaemia, angiogenic profile abnormalities appear, with early changes in the prevalence of anti-angiogenic status leading to endothelial dysfunction. Thus PIGF and VEGF levels are

lower than normal, and sFLT-1 and sEng levels are increased. sFLT-1 released from the placental circulation in large quantities will destroy the homeostasis of the maternal endothelium, resulting in hypertension, proteinuria and other systemic manifestations of preeclampsia. Also, the sFLT-1 factor is an antagonist of VEGF (Vascular Endothelial Growth Factor) on endothelial cells in kidney, brain and liver vessels. Some researchers have confirmed that the increased circulating level of the sFLT-1 factor predicts the onset of preeclampsia and is correlated with the severity of the disease.

The soluble Flt-1 is an anti-angiogenic circulating protein related to the receptor of the PlGF (placental growth factor) and VEGF fields (Vascular Endothelial Growth Factor), thus preventing the interaction with the endothelin receptors, causing endothelial dysfunctions. The role of these molecules was still unclear during our evaluations, with relative pitine data in the literature which is why we continued with the serum dosing of sFlt and PlGF and subsequently with the correlation of clinical data with the sFlt/PlGF ratio for patients with preeclampsia and eclampsia.

The data obtained by us were comparable to those in the literature, the ratio sFlt/PlGF being already included in the battery of tests used in the evaluation of the pregnant woman with preeclampsia. Because the sFlt-1/PlGF ratio represents an effective predictor of pre-eclampsia and of its severity. Thus, this angiogenic protein ratio may contribute, together with the well-known clinical and biological factors, to the stratification of the risk presented by hypertensive pregnant women; also, it could play an important role in what concerns the decision taken by the obstetrician related to the management of these pregnant women.

Decisions based on data supported by evidence will contribute to reduce the risk for the mother, as well as for the newborn baby.

Personal contributions

Published paper:

- Mihălceanu E, Nemescu D, Gavriluţ M, **Dimitriu DC**, Pangal A, Onofriescu M. *The correlation between markers of systemic inflammation and angiogenic markers in pre-eclampsia*. The Medical-Surgical Journal Society of Physicians and Naturalists Iași – România*,* 119(2), 2015: 473-483.
- Mihălceanu E, **Dimitriu DC**, Lazăr IT, Petre BA, Constantinescu G, Nemescu D, Scripcariu I. *The role of several angiogenesis peptides markers in the management of hypertensive pregnant women.* Revista de Chimie (Bucureşti-România), 69(6), 2018: 1509-1514. **Impact Factor – 1.605**.
- Vișan V, Scripcariu IS, Socolov D, Costescu A, Rusu D, Socolov R, Avasiloaiei A, Boiculese L, **Dimitriu C.** *Better prediction for FGR (fetal growth restriction) with the sFlt-1/PIGF ratio*, *Medicine*, 98(26), 2019: 16069. **Impact Factor – 2.028**.

3.2. Aim of the studies

The first study focused on the correlation between inflammatory parameters, represented by C-reactive protein (CRP) and blood pressure, on a group of pregnant women with pre-eclampsia (PE) and pregnancy induced hypertension (PIH) compared to a group of normal pregnant women.

Pre-eclampsia is a serious multisystemic syndrome, which represents one of the major causes of maternal, fetal and neonatal mortality and morbidity. Angiogenic factors contribute to preeclampsia molecular mechanisms, leading to hypertension and proteinuria. At the same time, it was intended to create a mathematic model to associate a predictor factor as PlGF, sFLT-1 or the PlGF/sFLT-1 ratio with the clinical parameters assessed in pregnant women with PE.

I have continued my research regarding the correlation between PE, hypertension and the modifications of the two angiogenic biomarkers, PlGF and sFLT-1, the results being published *in a second study*.

Considering the importance of careful maternal-fetal monitoring and therapeutic intervention at the right/optimal time in pre-eclampsia, to avoid fetal and maternal complications, this study evaluated the sensitivity and specificity of both usual and complex laboratory tests, which aims to evaluate some markers of angiogenesis, in the diagnosis and management of pre-eclamsia.

The aim of the study is to try to establish the levels of the sFLT-1 /PIGF ratio, as a prognostic tool in the patient with pre-eclampsia, depending on the influence of the cumulative risk factors. Moreover, in a third study, due to the fact that placental insufficiency that develops in pregnant women with pre-ecclamsia has consequences asupato the development of the fetus, the restriction of intrauterine growth being the most common situation.

We used the sFlt-1/PlGF ratio alongside the anthropometric data obtained from ultra-sound measurements to have checked the sFlt-1/PIGF ratio reduces the false positive rate of late fetal growth restriction (FGR) detection by ultrasound biometry.

3.3. Materials and methods

The first study was a retrospective, case-control study and focused on analyzing the clinical-progressive aspects of PE, by checking the correlation between the inflammatory parameters, represented by CRP and blood pressure, in a group of pregnant women (hospitalized between 2012-2014 – 138 pregnant women included in the study) with PE and PIH compared to a group of normal pregnant women.

The criteria for including them into the study group were: gestational age >20 weeks; high blood pressure and proteinuria, while the exclusion criteria were: gestational age <20 weeks; high blood pressure preexistent to pregnancy; spontaneous rupture of membranes; associated infectious pathology (chorioamnionitis, urinary infections, respiratory infections), seropositivity to HIV.

Laboratory tests were conducted on all pregnant women, the blood tests being focused on the parameters of the hepatic function (TGO, TGP, bilirubin, LDH) and renal function (urea, creatinine, uric acid), hematological parameters (thrombocytes, white globules), coagulation indexes (INR, Quick time, and APTT), of the inflammatory process (CRP, fibrinogen) and determining the serum concentrations of angiogenic markers (sFLT-1 and P1GF), by calculating their ratio (sFlt-1/P1GF) and establishing the appropriate statistical correlations.

Considering the clinical picture, meaning high blood preassure (HBP) associated to proteinuria and/or edemas, 3 study groups were created: PE group – 54 pregnant women (39.13%), aged between 19-41 years; PIH group – 34 pregnant women (24.63%) with high blood pressure values and/or insignificant proteinuria (<300 mg/24h) and/or edemas, aged between 22 and 44 years; NG group - 50 normal pregnant women (36.24%), aged between 16 and 41 years.

The second study. This research was applied on a study group of 138 pregnant women, between 2012 and 2018 in the "Cuza Vodă" Maternity Iași, who gave their free consent to enter the study, for which the PIGF and sFlt biomarkers were determined and the sFLT-1/PIGF ratio was calculated.

The patients were distributed in the following groups: the group represented by pregnant women diagnosed with hypertension (HTN) – including pregnant women who were diagnosed with HTN at hospital admission; this group included pregnant women suffering from medium and severe forms of preeclampsia (PE group), aged between 19 and 41 years old, and pregnant women diagnosed with non-complicated PIH (PIH group), aged between 22 and 44 years old.

The Control group – including a number of 50 normal pregnant women, aged between 16 and 41 years old. Baseline characteristics: The age of the pregnant women varied from 16 to 44 years old, registering a significantly higher average value (p=0.001) in case of pregnant women diagnosed with PIH (31.35 y *vs* 28.88 y in the group with patients diagnosed with PE and the gestational age at moment of diagnosis varied between 22 and 42 weeks, being significantly lower in case of patients suffering from PE (32.13 week) and PIH (33.74 week), compared to the Control Group (37.48 week) (p=0.001).

The blood pressure values varied in the PE group from 100/55 to 180/120 mmHg, the group average being the highest 153.44/92.5 mmHg, compared to the group of patients suffering from PIH (158.65/88.50 mmHg) or to normal pregnant women (120.40/72.56 mmHg) (p=0.001). Gestity and parity did not register significant discrepancies between the analyzed groups (p >0.05).

The third study was a prospective, case-control study, carried out between October 2017 and May 2018, (74 pregnant women, 37 patients and 37 control) in the same hospital as the other two studies, including pregnant women coming to the medical unit for their third trimester ultrasound (between 28 + 0 weeks and 34 + 6 weeks).

All patients underwent a third-trimester ultrasound scan with the estimation of fetal weight by measurement of bi-parietal diameter, head circumference, abdominal circumference, and femoral length, using Hadlock biometry curves.

Singleton pregnant women, with the estimated fetal weight at the 3rd-trimester US of <10% and as controls, pregnant women with estimated fetal weight between 10% and 90%, have been selected for the study.

The fetal growth restriction (FGR) definition was the one provided by American College of Obstetricians and Gynecologists (ACOG): "According to the guideline of the American College of Obstetricians and Gynecologists, a fetus with intrauterine growth restriction (IUGR) is a fetus with an estimated weight of less than the 10th percentile for gestational age." Exclusion criteria were multiple pregnancies and pregnancies that were not dated in the first trimester by CRL (crown rump length). Blood samples for sFlt-1/PIGF assessment were obtained from all the patients that were included in the study.

Some maternal characteristics were recorded: maternal age, racial origin, maternal weight and height with BMI calculation, method of conception (spontaneous, assisted conception requiring ovulation drugs, *in vitro* fertilization – IVF), cigarette smoking during pregnancy, patient's medical history (chronic HTN), diabetes mellitus, acquired or inherited thrombophilia, systemic lupus erythematosus or antiphospholipid syndrome.

Obstetrical history was also taken into consideration: gestation, parity, previous pregnancy with PE (yes/no), uterine apoplexy (UAP) (yes/no), previous children with FGR (yes/no) and data related with the first trimester: beta HCG (human chorionic gonadotropine) and pregnancy associated plasma protein A-1 (PAPP-A1) values (if available).

CapaUltrasound data were analyzed: first trimester dating of pregnancy by crown rump length (CRL); 2nd - and 3rd - trimester biometry (bi-parietal diameter – BPD, head circumference – HC, abdominal circumference – AC, femoral length – FL) using Hadlock curves, allowing the calculation of fetal weight and the weight percentile; Doppler parameters: uterine artery mean pulsatility index (PI) (UtAPI), and the presence of the notch, umbilical artery PI (UAPI), median cerebral artery PI (MCAPI); amniotic fluid index and placenta characteristics.

Pregnancy outcome was evaluated for gestational age at admission, gestational age at delivery, occurrence of maternal complications (severe PE, eclampsia, or hemolysis elevated liver enzymes low platelet count syndrome – HELLP, uterine apoplexy). The rate of cesarean deliveries (CD) was reported for fetal distress before/during labor compared to CD for other indications or vaginal delivery.

Neonatal outcome was analyzed according to neonatal weight and height, which allowed the calculation of Rohrer ponderal index and the classification in: small for gestational age (SGA) or appropriate for gestational age (AGA), using Lubchenco Growth Curves and Fenton Growth Charts for premature infants.

The following postnatal complications were also assessed: stillbirth; 5 minute and 10 minute Apgar scores below 7; cord blood pH<7, admission to the neonatal intensive care unit (NICU)/number of days for NICU hospitalization; respiratory distress (severe – requiring intubation and mechanical ventilation, medium – requiring noninvasive respiratory support – CPAP or mild – requiring supplemental oxygen administration; intraventricular hemorrhage; necrotizing enterocolitis (NEC); hypoglycemia; hyperbilirubinemia; patent ductus arteriosus or foramen ovalae.

The three studies included women hospitalized at the same medical unit: "Cuza Vodă" Clinical Hospital of Obstetrics and Gynecology of Iași. All the studies received the approval from the hospital's ethics commission. The processing of the biological evidence complies with the norms and legal procedures in force. The informed consents were signed by all the patients and control groups.

Laboratory evaluations:

The biochemical investigations used serum obtained after collecting the blood into "clot activator" type test tubes and spinning it at 4000 rpm, for 10 minutes. In determining the biochemical parameters we ocused on the parameters of the hepatic function (TGO, TGP, bili-rubin, LDH) and renal function (urea, creatinine, uric acid), it was used an automatic analyzer of Clinical chemistry, RX Imola model, along with calibrators and control serums.

For the hematological investigations total blood was used, collected on K3EDTA type anticoagulant using an automatic hematology analyzer (Celltac MEK – 6318K). For the coagulation indexes (Quick time, and APTT, INR) was used plasma collected on Na-citrate type anticoagulant using and analyzer HELENA-C.

The immunological investigations were made for the C-reactive protein using the chemiluminescence technique and a IMMULITE 2000 type automat. For the PlGF and sFlt-1 markers, it was used the chemiluminescence technique with the COBAS E411 ROCHE analyser. The detection limits for PlGF-1 were between 1-1.500 pg/ml, and for sFlt-1 were between 100-30.000 pg/ml.

Proteinuria was determined from the urine samples collected upon hospitalization, using the VITROS 950 dry chemistry automatic analyzer and diluting the samples accordingly.

The data were processed and assessed using statistic functions from SPSS 19 (first study), SPSS 17 (second study) and SPSS 18 (third study) at the significance threshold of 95% ($p<0.05$). The types of analyzed data required the different tests: F (ANOVA) test, χ^2 test (for both the first and the second studies), the Spearman correlation coefficient, the coefficient of determination (R2) and the sFlt-1/PlGF factor (for the first study); t-Student test, Mann-Whitney U test, chi-square test and in special cases, Fisher approximation (for the last study).

3.4. Results

HBP associated to proteinuria and/or edemas 3 study groups were created: PE group – 54 pregnant women (39.13%) with preeclampsia (PE), aged between 19 - 41 years; pregnancy induced hypertension group (PIH) – 34 pregnant women (24.63%) with high blood pressure values and/or insignificant proteinuria (<300 mg/24h) and/or edemas, aged between 22 and 44 years; normal pregnant women group (NG) – 50 pregnant women (36.24%) aged between 16 and 41 years.

Systolic blood pressure (SBP) was significantly higher in the case of pregnant women with PE ($p<0.001$), with variations from 120 to 180 mmHg, the group mean being of 153.44±18.95 mmHg. The diastolic blood pressure (DBP) was significantly higher in the

pregnant women from the group with PE ($p<0.001$), with variations from 70 to 150 mmHg, the group mean being of 92.50±14.83 mmHg.

In normal pregnant women, the DBP varied between 55 and 115 mmHg, the mean value of the group being of 72.56±11.09 mmHg mass index (BMI) indicated significant over weight (BMI=30.52 kg/m^2) in the patients from the PE group ($p=0.001$) and morbid obesity in 11.8% of the patients in PIH group compared to the witness group (NG).

The mean values obtained for the body mass index (BMI) indicated significant over weight (BMI=30.52 kg/m^2) in the patients from the PE group ($p=0.001$) and morbid obesity in 11.8% of the patients in PIH group compared to the witness group (NG). Depending of the BMI variation during pregnancy it was noticed that pregnant women with PE recorded body weight growth by more than 32%, while the pregnant women with PIH, the BMI growth was of almost 18%. Gestation and parity did not point out significant differences between the monitored groups ($p>0.05$).

The values of the biological markers indicated significantly higher differences for the PE group compared to the normal group (NG) for CRP ($p=0.001$), TGO ($p=0.001$), uric acid ($p=0.001$), serum urea ($p=0.005$) and proteinuria ($p=0.001$).

The C-reactive protein (CRP) was calculated in case of 56.3% of patients suffering from preeclampsia and of 73.5% of patients suffering from PIH; it has registered low values, but CRP average value was significantly higher ($p=0.002$) in case of patients suffering from PE (12 *vs* 6.72 mg/L). CRP varied from values under 6 up to 192 mg/l recording a mean value significantly higher in the group of pregnant women with pre-eclampsia compared to the group of pregnant women with PIH (35.89 *vs* 6.72 mg/l) ($p=0.001$).

The individual values of CRP in the group of pregnant women with PE varied from 6 to 192 mg/l, most pregnant women recording values in the reliability interval (RI 95%): 26.18-45.60 mg/l (42.6%), but 14.9% of the pregnant women with PE recorded values significantly higher of this parameter.Values for CRP depending by blood pressure and severity of pre-eclampsia (Table 3.XXI).

Table 3.XXI. The values of some statistical indicators depending of the severity of pre-eclampsia.

STUDY GROUP	N	MEAN	Std. deviation	Std. error	Reliability interval		Min.	Max.	p
					-95% Cl	+95% Cl			
Mean blood pressure (mmHg)									
Mild (moderate)	47	116	7.43	1.55	112.05	119.87	100	137	0.001
Severe	7	115.29	11.18	4.22	144.95	165.62	140	170	
Total	54	121.87	16.57	2.25	117.35	126.39	90	170	

Table 3.XXII. The values of some statistical indicators depending of the severity of pre-eclampsia (continued).

STUDY GROUP	N	MEAN	Std. deviation	Std. error	Reliability interval		Min.	Max.	p
					-95% Cl	+95% Cl			
C-reactive proteine (mg/L)									
Mild (moderate)	47	27.45	24.42	5	17.20	37.50	6	96	0.001
Severe	7	89.14	51.31	19.39	41.69	136.60	48	192	
Total	54	35.89	35.57	4.84	26.18	45.60	6	192	

PIGF

In the pre-eclampsia group, the individual values of PIGF recorded a variation in the range 16.37-942 pg/mL, with a significantly lower mean value (119.3 pg/mL) compared to the average value recorded in the control group (327, 57 pg/mL) or in the patients with high blood pressure- HBP - (129.13 pg/mL) (p=0.003) (Fig. 3.21).

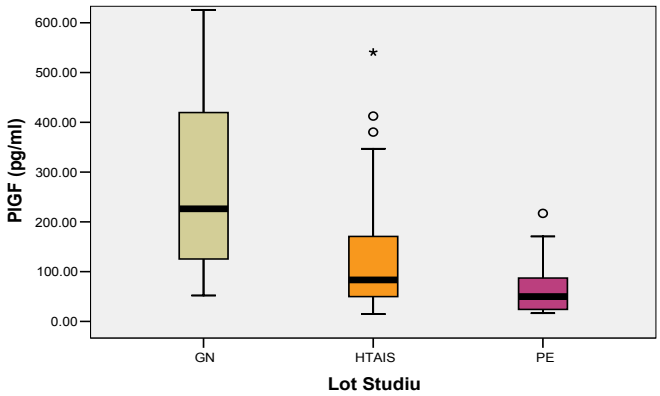


Fig. 3.21. Average PIGF values for study groups.

In the cases when the pregnant women developed PE, the mean value of PlGF was significantly lower (p=0.003) and sFlt-1 was significantly higher (p=0.001) compared to the NG group.

In the group of pregnant women with PE the individual values of PlGF recorded a variation between 16.37-942 pg/mL with a mean value (119.3 pg/mL) significantly lower if compared to the mean value recorded in the witness group (327.57 pg/mL) or the PIH group (129.13 pg/mL) (p=0.003).

Related to the age group, the mean PIGF values did not reveal statistically significant differences (p=0.704), although it is observed that patients under the age of 20 years have a median PIGF of approximately 200 pg/mL, and at age between 20 and 34 years the median exceeds the value of 100 pg/mL and at the age of over 35 years the median is within normal limits (Fig. 3.22).

The correlations between the individual PIGF values and the maximum recorded blood pressure values were indirect, moderate in intensity. Higher PIGF values were accompanied by low systolic (r=-0.359; R2=0.1771, p=0.001) or diastolic (r=-0.320; R2=0.1331, p=0.003).

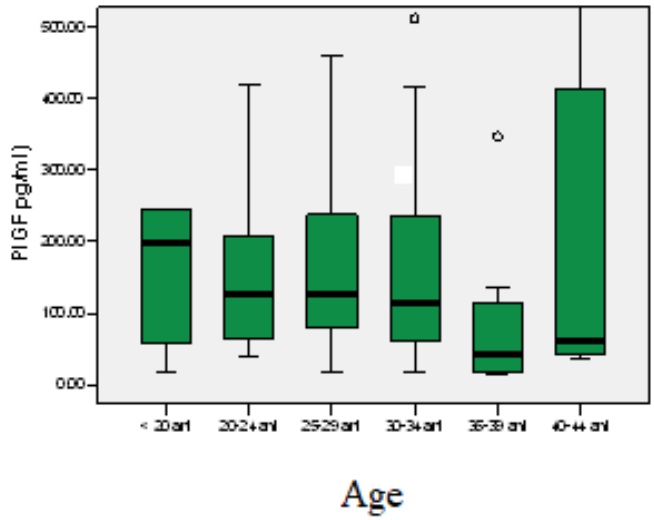


Fig. 3.22. Average PIGF values by age groups.

sFLT-1

sFLT-1 ranged from 1152 to 32708, with one variant over 92%, registering in the patients with a pre-eclamsie the highest mean value (14365±6464), significantly higher compared to the other study groups (p=0.001).

In pregnant women withpre-eclamsie, s-Flt ranged from 1804 to 27303 pg/mL, recording an average value of 14365 pg/ml. For pregnant women with HBP, the individual values recorded a wide variation in the range 1552-32708 pg/mL, with an average of the lot of 9892 pg/mL (Fig. 3.23).

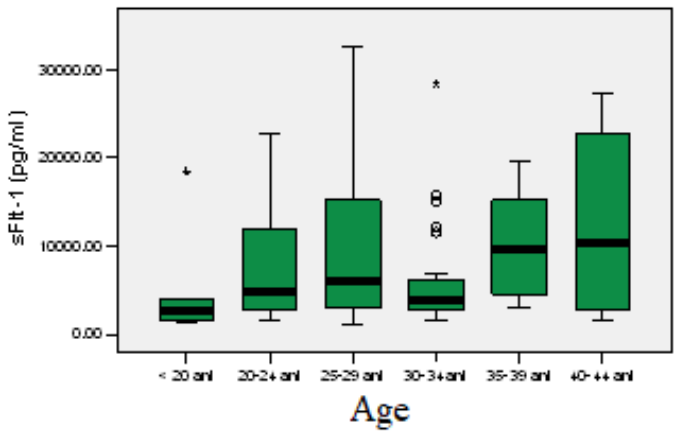


Fig. 3.23. Average sFlt-1 values by age groups.

The individual values of sFLT-1 recorded in normal pregnancies varied in the range152-6144 pg/mL, with an average value of 3278 pg/mL (Fig. 3.24).

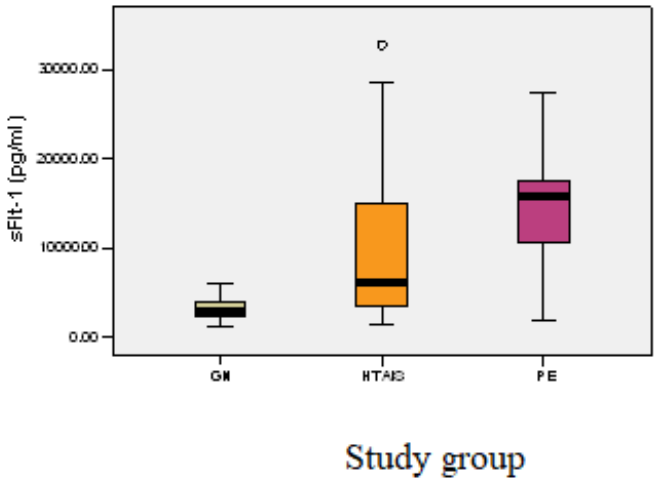


Fig. 3.24. Average values of sFLT-1 by study groups.

Depending on the gestational age, the mean value of sFLT-1 was significantly higher. In gestational weeks 25-28 (13867 pg/mL) ~ 29-32 (13033 pg/mL) (p=0.001) (Fig. 3.25).

The correlations between the individual values of sFLT-1 with the values of the maximum blood pressure recorded were direct, moderate in intensity, but statistically significant. Higher values of sFLT-1 were accompanied by increased values of systolic (r=+0.397; R2=0.3445, p=0.001) or diastolic (r=+0.430; R2=0.2755, p=0.001).

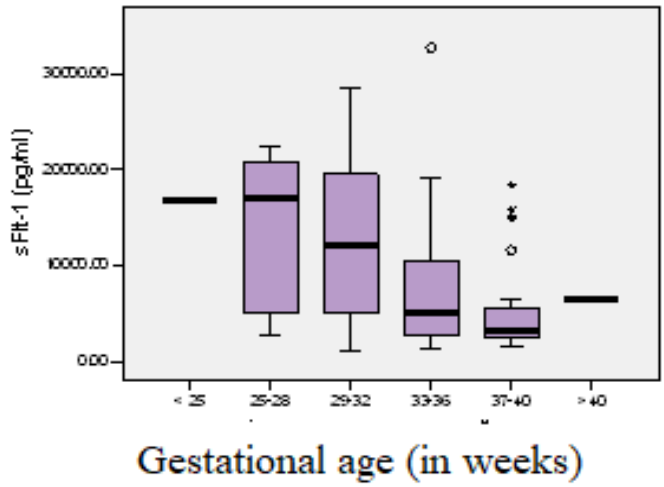


Fig. 3.25. Mean values of sFLT-1 in according to gestational age.

Ratio sFLT-1/PIGF

The values of the sFLT-1 /PIGF report showed the following variations (p=0.001):

- in the PE group, the range of values varies between 1.92-1124.56 and has the highest average value (417.62±350.73), especially at a gestational age of 37-40 weeks (about 600±200);
- in the group of patients with HTA, the ratio ranges from 2.87 to 1309.18, with an average to the group of 199.09±186.14;
- in the control group, the sFLT-1/PIGF ratio ranged from 0.88 to 110.64, being recorded the lowest mean value (23.70 ±17.02).

The sFlt-1factor was significantly correlated with age, the mean BP and the high levels of LDH and AST (r=0.784; p=0.046), being a better pre-eclampsia predictor than PlGF. The values obtained for the sFlt-1 factor varied from 1152 to 32708 pg/mL with a variation of more than 92%, recording in the PE group the highest mean value (14365±6464), significantly higher if compared to the other study groups (p=0.001). The sFlt-1/ PlGF ratio was significantly higher at the group of pregnant women with PE (p=0.001) compared to the NG (Fig. 3.26).

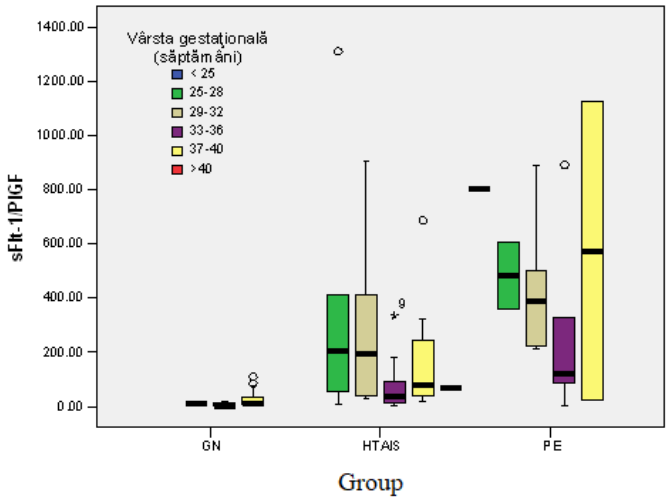


Fig. 3.26. Average sFLT-1/PIGF ratio in study groups between gestional ages on diagnosis.

In patients with pre-eclamie, there is a direct correlation, moderate in intensity, between systolic blood pressure (TAS) and sFLT-1/PIGF (r=+0.555; p=0.026), but the values of increased diastolic blood pressure (TAD) are associated with elevated sFLT-1 /PIGF levels in only 18.5% of patients (r=+0.185; p=0.493). In pregnant women in the HTAIS group, the correlations between TAS (r=+0.014; p=0.937) and TAD (r=+0.105; p=0.555) with the sFLT-1/PIGF ratio were not statistically significant.

In patients with pre-eclamsia AST is significantly correlated, with a direct correlation of moderate intensity with sFLT-1 (r=+0.535; p=0.033), but with PIGF (r =-0.070; p=0.796) or the sFLT-1 /PIGF ratio (r=-0.031; p=0.909) the indirect correlations were not statistically significant (Fig. 3.27).

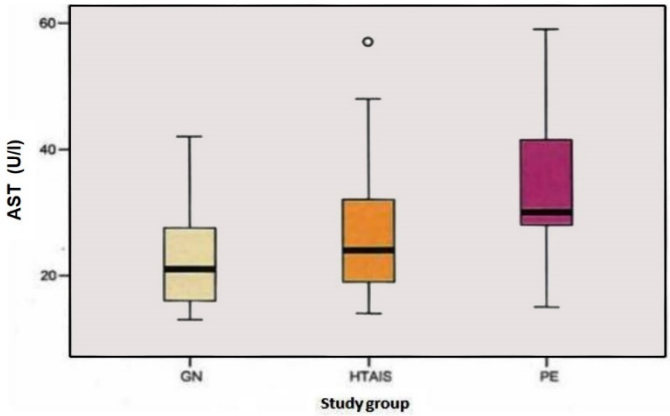


Fig. 3.27. Average values of AST on study groups.

In patients with pre-eclamsie, ALT is significantly correlated, with moderate intensity direct correlation with sFLT-1 (r=+0.532; p=0.034), but with P1GF (r=-0.066; p=0.808) or

the sFLT-1/PIGF ratio (r=-0.026; p=0.925) the indirect correlations were not statistically significant (Fig. 3.28).

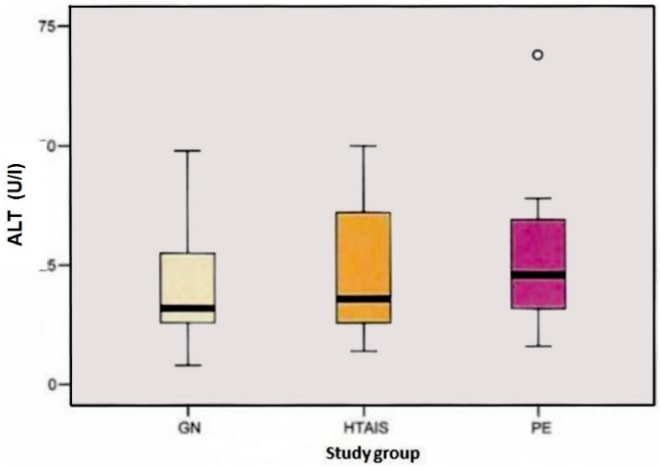


Fig. 3.28. Average values of ALT on study groups.

Increased urine protein levels correlate with increased sFLT-1 /PIGF ratio in 44.8% of PE patients (r=+0.448; p=0.049) and 32.5% of patients with HTA (r=+0.325; p<0.060).

Within this research, the following parameters were evaluated as markers for pre-eclampsia diagnosis: the systolic arterial pressure/the diastolic arterial pressure (SBP/DBP), the proteinuria, the creatinine, the urea and the uric acid at the serum level, the number of thrombocytes, the AST, ALT, LDH enzyme markers, as well as the sFlt-1 and PlGF angiogenic proteins.

The ROC curve (Fig. 3.29) has shown that proteinuria (AUC=0.849), AST (AUC=0.664), the increased SBP/DBP values (AUC=0.683/0.631), the serum creatinine (AUC=0.674) and the sFlt-1/PlGF ratio, at a cut off value=200, are effective preeclampsia predictors.

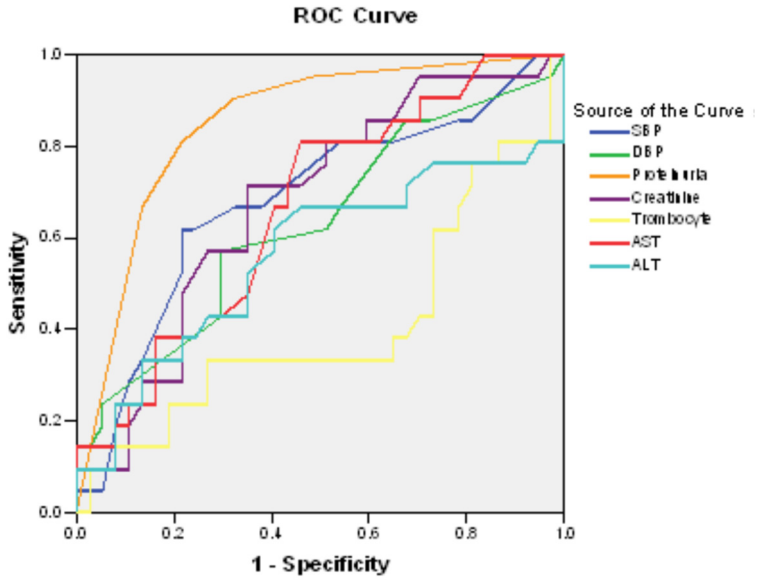


Fig. 3.29. The variation of markers as predictors of preeclampsia in case of pregnant women.

It should be noted that the sFLT-1/PIGF ratio is the best predictor of pre-eclamsia-with an accuracy of 72.3%, but sFLT-1 is a good predictor as the accuracy reaches a level of 61.6%, an aspect that it is not confirmed by the accuracy of PIGF (30.8%) (Fig. 3.30).

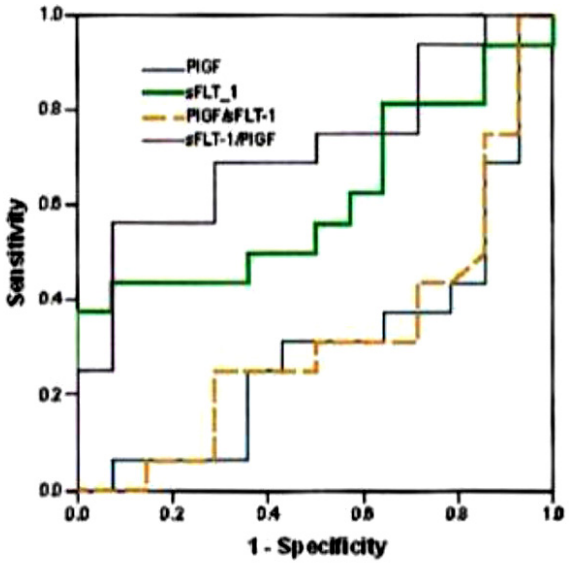


Fig. 3.30. ROC curve – optimal values for angiogenic analyzes.

For our study the population characteristics are presented in the Table 3.XXIII.

Table 3.XXIII. Characteristics of the study population of pregnant women from the FGR group vs control group.

Characteristics	FGR Group N = 37 [CI 95]	Control group N = 37 [CI 95]	Statistical test applied	P
Maternal characteristics				
Maternal age (years)	29 [26–34]	29 [24–33]	Mann Whitney test	.92
Maternal weight (kg)	75.23 ± 12.56	72.15 ± 12.55	Student *t* test	.312
Maternal height (cm)	162.34 ± 4.62	164.4 ± 5.56	Student *t* test	.09
Maternal BMI	28.50 ± 4.33	26.64 ± 4.11	Student *t* test	.072
Racial origin	37/37	37/37		–
Caucasian				
Smoking	6/37 (16.2%)	10/37 (27%)	Chi-square test	.20
Chronic hypertension	5/37 (13.5%)	1/37 (2.7%)	Fisher test	.19
Diabetes mellitus	1/37	0/37	Fisher test	.49
SLE or APS	1/37	0/37	Fisher test	.49
Obstetrical history				-
Mode of conception	2/37 IVF	1/37 IVF	Fisher	.61
- Spontaneous				
- Ovulation drugs = 0				
- In-vitro fertilization				
Gestation	1 [0–3]	1 [0–9]	Mann Whitney test	.54
Parity	0 [0–2]	1 [0–3]	Mann Whitney test	.045
Previous pregnancy-induced hypertension	4/37 (10.8%)	2/37 (5.4%)	Fisher	.43
Previous PE, E, HELLP, UAP	6/37	0/37	Fisher	.03
FGR at previous pregnancies	4/37	0/37	Fisher	.51

No significant differences were found between participants and controls for maternal age, maternal BMI, gestational age at the moment of recruitment, racial origin (all subjects being Caucasians), smoking and alcohol consumption (no alcohol users among participants or controls), personal history of chronic hypertension, diabetesmellitus, SLE or APS, and for the obstetrical history data mode of conception (spontaneous or in-vitro fertilization IVF), gestations, parity, and previous hypertension induced by pregnancy or preeclampsia and its complications, eclampsia, HELLP syndrome, and uterine apoplexy.

Data are presented as median (interquartile range), mean ± standard deviation, or n (%).

Comparisons between outcome groups were calculated using chi-square test or Fisher exact test for categorical variables and Mann–Whitney U test or Student t test for continuous

variables. P<0.05 was considered statistically significant. APS=antiphospholipid syndrome; GA=gestational age; SLE=systemic lupus erythematosus; PE=preeclampsia, E=eclampsia, HELLP=hemolysis, elevated liver enzymes, low plateletcount; UAP=uterine apoplexy.

Was estimation of biometrical age, weight percentile, pulsatility index (PI) of the uterine arteries and the presence/absence of the notch, PI for umbilical artery, cerebral artery and the cerebro-placental index value, amniotic fluid index (AFI); cardio tocogrphy (CTG) data including abnormal variability and the presence of decelerations; some first trimester biochemical marker values PAPPA1 (MoM) and beta HCG (MoM) (when available); as well as the angiogenic marker values (sFlt-1, PIGF) and the sFlt-1/PIGF ratio.

Average PIGF level among pregnant women with FGR was significantly lower than that of the control group (86.6 and 459.3, p=0.001). The average sFlt1 levels in pregnancies complicated by FGR was higher than among normal pregnancies (6394 and 2402, p=0.001). The sFlt-1/PIGF ratio among FGR *vs* normal pregnancies was (103.6 *vs* 5.20), significantly higher (p=0.001) (Table 3.XXIV).

Table 3.XXIV. Ultrasound (US) data at the moment of recruitment and CTG data and biochemical markers of the assessed pregnancies.

Characteristic	FGR group N=37	Control group N=37	Statistical test applied	*P*
First trimester PAPPA1 (MoM)	0.71±0.56	0.93±0.50	Student *t* test	.23
First trimester beta HCG (MoM)	1.66±2.04	1.28±0.75	Student *t* test	.48
GA at inclusion (weeks+days)	32.8[32.2–33.4]	32.9[32.6–33.3]	Mann Whitney test	.48
US weight percentile at the moment of inclusion in the study	6.4±3.36	46.7±21.58	Student *t* test	<.001
PI-UA >1of at least one UA	10/37 (27%)	5/37 (13,5%)	Fisher	.21
Notch presence at least to one UA	9/37 (24.3%)	1/37 (2.7%)	Fisher	.04
PI umbilical artery>1	22/37 (59.4%)	8/37 (21.6%)	Chi-square	<.001
Null or inverted end diastolic flow umbilical artery: Absent/ negative=pathologic	2/37 (5.4%)	0/37 (0)	Fisher	.494
PI cerebral artery PI< 1.5=pathologic	19/30 (63.3%)	10/24 (41.6%)	Chi-square	.113
Cerebro-Placental Index<1	10/37	0/37	Fisher	.28
Amniotic index	15/37 (40.5%)	5/37 (13.5%)	Fisher	<.001
Cardiotocography at the moment of fetal extraction				–
Abnormal variability	7/37	0/37	Fisher	.011
Presence of decelerations	4/37	0/37	Fisher	.11
PIGF (ng/mL)	86.6 [42.2–155.35]	459.3 [276.3–1387]	Mann Whitney test	<.001
sFlt-1 (ng/mL)	6394 [3703.5–10187.5]	2402 [1491–3098]	Mann Whitney test	<.001
sFlt-1/PIGF ratio	103.6 [29.2–194.2]	5.20 [1.54–9.34]	Mann Whitney test	<.001

The ROC curve (Fig. 3.31) was calculated splitting the enrolled cases into SGA (pathological condition) and AGA/LGA (normal condition), according to the neonatal evaluation (using the neonatal growth curves Lubcenco and Fenton for preterm newborns). We considered the sFlt-1/PIGF ratio as a variable.

We found a cut-off value of 36.065 with a sensitivity of 86.4% and a specificity of 80.8%, very close to the cutoff for preeclampsia found by Zeisler. Because pre-eclampsia is associated with high values of the sFlt1/PIGF ratio we recalculated the ROC curve after the exclusion of patients who developed PE and its complications (pre-eclampsia, HELLP syndrome, uterine apoplexy), not to bias the results.

The new ROC curve is presented in figure 3.32, after the exclusion of 5 participants, 4 with preeclampsia and one with uterine apoplexy. No participants with eclampsia or HELLP syndrome were enrolled in our study.

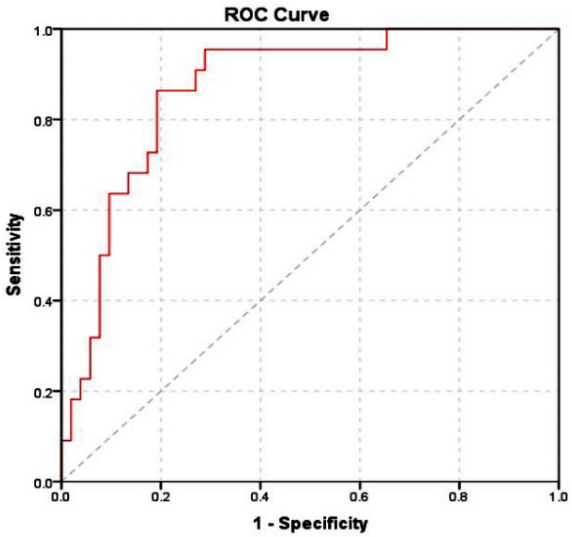


Fig. 3.31. Receiver-operating characteristics curve calculating the cutoff values for the sFLT-1/PIGF ratio in the detection of SGA including all casses and controls.

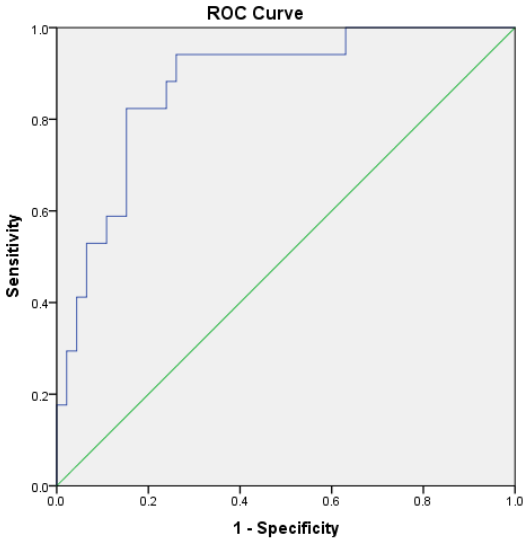


Fig. 3.32. Receiver-operating characteristics curve calculating the cutoff value for the sFLT-1/PIGF ratio in the detection of SGA when PE and its complications (pre-eclampsia, HELLP syndrome, AUP) (n=5) are excluded.

3.5. Discussions

Nowadays there isn't any single objective test to identify PE or to quantify the risk of certain severe complications, the diagnosis relying only on the clinical laboratory parameters evaluated in dynamics. Experience shows that early identification and the monitoring of the mother and fetus are useful and hence the prediction of PE becomes very important.

Early identification of PE, not only based on the inventory of risk factors, but also with the use of some markers might indicate better the moment suitable for beginning the treatment, its action and the moment to remove the child. Because of the fact that these modifications appear a long time before the apparition of the clinical signs of the disease, recent research focus on the endothelial dysfunction as a physiopathological mechanism of PE.

Many researchers (Dragan *et al*., 2017) noticed that the inflammatory maternal answer in PE and especially in severe PE is exaggerated (Crispi *et. al*., 2008), in this context, being considered that PE, at a given moment in its evolution, borrows a link of the systemic inflammatory answer that combines with the endothelial dysfunction.

The increase of CRP indicates the presence of an inflammatory, destructive, infectious or non-infectious process. In the *first study* it was demonstrated that the plasmatic level of CRP is significantly and directly correlated with systolic blood pressure (SBP) and diastolic blood pressure (DBP). Can used the mean arterial pressure as a severity indicator and he demonstrated the direct association with the inflammatory reaction.

This result joins the studies that support the affirmation that CRP is an effective marker for the apparition of PE and it correlates significantly with the severity of the disorder. Carl *et al*., 2008 demonstrated that a value of more than 3 mg/L is a good predictor for cardio-vascular and inflammatory risk in women with PE/eclampsia antecedents.

A prospective study, initiated by Behboudi *et al*., 2010 on a group of 778 pregnant women, established a reference value of 4.5 mg/dL for CRP in the first trimester of pregnancy as a predictive factor for PE, and Bita, 2010 with a study on 400 pregnant women established the threshold over which PE can be predicted, in the first trimester of pregnancy of more than 5 mg/L.

Other authors pointed out the relationship between CRP and the clinical and biochemical parameters from PE; the high levels of hemoglobin, creatinine, TGO, TGP, LDH, blood urea and proteinuria were associated with high levels of CRP. The high CRP values are a useful parameter in assessing the severity risk of PE in pregnant women with high body mass index in the third trimester of pregnancy (Stefanovic, 2009). In the first study it was noticed a risk of 6.71 times higher of severe PE in obese patients.

Considering the importance of carefully monitoring the mother and the fetus and the therapeutic intervention at the best moment in the process of PE, to avoid fontal and maternal complications, in the first study it was assessed the sensitivity and the specificity of some laboratory tests, including the usual ones, that focus on assessing various angiogenesis markers in PE diagnosis and management.

The evaluation of the ratio of circulating angiogenic protein – sFlt-1/PlGF may contribute to differentiate PE manifested by HTN or proteinuria from other conditions (systemic lupus erythematosus) (Gilbert *et al*., 2014). The higher the sFlt-1/PlGF ratio the better can it assess the risk of premature birth and it represents a potential prognosis parameter in PE monitoring.

In *the second study*, it was noticed that PlGF and sFlt-1 levels are simultaneously affected in PE; thus, the sFlt-1/PlGF ratio represents now a predictor element superior to the individual analysis of these markers, characterized by a high specificity level.

Pre-eclamsia, this systemic vascular disease, can affect many organs, causing severe complications for both the fetus and mother. Although the exact causes leading to the onset of pre-eclampsia are not known, however, it has been found that placental dysfunction plays an important role in its occurrence, correlating with subsequent maternal endothelial dysfunction (Matsuo *et colab*., 2007, Saftlas *et colab*., 2003).

Following examination of the placenta in pregnant women with severe pre-eclamsia, the presence of infarcts, fibrinous necrosis of the walls of the blood vessels, thrombosis and signs of chronic inflammation were frequently found (Nilsson *et colab*., 2004).

In pre-eclamsia, the cytotrophoblastic invasion is incomplete, the cytotrophoblastic cells being present only in the superficial layers of the uterine endometrium (Burke *et colab*., 2013). Therefore, vascular resistance will increase significantly, as the spiral arteries of the endometrium fail to be properly remodeled (Lyall *et colab*., 2013). Alternatively, pregnant

women with old vascular diseases may develop signs and symptoms of the pre-eclamsia, even if sFLT-1 values are low.

Proteinuria refers to the excretion of whole proteins. The renal modifications that may be noticed in case of PE are generated by a glomerular particular lesion, characterized by the endothelial proliferation of capillary vessels. These lesions, associated to the decrease of the renal plasma flow (RPF), because of vasoconstriction, lead to the decrease of the glomerular filtration (GFR) by about 25% compared to normal pregnancy. Thus, in case of PE, the values corresponding to urea and creatinine can be approximately equal or slightly higher compared to the valued registered in normal pregnancy. After giving birth, the glomerular changes tend to reach relatively fast the normal level, once HTN and proteinuria are stabilized.

The renin-angiotensin-aldosterone system is also affected in pre-eclamsia. Unlike normal pregnancies, whose vascular response decreases in the presence of vasoactive peptides, such as angiotensin II and epinephrine, in pregnant women who develop preeclampsia, the vascular response is much too strong (III) (Kleinrouweler *et colab*., 2012).

There are studies in which agonist antibodies have been identified on angiotensin II receptors. This type of antibody was subsequently injected into pregnant female mice, and these developed hypertension, proteinuria, and increased levels of sFLT-1 and sEng (Umapathy *et colab*., 2019).

Renal changes in pre-eclamsia are explained by a particular glomerular lesion characterized by endothelial proliferation of the capillaries. Thus, in pre-eclamsia, urea and creatinine may have approximately equal or slightly increased values compared to normal pregnancy, but the degree of proteinuria varies widely between minimal and nephrotic values (Verlohren *et colab*. 2010).

The hepatic damage in case of PE depends on the disease severity; in general, the values corresponding to transaminases and to the lactate dehydrogenase are moderately increased, except for the outbreak of the HELLP syndrome, when their values are significantly higher, (Abraham *et al*., 2003).

Also, pregnant women suffering from PE have a high risk of death caused by cardiovascular factors and an extremely high risk in case of those who experienced an early manifestation of the disease (Merih *et al*., 2012). Even if the acute manifestation of the disease is more dangerous for the mother, PE may also affect the fetus, leading to an increased risk of spontaneous preterm birth, fetal growth retardation, oligohydramnios, as well as to an increased risk of perinatal death (Lyall *et al*., 2013).

The use of ultrasonography (US) helps to evaluate the fetus condition, in particular growth retardation, as well as the extent to which fetal circulation is affected (Kawabata *et al*., 2006). Certain pregnant women are diagnosed with the socalled atypical PE (which does not involve HTN or proteinuria) and their condition worsens unexpectedly (Bersinger *et al*., 2002).

Regarding the angiogenic markers, the results obtained within the second study have indicated a significant increase of the serum concentration corresponding to the sFLT-1 anti-angiogenic factor of more than 4 times for the PE group and of more than three times for the HTN group, compared to the control group. The average values obtained for the PlGF parameter have shown a decrease of more than 2.5 times in case of the PE group, as well as in case of the HTN group, compared to the control group.

FGR is a condition responsible for many poor neonatal outcomes, requiring hospitalization in a neonatal intensive care unit (NICU). During pregnancy, FGR status is suspected initially by serial symphysis-fundal height measurements (Lindhard,1990). But the performance of this screening method is poor, with less than a 30% detection rate (Goto, 2013). Another promising approach is to add serum biomarkers when the suspicion of FGR is raised by third trimester scan biometry and maternal factors. The first biochemical markers analyzed were those performed for other indications, such as fetal aneuploidies.

Indeed, increased levels of transaminases are a clinical marker of severity. Liver rhinoscopic examination may reveal periportal hemorrhage, ischemic injury, and fibrin deposits (193). In this study, the determination of AST constituted a test with a sensitivity (Se) higher than the specificity (Sp) for AST values of 275 IUIL (Se=0.812; Sp=0.665).

Disorders in these balance factors can lead to aberrant placental vascular development. Their release into the maternal circulation contributes to the adaptation of the maternal cardiovascular system to pregnancy. Many issues in this field such as assay methodology and lack of data considering different placental cell types mean that the physiological roles of these factors in the maternal and placental circulations are making them a misnomer (Umapathy *et colab*., 2019).

As mentioned before, many studies confirmed the role of angiogenic fraction (sFlt-1/PIGF ratio) in the detection of PE, but fewer studies have analyzed the role of placental angiogenic factors in predicting other poor pregnancy outcomes due to placental insufficiency, for example, uterine apoplexy (Gaccioli *et al*., 2018), fetal growth restriction (Odibo *et al*., 2006), in utero fetal death, early pregnancy loss (Dugoff *et al*., 2005) and some cases of preterm delivery.

Also, an important number of studies suggested that adding angiogenic biomarkers to the routine third-trimester scan and maternal risk factors improves the detection rate of SGA. Thus, Bakalis *et al*. 2015, in a routine screening for delivery of SGA neonates by a combination of maternal characteristics, medical history (maternal factors) and EFW from ultrasound biometry performed at 30 to 34 weeks' gestation, found a 10% false-positive rate (FPR), 80%, 87%, and 92% of SGA neonates delivered <5 weeks following assessment with a birth weight <10th, <5th, and <3rd percentiles, respectively; the respective detection rates for SGA neonates delivering ≥5 weeks following assessment were 53%, 58%, and 61% (Muttukrishna *et al*., 2011).

The results obtained in this study indicated a significant increase in the serum concentration of the antiangiogenic factor sFLT-1 more than 4 times for the PE group and over 3 times for the HTA group compared to the control group (Hendrix *et colab*., 2020).

The average values obtained for the PIGF parameter showed a decrease of more than 2.5 times in both the PE group and the HTA group compared to the control group. On the other hand, the results obtained for the studied groups showed that the sFLT-1/PIGF ratio represents a good predictor of preeclampsia.

The third study confirmed the idea that by adding angiogenic biomarkers (sFLT1/PIGF ratio) to ultrasound biometry, we can increase the sensitivity of screening for late SGA. When we used ultrasound biometry alone for the estimation of fetal weight $<10^{th}$ percentile, the sensitivity was 44.4% with a specificity of 89% for a false-positive rate of 10%. When we combined the ultrasound EFW <10th percentile with the sFLT-1/PIGF ratio >38, the sensitivity became 84.21% with a specificity of 84.31% for an FPR of 10% (Herraiz *et al*., 2018).

The identification of IUGR or restriction as a separate entity was discussed in the mid-20th century, when the age at birth and the birth weight were overlapping concepts. The World Health Organization then recommended that a weight of less than 2,500 g at birth means prematurity.

The scrrening for IUGR represents a central interest point for obstetricians and neonatologists because this condition is associated to maternal and fetal mortality and morbidity. Even if there is a set of risk factors for IUGR, their prediction capacity is still small.

Some of the risk factors are: smoking, the mother's weight and low height, preexisting cardiovascular disorders. All these are generators of reactive oxygen species through the generated pathophysiological mechanisms.

The endoltelial dysfunction and the local inflammation are considered by certain central elements in the pathogenesis of placental ischemic disease. The presence of the

vascular cell adhesion molecules VCAM, the soluble intracellular adhesion molecule-1 (sICAM-1) and E-selectin, in this process is also known in the case of these pathologies. The increased levels in the circulating blood in pregnant women may indicate the presence of endothelial dysfunction, but there are still difficulties in the dosing process.

The prediction of placental ischemic disease is a challenge for any clinician in the context in which there is a special clinical polymorphism, whereas the etiopathogenic events are disparate in time and space. For the diagnosis of PE the markers have been standardized to measure the severity of the disease as well as to assess the placental imbalance, but for IUGR, the placental abruption and especially the early identification of these pathologies, many studies are still ongoing.

The practical utility of the angiogenic fraction: sFLT1/PIGF ratio, has already been demonstrated in preeclampsia screening and Zeisler *et al*., 2016, in the PROGNOSIS study, showed its predictive value for preeclampsia, finding a cut-off value of 38. There are many studies confirming the role of angiogenic fraction (sFlt-1/PIGF ratio) in the detection of pre-eclampsia (Benn *et al*., 1996, Caillon *et al*., 2018), some of them finding the same cutoff value of 38 as in our study (Gaccioli *et al*., 2018).

When adding an angiogenic biomarker at the previous screening, at a 10% false-positive rate, the prediction rate arises at 85%, 93% and 92% in the detection of SGA neonates delivering <5 weeks following assessment with birthweight $<10^{th}$, $<5^{th}$ and $<3^{rd}$ percentiles, respectively; the respective detection rates of combined screening for SGA neonates delivering ≥5 weeks following assessment were 57%, 64% and 71% (Signore *et al*., 2006).

Other studies that investigated the role of the sFlt1/PIGF ratio to identify the fetal growth restriction did not exclude the association of FGR with other pathologic outcomes connected to placental insufficiency, such as preeclampsia, eclampsia, HELLP syndrome, uterine apoplexy that can bias the results.

Our study demonstrated that the sFlt1/PIGF ratio is a useful biochemical marker in identifying FGR cases. More than that, our study calculated the cutoff value for this ratio of 36.05, very close to the cutoff of the same ratio when screening for PE (Komwilaisak, *et al*., 2017). This cutoff value does not change if we consider all cases of SGA, including those with associated preeclampsia and/or its complications to the ongoing pregnancy or if we consider only FGR cases without associated preeclampsia. We designed an evaluation algorithm in according to sFlt-1/PlGf ratio, for a cutoff of 38 (Fig. 3.33).

The third trimester screening for PE or SGA does not allow us to apply primary prevention because the administration of low-dose aspirin after 16 weeks does not prevent the subsequent development of PE or SGA (Wallner *et al*., 2007, Roberge *et al*., 2012). So, the objective of the screening for SGA in the third trimester (28-35 weeks) is to identify a high-risk group that needs closer monitoring, referral for a third-degree pregnancy, and a better evaluation of the proper time of delivery (Yu *et al*., 2003).

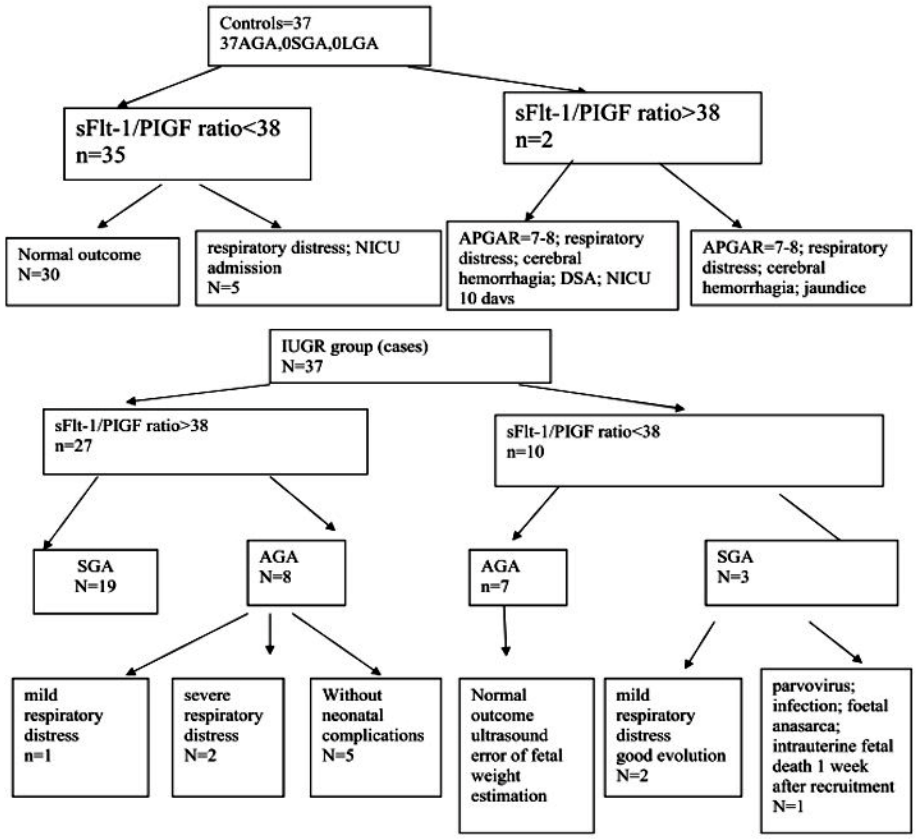


Fig. 3.33. Flow chart representing the outcome of pregnancies in participants enrolled, according to sFlt-1/PlGf ratio for a cutoff of 38.

3.6. Preliminary study to identify specific proteins as markers in placental dysfunction

Pregnancy disorders like hypoxia, pre-eclampsia, intrauterine growth restriction, or pre-term labor, which mostly contribute to profound morbidity and life-threatening complications for both the mother and the fetus, are characterized by placental malfunction.

To get deeper insights in disease development and to identify proteins that are potential targets for therapy, it is increasingly important to investigate the differences of proteins expressed during the physiological and the pathological development of the placenta.

The human placenta is a short-lived organ of embryonic origin that mediates the exchange of nutrients, hormones, growth factors, gases, immunoglobulins, and ions between the mother and the fetus. The maternal blood enters the placenta and encircles the microvillous membranes that are located on the apical side of a specialized cell monolayer. This multinuclear, syncytial monolayer, the syncytiotrophoblast (STB) comprises polarized endothelial cells, which form the barrier for the exchange between the mother and fetus.

As a basic technique after the isolation and separation of proteins from complex biological samples by electrophoretic methods, the exact identification of proteins is desired (Oveland *et al*., 2015). For this, the gel strips bands are first excised, followed by destaing enzymatic digestion and the resulted peptides are analyzed by mass spectrometry. The masses of m/z ions in the mass spectra are used to search for protein identity in databases such as UniProt or SwissProt (Smith *et al*., 2008).

Quantitative proteomics is an approach to obtain quantitative information about proteins in a specific sample and a control one. Compared to qualitative or semi-quantitative proteomics,

this approach can provide more insight into the effects of a specific stimulus, such as a change in the expression level of a protein and its posttranslational modifications, or to a panel of proposed biomarkers in a given disease state. Proteomics methodologies, along with a variety of bioinformatics approaches, are a major tool in quantitative proteomics (Chen *et al*., 2016).

The primary application of a biomarker panel is that it serves as a molecular indicator of the severity of a disease or its early response to treatment. In this way, biomarkers enable the application of precision medicine, an approach that tailors specific interventions to those individuals that would most benefit (Wiktorowicz *et al*., 2016).

3.6.1. Aim of studies

The aim of this study is to identify and characterize protein structures with specific modifications that could be benchmarks for anticipating possible identifiable changes, in an early stage of triggering changes that can lead to pathological situations with serious consequences. Proteomics, as a large-scale study of a set of proteins produced in an organism called a proteome, is the most current way to identify and characterize proteins.

In the study we took placenta fragments from pregnant women without any overt pathology and placentas from patients who were diagnosed with IUGR. The protocol for processing the placenta samples was elaborated and optimized in order to be able to store and prepare the protein solutions for further analysis by mass spectrometry.

3.6.2. Materials and methods

Triturate 1 g of the sample and immerse in 10 ml of PBS and homogenize for 15 min (5 min x 3 times) at 200 rpm (speed). The obtained solution was transferred to eppendorf tubes and subjected to centrifugation for 30 min at 14000 rpm and 4°C. Separate the supernatant from the processed samples and freeze at -20°.

Protein concentration was measured by using a NanoDrop UV/VIS spectrophotometer. For each analyzed sample, was used 45 µg of the total protein extract distributed on each mini well.

3.6.3. Results

Preliminary results show that for both normal placentas (samples 2 and 5) and the ones collected from patients previously diagnosed with IUGR (samples 1, 3, 4, 6 and 7), several protein bands with a large molecular mass scale between 15 kDa and 100 kDa) were observed, according to figure 3.34. The sensitive staining by Comassie Brillant Blue reveals that the protein samples contain proteins with different abundance and almost reproductibility between the extractions. The major protein band it seems to be albumin which should be depleted by next protocol we will performed for protein extraction. The lower protein bands seems to have different abundance while the proteins appeared by higher masses are more similar in all samples.

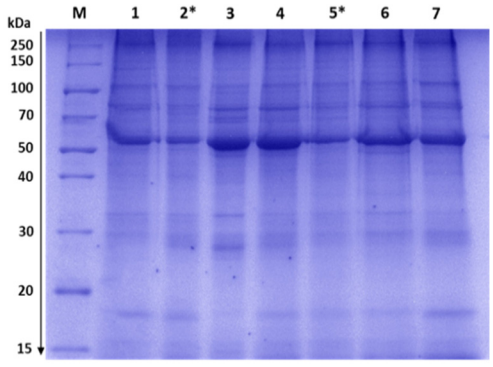


Fig. 3.34. The SDS-PAGE protein bands from normal placentas and abnormal placentas.

3.6.4. Discussions

As we see in the figure there are some bands (proteins) little highlighted at the limit of detection of staining with Coomassie Brilliant Blue G-250, which indicates a higher complexity of the extracted proteins than what was seen in this experiment.

There are some differences between the separate homogenates, namely the band of approx. 60 kDa is more obvious in pathological conditions than in samples from healthy pregnant women whereas the bands around the 30 kDa control vary and do not show constant intensities between samples. The use of albumin depletion protocols will help us in the next experiments to separate much better the low abundant placenta proteins, that are relevant for our main objective.

In the future, we will also consider the separation of these homogenates by the 2D-gel technique that allows the loading of the gels with 200-300 μg of homogenate and the separation will be made according to two dimensions, the isoelectric one and then the molecular mass. This complex separation method will provide an overview of protein isoforms that can be relevant to the IUGR pathology with respect of their posttranslational modification. Also, we will perform different extraction protocols for a better coverage of proteins contained in placental membrane. Both, top-down and bottom up approaches will be employed to identify and charactezise proteins as a markers for this pathology. Nano-LC-ESI-MS/MS, known as the best hybrid tehnology for direct proteomics studies will be used in collaboration with international recognized mass spectrometry laboratory from Germany and Russia.

3.6.5. Conclusions

PE is a serious multisystemic syndrome, which represents one of the major causes of maternal, fetal and neonatal mortality and morbidity. CRP is an inflammation marker but in PE it was not proved that it can be used in the clinical practice on a daily basis.

Angiogenic factors contribute to preeclampsia molecular mechanisms, leading to HTN and proteinuria. According to the specialized literature, molecular markers, such as the soluble fms-like tyrosine kinase-1 (sFlt-1) and the proangiogenic placental growth protein (PlGF), have significantly modified values, preceding by several weeks the emergence of pre-eclampsia signs and symptoms. In this respect, these markers can be successfully used in order to identify patients presenting a high risk of early preeclampsia manifestations (<34 weeks).

The sFlt-1/PlGF ratio represents an effective predictor of PE and may contribute, together with the well-known clinical and biological factors, to the stratification of the risk presented by hypertensive pregnant women; also, it could play an important role in what concerns the decision taken by the obstetrician related to the management of these pregnant women.

The higher sFLT-1/PIGF ratio can evaluate the risk of preterm birth. The sFLT-1/PIGF report is a potential prognostic parameter in monitoring preeclampsia. The results of our study confirm the importance of deterring these markers for the diagnosis and monitoring of hypertensive pregnancies and at the same time to emphasize that the sFLT-1/PIGF ratio is a good predictor of preeclampsia. Therefore, the obstetrician could have the possibility to promptly direct pregnant women from the increased risk group to the competent specialized health care facilities.

In the prediction of IUGR it is very important to combining Doppler indices with biochemical and clinical parameters due to the insufficient predictive value of each marker alone. The combined approach reflects various pathological pathways: Doppler ultrasound of the uterine arteries shows inadequate invasion of the spiral arteries and impaired secretory placental function is reflected by the disturbances of biomarker levels.

The protein bands highlighted in the gels presented above by the Coomassie Brilliant Blue G-250 staining technique will be analyzed in future studies by mass spectrometry methods for

their exact identification and characterization. The primary purpose of this future research is to identify possible biomarkers that may make the difference between normal/pathological, in the case of the present IUGR study.

Pre-eclampsia is associated with IUGR and models of prediction by combining ultrasound assessment of uterine arteries, biochemical markers and maternal history were published concerning the assessment of both conditions, but the need for the development of a model for prediction of small for gestational age without preeclampsia emerged.

Taking all these steps, the obstetrician will be more effective in what concerns PE treatment. Decisions based on data supported by evidence will contribute to reduce the risk for the mother, as well as for the newborn baby. The use of the sFlt1/PIGF ratio is also useful in other pathologies related to placental insufficiency, one of them being FGR.

CHAPTER 4

RELATIONSHIP BETWEEN BIOCHEMICAL AND MOLECULAR MARKERS FOR ASSESSMENT OF THE PATIENT WITH CARDIOMETABOLIC SYNDROME

4.1. Introduction

The research teams of which I was a part, before starting doctoral studies, had as main objective the study of cardiovascular pathologies and especially changes in the vascular endothelium. The experience gained helped me to carry out with my fellow preclinical and clinical studies and investigations on patients with cardiometabolic syndrome, making correlations between electrophysiological, biochemical parameters and clinical manifestations (Perk *et al*., 2012), a constellation of cardiovascular risk factors with a direct impact on the initiation and progression of atherosclerotic cardiovascular disease.

Several large studies showed that subjects with cardiometabolic syndrome (CMetS) are prone to adverse cardio-vascular events. Such a patient is vulnerable, prone to acute coronary syndrome, sudden death, or stroke, due to vulnerable atherosclerotic plaque (prone to disruption or thrombosis), vulnerable blood (prone to thrombosis) or vulnerable myocardium (prone to arrhythmia) (Ford, 2005, Byrne, 2006).

Due to the importance and major implications on the health of patients with cardiometabolic syndrome we investigated the presence of polymorphisms at the level of the LPL gene (lipoprotein lipase), the first conducted in Romania, study we conducted within the frame-work of a university-funded grant, entitled "Study of the prevalence of two poly-morphisms of the lipoprotein lipase gene in patients with cardiometabolic syndrome".

An essential role in the metabolism of plasma lipoproteins is performed by lipoprotein lipase (LPL). This enzyme is involved in the catabolism of chylomicrons and very low densitity lipoprotein (VLDL) by catalyzing the hydrolysis of triglycerides (TG) contained in these particles, thereby providing fatty acids to tissues as an energy source or for storage (Sagoo *et al*., 2008) LPL requires a specific cofactor, apolipoprotein C-II, as an essential activator (Wang, Eckel, 2009).

During LPL-mediated hydrolysis of TG-rich lipoproteins, surface lipids and apo-lipoproteins are transferred to (high density lipoprotein) HDL, so an additional role of LPL is to contribute to HDL particle formation (Goldberg, 1996). LPL is distributed in a wide variety of extrahepatic tissues, mainly in the adipose tissue, skeletal muscle, and myocardium.

The enzyme acts at the luminal surface of blood vessels, being anchored to vascular endothelium via heparan sulfate proteoglycans (Spence *et.al*., 2003, Mead, *et.al*., 2002). The enzymatic activity of LPL is regulated in a complex, tissue-specific manner in response to energy requirements and hormonal changes.

Personal contributions

Internal Project – Project Manager

"Study of the prevalence of the second polymorphisms of the lipoprotein lipase gene in patients with cardiometabolic syndrome" – Project UMF contract nr. 29232/2013.

Internal project – Member

"The role of quantifying the degree of subclinical atherosclerosis in patients with cardio-metabolic syndrome, establishing the risk class and therapeutic behavior" – Project UMF contract nr.28212/2011.

Published paper:

- Mitu F, Mitu O, **Dimitriu C**, Dimitriu G, Mitu M. *Significance of arterial stiffness and relationship with other noninvasive methods for subclinical atherosclerosis assessment in patients with metabolic syndrome*, The Medical-Surgical Journal Society of Physicians and Naturalists Iaşi - România, 117(1), 2013: 59-64.
- **Dimitriu DC**, Mircea C, Pricop C, Mitu O, Stănescu R, Hăncianu M, Petrescu-Dănilă E. *Small LDL: a helpful particle in monitoring patients with metabolic syndrome*. Farmacia, 64(2), 2016: 294-297. **Impact factor – 1.348**.
- Petrescu-Dănilă E, Pricop C, Mitu F, Leuștean L, Mitu O, Voicu PM, Bordeianu G, **Dimitriu DC**. *Study on the Prevalence of Asn291Ser Mutation in the Lipoprote in Lipase Gene in a Population with Cardiometabolic Syndrome from North East Romania*. Revista de Chimie, Bucharest, 67(3), 2016: 496-499. **Impact factor – 1.232**.

4.2. Aim of the studies

The first study based on the measurement of arterial stiffness as an accurate method of assessment of endothelial dysfunction, together with other noninvasive methods, in the diagnosis of atherosclerotic burden in patients with metabolic syndrome (MetS).

The aim of *the second study* was to evaluate the sLDL levels among the other lipoprotein subclasses, and correlate them with carotid intimamedia thickness, in order to assess the risk of coronary heart disease in patients with metabolic syndrome, while the aim of *the third study* was to assess the frequency of the Asn291Ser mutation, one of the common variations in the LPL gene, in a group of patients with cardiometabolic syndrome from North East Romania, and to make a correlation between the carrier status for this mutation and the plasma levels of triglycerides, HDL cholesterol and small dense LDL.

4.3. Materials and methods

The first study included 38 patients with MetS (18 men, 20 women) – group S, and 25 control patients (14 men, 11 women) – group C. Patients in group S met the MetS criteria according to National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) criteria: fasting glucose ≥110 mg/dl or known type 2 diabetes mellitus (type 2 DM); waist circumference (WC) ≥102 cm in men and ≥88 cm in women; serum triglycerides ≥150 mg/dl; HDL <40 mg/dl in men and <50 mg/dl in women; arterial pressure ≥135/80 mmHg or treatment for arterial hypertension (AHT).

Patients were included in group C when at least three MetS criteria were not present.

The exclusion criteria were: secondary AHT, significant chronic renal disease, major cardiovascular complications (myocardial infarction, stroke, heart failure, cardiac or peripheral revascularization).

The following investigations were made: medical history and clinical examination; electrocardiography; ankle-brachial index (ABI). Echocardiography studied markers of left ventricular hypertrophy (LVH), systolic and diastolic function, aortic and valvular atherosclerosis. Carotid ultrasound (CU) measured intima-media thickness (IMT) of the distal common carotid artery and bifurcation and revealed atherosclerotic plaques.

The biochemical tests included determinations of: fasting blood glucose, cholesterol (Col), HDL cholesterol, triglycerides (TG), calculation of LDL cholesterol with the formula

Col – HDL – TG/5, C reactive protein (CRP), fibrinogen (Fb), plasma urea and creatinine, hepatic enzymes – alanin-aminotransferase (ALAT), aspartataminotransferase (ASAT).

Regarding arterial stiffness, the following parameters were measured: brachial augmentation index (AIXbr), aortic augmen-tation index (AIXao), aortic pulse wave velocity (PWVao), central pulse pressure (PPao), and systolic blood pressure (SBPao).

The second study was conducted between April 2014 and March 2015 on 102 patients with MetS (72 men and 30 women), diagnosed according to NCEP ATP III criteria. The patients included in the study presented changes only for the lipid parameters values, the glycemia levels being in the normal range. The test panel included biochemical markers of lipid profile: TG, total cholesterol, HDL-cholesterol, LDL-cholesterol, sLDL.

Although all LDL particles are considered to have atherogenic potential, sLDL is associated with the highest cardiovascular risk (Packard *et al*., 2000). The carotid ultra-sound examination was performed by a sonographer, using an ESAOTE MyLab50 with 2.5/3.5 MHz probe. The quantification of the atherosclerotic process was made measuring the ejection fraction (LVEF), of the left ventricle mass (LVM) and aortic atheroma.

The third study evaluated 76 patients with cardiometabolic syndrome (55 males and 21 females) presenting abnormal values of the lipid parameters and the following three criteria: abdominal obesity, changes of at least one of the lipid parameters and changes of the arterial blood pressure.

The patients were selected in this way, based on the fact that in the scientific literature dyslipidemia in the cardiometabolic syndrome is considered to be one of the most important risk factors in the pathogenesis of atherosclerosis (Stancáková *et al*., 2006). For the selected patients we searched for the presence of Asn291Ser mutation in the lipoprotein lipase (LPL) gene.

The result of this LPL action is the increased retention and accumulation of lipoproteins in the arterial subendothelial matrix (Seo *et al*., 2000). Such trapped lipoproteins are more susceptible to atherogenic modification, and they are more rapidly taken up by macrophages, favoring foam cell formation (Medh *et al*., 1996, Clee *et al*., 2000).

The inclusion of patients in studies was achieved by diagnosis in according to National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) criteria:

- abdominal obesity characterized by waist circumference >102 cm for males and >88 cm for females;
- fasting glycemia >110 mg/dL or confirmed diabetes mellitus;
- serum triglycerides ≥ 150 mg/dL;
- HDL cholesterol <40 mL/dL in males and <50 mg/dL in females;
- arterial blood pressure ≥135/80 mmHg (Expert Panel on Detection).

To ascertain the diagnosis of cardiometabolic syndrome, three of the five criteria must be met.

The researchs has been conducted with the approval of the Ethics Committee of the University of Medicine and Pharmacy "Grigore T. Popa" – Iaşi and in accordance to the European Communities Council Directive 86/609/EEC, with the informed consent of the patients.

The determinations of biochemical parameters were made by using enzymatic dosing methods with RANDOX kits with calibration and control serums. All measurements were made on Imola/Daytona Plus automatic analyzer.

The values of the main lipid parameters were determined in serum samples, after an overnight fast. Triglyceride measurement was performed by a spectrophotometric assay using specific enzymes (Fossati and Prencipe method, 1982) coupled with the Trinder's reaction. Total cholesterol was measured using an enzymatic spectrophotometric assay.

For the determination of cholesterol fractions specific reagents were used and the following steps were followed. (a) HDL cholesterol: selective precipitation of chylomicrons, VLDL and LDL particles with phosphotungstic acid and $MgCl_2$, followed by centrifugation and measurement of HDL cholesterol in the supernatant. (b) LDL cholesterol: a direct enzymatic cholorimetric method was used.

This is based on the selective micellar solubilization of LDL cholesterol with a non-ionic detergent coupled to the use of a carbohydrate compound that interacts with VLDL and chylomicrons and prevents the reaction of cholesterol present in these particles with the reagents used in the assay (Martin *et al*., 2013).

This allows the selective measurement of LDL cholesterol in serum. This method meets the NCEP (National Cholesterol Education Program) requirements of having a total analytical error ≤12% (Martin *et al*., 2013). The serum concentration of small dense LDL particles (sLDL) was also determined, taking into account the atherogenic role of this lipoprotein fraction. A "two step" technique was performed using surfactant and specific enzymes.

The studied inflammation markers were C protein reactive (CRP) and serum fibrinogen (Fb). CRP was measured by immunoturbidimetric method on a Imola analyzer. The serum fibrinogen assay is the Clauss method.

The Asn291Ser mutation in the LPL gene was detected by mismatch polymerase chain reaction (PCR) followed by Rsa I restriction digestion, according to the method of (Zhang *et al*., 1995), adapted (regarding the PCR amplification program) in our laboratory. The following steps were performed:

a. DNA extraction (using a GeneJET purification kit and an automated Magnesia 16 magnetic bead extraction system; the OD280/OD260 ratio was an indicator of nucleic acid purity);
b. DNA amplification (Exon 6 of the LPL gene was amplified using a 5'-PCR primer located in intron 5 near the 5' boundary of exon 6-5'-GCCGAGATACAATCTTGGTG- 3' and a 3'-mismatch PCR primer located in exon 6 near the Asn291Ser mutation-5'-CTGCTTCTTTTGGCTCTGACTGTA-3'; PCR amplification reactions were performed with 5 μL of genomic DNA in PCR buffer containing 2 mmol/L MgCl2, 0.2 mmol/L of each dNTP, 1 μmol/L of each primer, and 0.6 U Taq DNA polymerase);
c. Restriction digestion of the PCR product (the PCR product – 10 μL was digested with 5 U Rsa I enzyme, from *Rhodopseudomonas sphaeroides*, 2 μL 10x buffer Tango, and 18 μL nuclease-free water at 37°C for 1 h; the digested fragments were separated on 2% agarose gels).

The carotid ultrasound examination was performed by a sonographer, using an ESAOTE MyLab50 with 2.5/3.5 Hz probe. The quantification of the atherosclerotic process was made measuring the ejection fraction (LVEF), of the left ventricle mass (LVM) and aortic atheroma. Statistical analyses were performed using SPSS/ v. 20, (t-Student test, Mann-Whitney U test).

4.4. Results

Analysis of *arterial stiffness parameters* showed that PWVao was 10.06 m/s in group S, and significantly lower (p=0.0001), 8.29 m/s, in group C. According to the reference values, in 64% of the controls it was optimal, in the remaining 36% being normal or elevated. In group S, in 50% it was normal, in 34.2% elevated and in 13.1% pathologic, above 12 m/s (with a maximum of 17.1 m/s) (Fig. 4.35).

The average values along with standard deviation for age, lipid profile (cholesterol, LDL, sLDL, HDL, TG, LDL/HDL ratio) and cardiac function parameters (left ventricular ejection fraction – LVEF and left ventricular mass LVM) by sex are shown in Table 4.XXV.

Table 4.XXV. Mean values of the studied parametres.

Parameter	Groups	
	Men (mean ± standard deviation)	**Women** (mean ± standard deviation)
Age (years)	48.056 ± 15.615	52.115 ± 11.582
Cholesterol (mg/dL)	248.842 ± 67.894	251.963 ± 54.665
HDL (mg/dL)	50.505 ± 7.759	45.367 ± 12.896
LDL (mg/dL)	165.789 ± 55.090	156.913 ± 51.197
sLDL (mg/dL)	61.558 ± 26.805	65.856 ± 28.612
Triglycerides (mg/dL)	344.210 ± 239.970	426.993 ± 254.041
LDL/HDL ratio	3.239 ± 0.861	3.581 ± 1.1929
LVEF (%)	65.500 ± 6.833	60.230 ± 10.497
LVM (g)	216.333 ± 57.109	271.760 ± 80.684

The evaluation of the lipid parameters was performed in relation to the reference ranges for the age groups that were included in the study (Fig. 4.35).

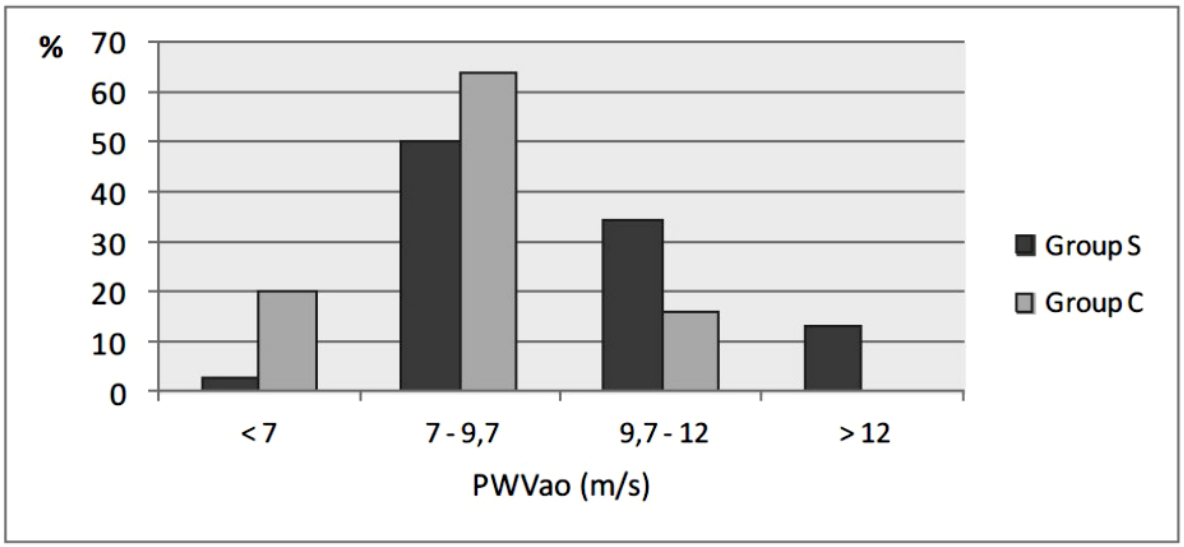


Fig. 4.35. The prevalence of PWVao according to value categories Echocardiography revealed aortic atherosclerotic alterations in almost all patients with MetS (97.4%), and in most controls (80%). Diastolic dysfunction, assessed by E/A ratio <0.9, was present in 68.4% group S and 52% group C patients.

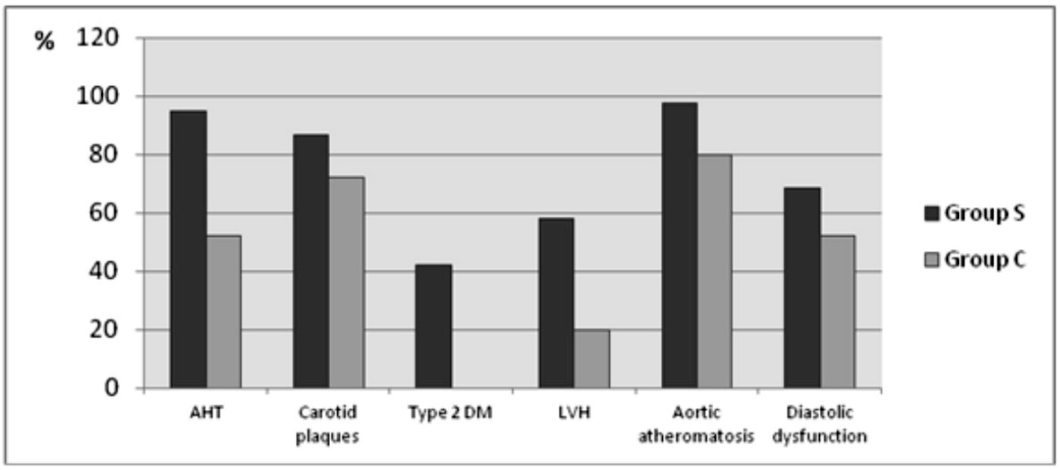


Fig. 4.36. Prevalence of different parameters in the two study groups.

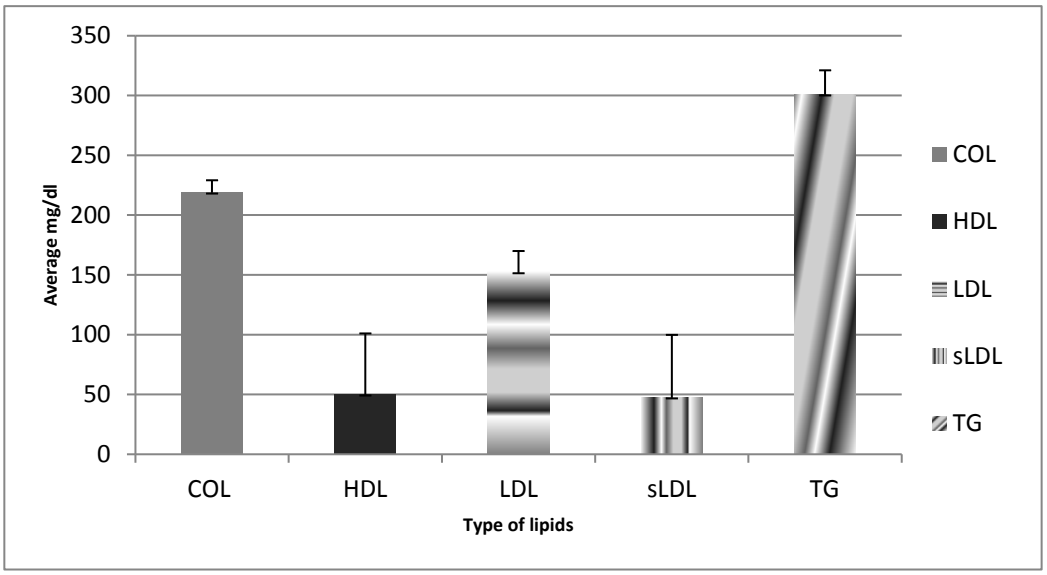


Fig. 4.37. The variation of the lipide profile according to average and standard deviation.

To detect this mutation we used a mismatch PCR primer as the 3'-PCR primer together with the normal 5'-PCR primer. DNA amplification of exon 6 with the two primers generates a 238-bp fragment.

Thus, in the PCR fragment from the mutant allele, a recognition site for the *Rsa* I restriction endonuclease will be created: 5'-GTAC-3' (G_{1127} from the Asn291Ser mutation and C_{1130} from the mismatch). As a consequence, this 238-bp fragment will be cleaved into a 215-bp fragment and a 23-bp fragment. The PCR product from the normal allele will have a 5′-ATAC-3′ sequence in this region and cannot be cleaved by the *Rsa* I enzyme, therefore remaining as a single 238-bp fragment (Fig. 4.38).

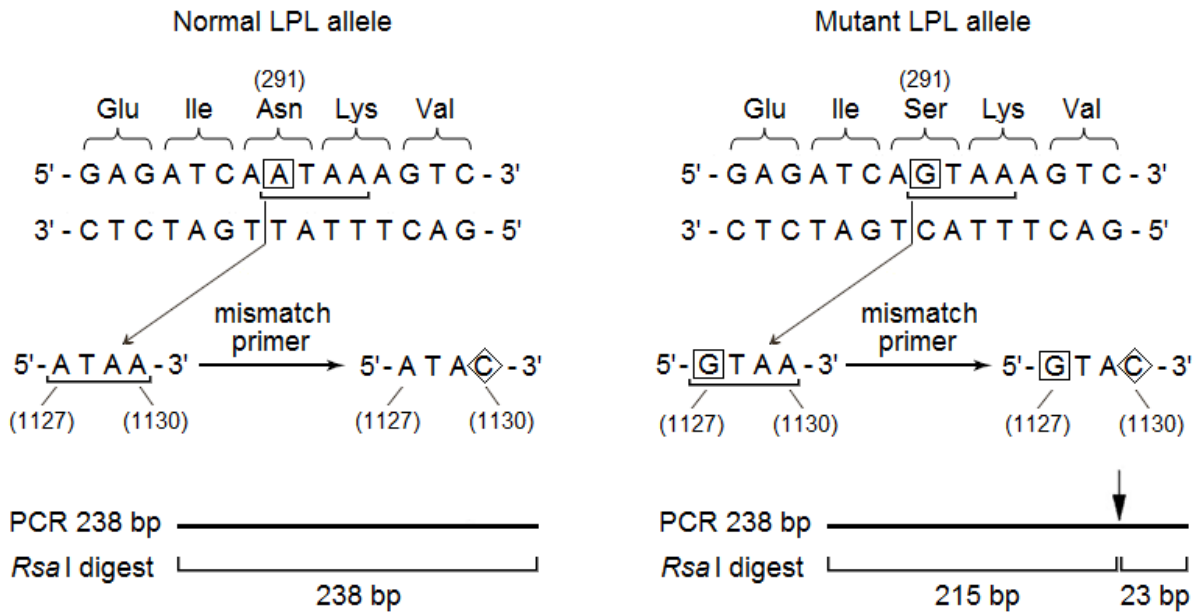


Fig. 4.38. The sequence of exon 6 of LPL gene containing the A to G substitution at nucleotide 1127, and the products of *Rsa* I digest of the PCR product of exon 6, for the normal and the mutant allele.

In our population sample we found two subjects that were heterozygous for the LPL Asn291Ser mutation (one female and one male – Fig. 4.39 and Fig. 4.40 respectively), corresponding to a carrier frequency of 2.63%.

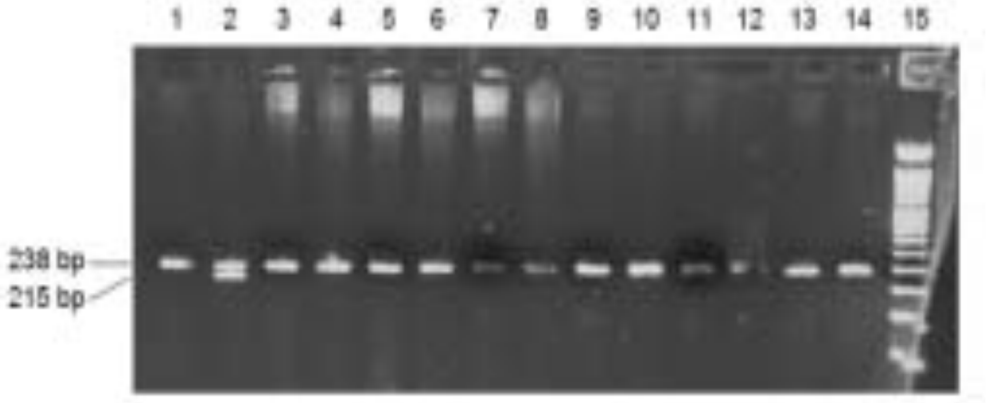


Fig. 4.39. Detection of the Asn291Ser mutation by PCR and Rsa I restriction digestion for the female patient. Lane 2: heterozygote; lanes 1 and 3-14: normal; lane 15: MW marker.

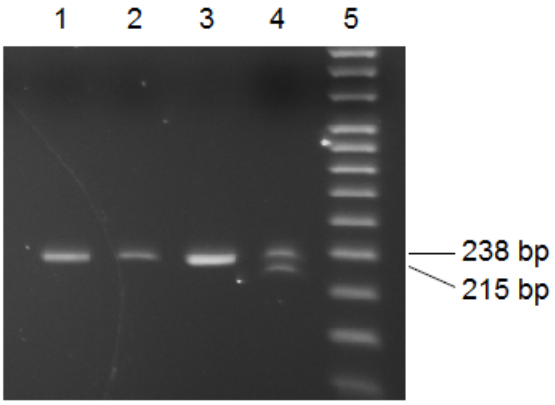


Fig. 4.40. Detection of the Asn291Ser mutation by PCR and *Rsa* I restriction digestion for the male patient. Lane 4: heterozygote; lanes 1-3: normal; lane 5: MW marker.

4.5. Discussions

In the first study, the analysis of the clinical and biochemical features related to the criteria used to define MetS showed, as expected, higher WC and BMI in the CMetS group. WC is a better predictor of cardiometabolic risk than BMI. Type 2 DM was present in about half of MetS group and this could be explained by insulin resistance (Simon *et al*., 2006).

The risk of developing DM is three times higher in CMetS compared to general population. AHT was present in almost all CMetS patients and doubled the cardiovascular risk (Naghavi *et al*., 2006, Janssen *et al*., 2004). LVH is more frequent in CMetS and is an independent cardiovascular risk factor. IMT is another marker of atherosclerosis, together with carotid plaques, and their presence has to be interpreted as atherosclerotic disease, even if asymptomatic and adequate measures must be taken (Vlachopoulos *et al*., 2010, O'Leary *et al*.,1999).

Mean serum cholesterol is higher in CMetS patients and increases cardio metabolic risk. In this study there were not significant differences in HDL and TG between the two groups; this can be partially explained by the relative small groups, but also by the fact that most patients were under hypolipemiant treatment (Mancia *et al*., 2008). The finding of higher CRP levels is in agreement with other studies that showed a correlation between the inflammation markers and MetS (Rutter *et al*., 2004).

Focusing on the results of arterial stiffness in MetS patients, it could be asserted that PWVao is the parameter that correlates best with the presence of MetS, followed by SBPao. The differences between groups could be explained in part by the higher prevalence of AHT in the MetS group. Arterial stiffness is known to be a marker of early endothelial dysfunction, which is influenced by several factors, not only by elevated arterial pressure levels (Eur Heart, 2010).

In the second study, the correlations between age and ventricular dysfunction, as well as between ventricular dysfunction and sLDL levels (Gentile *et al*., 2008, Gentile *et al*., 2013), suggest that elevated levels of sLDL contribute to the onset of cardiac disease (Hirayama *et al*., 2012).

Our data are consistent with the Hulthe, 2000, study which stated that patients with risk factors for metabolic syndrome had a higher concentration of sLDL and a higher value for intima-media thickness of the carotid and femoral artery. sLDL levels increase in parallel with visceral adiposity and TG levels (Srisawasdi *et al*., 2015), but independent of age and other lipid parameters. The presence of atherosclerotic plaques in 94.7% patients, including some with normal HDL-cholesterol or LDL-cholesterol levels, but with increased sLDL fraction, proves the importance of sLDL evaluation in order to identify subclinical atherosclerosis (Manjunath *et al*., 2013).

Among all measured lipid markers, the correlation of age with sLDL values in patients with metabolic syndrome, especially in young subjects, could be a useful predictive factor in monitoring patients with cardiovascular risk. The atherogenic potential of sLDL particles is due to their ability to penetrate the arterial wall (Nambi *et al*., 2010), low affinity for LDL receptor, increased plasma halflife and high susceptibility to glycation and oxidative stress (Younis *et al*., 2009).

More than 100 naturally occurring mutations in the LPL gene have been identified in humans, the majority being single nucleotide polymorphisms. Many of the mutations in LPL gene are associated with a decrease in LPL catalytic activity to various degrees, and they have been shown to play a major role in the development of dyslipidemia, thus leading to premature atherosclerosis and increased risk of CHD (Johansen *et al*., 2011).

Among the mutations reducing LPL activity, Asn291Ser is one of the most common, the frequency of heterozygous carriers being estimated as ranging between 2% and 5% in Caucasian populations (Merkel *et al*., 2003). Several studies have shown that the Asn291Ser variant in the LPL gene is a risk factor for dyslipidemia, characterized by hypertriglyceridemia and low HDL cholesterol levels (Ghibu *et al*., 2015).

In the third study, it was searched the presence of the LPL Asn291Ser mutation in 76 patients with cardiometabolic syndrome. To detect this mutation we used a mismatch PCR primer as the 3'-PCR primer together with the normal 5'-PCR primer. DNA amplification of exon with the two primers generates a 238-bp fragment (Wittrup *et al*., 1999).

The human LPL gene is located on chromosome 8p22, spans about 30 kb, and consists of 10 exons. It encodes a 475 amino acid polypeptide that yields a 448 amino acid mature protein after cleavage of a 27 amino acid signal peptide.

In view of its critical role in lipo-protein metabolism, LPL is a strong candidate gene for atherogenic lipid profiles and coronary heart disease (CHD) (Byrne, Wild, 2011). To detect this mutation we used a mismatch PCR primer as the 3'-PCR primer together with the normal 5'-PCR primer.

The Asn291Ser substitution in the LPL protein is caused by an A to G mutation located at nucleotide 1127 in exon 6 of the LPL gene. The use of a mismatch primer generates a C instead of the normal A (the mismatch) at nucleotide 1130 in the PCR fragments amplified from both the mutant and normal alleles.

Thus, in the PCR fragment from the mutant allele, a recognition site for the Rsa I restriction endonuclease will be created: 5'-GTAC-3' (G1127 from the Asn291Ser mutation and C1130 from the mismatch). As a consequence, this 238-bp fragment will be cleaved into a 215-bp fragment and a 23-bp fragment.

In this study were found two subjects that were heterozygous for the LPL Asn291Ser mutation, corresponding to a carrier frequency of 2.63%, which is lower compared to that reported by other studies. For exemple, the Asn291Ser mutation has been found with a carrier frequency of 5.2% in a group of 807 Dutch patients with CHD (Reymer *et al*., 1995), 4.5% in a group of 899 men from the United States with CHD and low HDL cholesterol levels (Brousseau *et al*., 2004) and 3.3% in a group of 721 Australian subjects with CHD (Van Bockxmeer *et al*., 2001).

These differences might be explained by the fact that our group was significantly smaller compared with the others. Moreover, it was found that the female carrier subject had a serum TG level increased by 9.8% and an HDL cholesterol level decreased by 5.41 mg/dL compared with the corresponding mean values of the female subgroup; these results are in accordance with those reported by several studies.

A meta-analysis performed by (Hu *et al*., 2006) on 21 studies published up to 2004 and including ~19000 subjects revealed that the Asn291Ser variant in the LPL gene was a risk factor for dyslipidemia, characterized by hypertriglyceridemia and low HDL cholesterol levels.

On the other hand, the male carrier subject of our study had a serum level of HDL cholesterol decreased by 9.12 mg/dL compared with the mean value of the male subgroup, but the TG level did not differ from that of the corresponding subgroup; these results are in accordance with those reported by (Ferencak *et al*., 2003).

LDL subfraction has been shown to coexist with elevated TG and low HDL cholesterol levels and to be associated with increased cardiovascular risk. The sLDL particles are more easily oxidized, have lower affinity for the LDL receptor, and are taken up more easily by arterial tissue, having a higher degree of retention in the arterial wall (Nikolic *et al*., 2013).

Both patients from our group that were carriers of the Asn291Ser mutation had sLDL levels higher than the mean value of the coresponding subgroup (by 15 mg/dL for the female carrier and by 10 mg/dL for the male carrier). Being associated with a decrease in LPL activity, the Asn291Ser mutation leads to deffective VLDL catabolism and an elevation of VLDL concentration.

Our results are in accordance with those reported by (Lopez-Ruiz *et al*., 2009), who found that patients with familial combined hyperlipidemia also carrying the Asn291Ser mutation showed a smaller average diameter of LDL particles and were at higher cardiovascular risk.

4.6. Conclusions

In the first study, it was highlighted that increased arterial stiffness is an early finding in patients with MetS compared with controls.

The most reliable parameter is PWVao, followed by SBPao; AIXbr and AIXao have a less predictive value. Other markers of early atherosclerosis in CMetS are carotid IMT and LVH. Of the biochemical investigations, CRP and cholesterol have better predictive value while LDL and TG have less predictive value.

The results of *the second study* showed that sLDL could be a valuable marker for the risk of coronary heart disease, better than the LDL levels, in patients with metabolic syndrome. The value of this marker is increased and statistically correlated with age and TG levels. sLDL can bring its contribution not only in the evaluation of atherosclerosis risks, but also in the therapy individualization and monitoring.

The third study proved that one of the most common LPL mutations, Asn291Ser, may be a factor in the development of atherogenic dyslipidemia associated with cardiometabolic syndrome, characterized by hypertriglyceridemia, low HDL cholesterol levels and increased sLDL levels.

However, as far as we know, our study is the first one that reports the frequency of the Asn291Ser mutation, a common variation in the LPL gene, in a group of subjects with cardiometabolic syndrome from North East Romania. We consider the initiation of such a research, in which we optimized the method for detecting this mutation, as being appropriate

We consider that the identification of such a genetic factor, that increases the cardiovascular risk, will help the patient, as well as his physician, to focus on decreasing or eliminating the modifiable risk factors (such as smoking or obesity), thus favoring therapeutic efficiency.

SECTION II

PERSPECTIVES – NEW RESEARCH DIRECTIONS

Future projects will be continued on three main directions

II.1. Study of specific proteins involved in pre-eclampsia – eclampsia and intrauterine growth restriction by proteomic and genomic analysis

II.1.1. Aim of the study

Proteomics is the large-scale study of a set of proteins produced in an organism called a proteome. The proteome is not constant; it differs from cell to cell and changes over time. To some extent, the proteome reflects the basic transcript. Thus, proteomics can provide significant biological information for many biological problems.

Proteomics is used to investigate protein expression, its stability, changes (*e.g.* post-translational changes (PTM), such as phosphorylation), the movement of proteins between subcellular compartments, and the involvement of proteins in metabolic pathways and how proteins interact with each other. Recently, several high-performance technologies have been developed for the thorough investigation of proteomes. The most commonly used are mass spectrometry (MS) techniques such as Tandem-MS and gel-based techniques such as differential in-gel electrophoresis (DIGE).

These technologies generate huge amounts of data so that databases are essential for recording and storing the results obtained. Links between the results obtained and existing knowledge can be subsequently made by researchers. The four major databases related to proteomic research (UniProtKB, IntAct, Reactome and PRIDE) allow fast search of data from the genetic sequence.

In many ways, proteomics runs in parallel with genomics. The starting point for genomics is a gene to make deductions about its products (*i.e.* proteins), while proteomics starts with the functionally modified protein and works back to the gene responsible for its production.

The proteome is not constant; differs from cell to cell and changes depending on age or lifestyle. To some extent, the proteome reflects the basic transcriptome, so proteomics can provide significant biological information for many medical problems. The term proteomics covers a variety of methodologies and procedures that are aimed at the identification of proteins and ideally their quantification in specific biological samples. Monitoring posttranslational protein modifications that occur in many proteins which may be crucial for regulation of protein function is also a component of a complete proteomics analysis.

The human placenta is a complex and vital organ that mediates the selective transfer of solutes and gasses between mother and fetus. Additionally, the placenta produces hormones and other factors that support pregnancy and provides a barrier to the maternal immune system. Proteomics analysis of the human placenta, whether normal or diseased, is at an early stage of development.

The new paradigm of the mother-fetus medicine refers to an inverted pyramid of the pre-natal consultancy and draws the attention to the risk assessment ever since the first pregnancy trimester, with the implementation of a prevention program for pregnant women at risk. The performance of tests and screening methods is in a continuous dynamic, as they are perfectible systems and have the role of increasing the performance of the screening. However, the need

to include new elements that can improve the diagnostic value is fully justified, especially since traditional prediction methods have difficulty in classifying risk groups and provide an optimistic prediction.

The main purpose of proteomics is to identify new markers for screening, diagnosis or therapeutic purposes for various diseases. The applicability of proteomics in obstetrics is high, at present the research is largely directed towards the identification of markers in the maternal blood that can be used in the prenatal screening. Moreover, the proteomic study on bioactive fragments of the type IV collagen, a well-known component at the level of placental extracellular matrix and not only, respectively the implementation of artificial intelligence algorithms, provides a dynamic research perspective.

In Romania, very often, the screening for pre-eclamsia or intrauterine growth restriction is performed only according to the maternal risk factors, without taking into account the markers from the sonographic exploration and the serum ones.

The determination of the serum markers along with other compounds that can be determined by current laboratory techniques, sometimes at a high cost, can help to establish correlations between predictors, which enables finding adaptive and efficient diagnosis algorithms in the early and realistic diagnosis of the complications caused by placental ischemia.

II.2. New approaches for diagnosing lysosomal storage diseases

II.2.1. Aim of the study

Lysosomal Storage Diseases are a group of >70 genetic and partially biochemical disorders. These disorders, apart from bringing down the quality of life of the proband, severely affect the mental and emotional status of the relatives who constantly manage these patients and live with them through it (Safary *et al*., 2018). An appalling number of such cases increase the genetic burden on the economy, eventually leading to its deterioration. A group of such genetic disorders due to malfunctioning of enzymes within the lysosomes are called as lysosomal storage defects (Ferreira, Gahl, 2017).

The proteins within the lysosomal compartment belong to various families such as hydrolases, ceramidases, acid phosphatases, sulfatases etc. which need an optimum temperature and an acidic environment to vitally functioning inside the cell (Mohammad, Samie, 2014).

Any malfunctioning in these vital players eventually results in an accumulation of undegraded substrates in various tissues of the body as inclusions and thereby manifests as lysosomal storage disorders. Usually a hydrolase enzyme, residual enzyme activity can range from 0% to ~30%. Enzyme deficiency leads to substrate accumulation and to cellular dysfunction and irreversible organ damage. Due to a low prevalence, knowledge about rare diseases is sparse both in society and among healthcare professionals (von der Lippe, 2017).

Besides the expensive and not accesible genetic tests, biochemical tests can be easy performed, for the evaluation of enzyme activity, using fluorimetric methods with the aid of dry blood spots (DBSs). Our research aim is to synthetise the new synthetic organic substrate with different alkyl group, in order to determine enzyme activity by using both florimetric and mass spectrometric (MS) in four rare diseases (Gaucher, Fabry, Pompe, Mucopolizaharidoza VI). The development of a new method for simultaneously diagnosis of several LSDs, by a new born multiplex screening can be successfully realized using MS.

The MS-multiplex approach presents several advantages: (i) the low quantity of blood sample (μL) collected from new borns; (ii) fast measurements and (iii) data accuracy. Also, a dynamic investigation of enzyme activity's stability during DBSs storage represents an essential study in diagnosing these LSDs (Fig. II.1.41). We believe it is crucial to develop new

strategies or to improve the existing methods to quickly conduct neonatal screening for lysosomal storage diseases.

The determinations for the enzyme deficiency in the case of Fabry, Pompe and MPS6 diseases were made as a result of a project carried out by "Al. I. Cuza" University of Iași, "Characterization by bio-affinity and mass spectrometry methods of pathophysiologically modified proteins, acronym ModProtical", project code PN-II-RU-TE_2014-4-0920, project director Associate Professor Alina Brandusa Petre with counseling and verification support for data validation laboratory at the University of Konstanz.

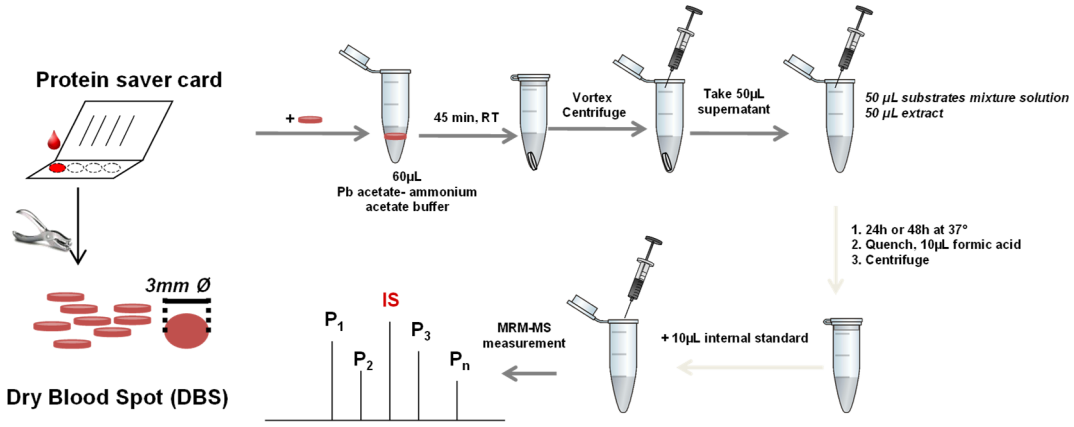


Fig. II.1.41. LSD Diagnostics using DBSs –multiplex assay.

Within this study direction, we performed a number of approximately 100 samples from newborns from "Cuza Vodă" Obstetrics and Gynecology Hospital Iași according to the work protocol established between the university and the hospital. The study was accepted and received the approval of the Ethics Commission within the UMF "Grigore T. Popa" UMF of Iași as well as the ethics commission of the hospital. A leaflet was sent to mothers with data and information on rare lysosomal diseases as well as the blood sampling technique for this type of test.

Some of the results were presented and communicated at national and international scientific events and as we acquire new and more data we will establish our own cut-off values (North-East region of the country) for the level of enzyme activities involved correlated with data in the literature and we hope to lay the foundations together with the fellow geneticists from a department of testing, evaluation and genetic counseling for families with children affected by such innate errors of metabolism.

Personal activity

Published paper:

- **Dimitriu DC**, Ion L, Gorduza EV, Augustin G, Petre B A. *Enzyme activity determination by fluorimetric methods in some lysosomal storage diseases*. Revista Română de Medicină de Laborator, Supliment la Vol. 26, Nr. 2, p. 103-104, 2018.
- Petre BA, Ion L, **Dimitriu C**, Maeser S, Kleinekofort W, Bulei C, Przybylski M. *Clinical Diagnostics of Lysosomal Storage Diseases in DBS Using New Substrates by MRM-MS* – prezentare poster 67th ASMS Conference On Mass Spectrometry, Atlanta, Georgia, USA, , 2019 June 2-6.

II.3. Molecular Biochemistry Studies – Analysis of the mutational profile of pancreatic cancer in Romania

These studies aim at «Analysis of the mutational profile of pancreatic cancer in Romania using the next generation sequencing of the entire exome».

II.3.1. Aim of the study

The use of the new generation sequencing technology of the entire exome to identify the mutational profile for a considerable group of patients. The current diagnosis and treatment of pancreatic cancer are not yet guided by changes at the molecular level in individual patients, and the detailed mechanisms responsible for the particular aggressiveness of this type of cancer are incompletely known. From a technical point of view, the obvious desmoplastic reaction further complicates the genomic analyzes as well as the molecular stratification of the patients.

The complex project aims at identifying the genomic changes responsible for the evolution and metastasis of ductal pancreatic adenocarcinoma in Romania. The aim of the project is to generate information that will help to improve the quality of life of Romanian patients by translating the scientific results obtained.

Unlike other cancers that have made significant progress in diagnosis and treatment (often targeted, guided by molecular analysis), the pancreatic cancer continues to have a uniform gloomy prognosis, with an average 5-year survival rate of 5%. Moreover, if at the EU level in the period 2008-2012 there was an increase in the incidence of cancer by 6.83% and mortality by 2.83%, in Romania in the same period there was an increase in the incidence by 12.03% and mortality by 4.21%, double comparing to the EU.

In Romania, according to Globocan (2012), pancreatic cancer is at alarming levels, in 2012 there were 3082 new cases, with 2782 deaths, placing deaths from pancreatic cancer on the 6^{th} position of total cancer deaths, with a tendency to become the second leading cause of cancer death by 2030 (Ying *et al*., 2016).

One goal of these translational studies is to customize developing therapies that target molecular pathways which lead to the progression of pancreatic cancer. Tissue and blood-based biomarkers developed from this project will be successfully validated in future clinical trials and will be developed in collaboration with industry partners (eg. Merck and Targos Gmbh).

Another objective of the project is to integrate the Romanian groups, components of the consortium in European networks with competence in translational studies in the field of cancer (EATRIS, ECRIN).

II.4. The didactic activity in addition to the scientific one

The research directions that I have approached as well as the ones that will be my future preoccupations are in close interdependence with my ambition to train the students from the Faculty of Medicine and also the residents from the specialty of Laboratory Medicine. I am preoccupied every year to update the clinical biochemistry courses with the latest data on the biochemical explorations in various pathologies.

I collaborate with colleagues from the other Universities of Medicine in our country within the events organized annually by the Romanian Association of Laboratory Medicine, and we try to harmonize the content of the courses and teaching materials with the topic of clinical biochemistry addressed to residents in the specialties of Laboratory Medicine and Medical Microbiology.

I am part of the author team for the 4^{th} and 5^{th} editions of the treatise on Medical Biochemistry - Clinical Implications under the guidance of Professor Minodora Dobreanu PhD. It is a reference book for specialists in medical laboratories, but also a very useful tool

for doctors in other specialties who will understand the mechanisms and biochemical changes in certain pathologies.

The continuing medical training is not only a desideratum, but also a constant preoccupation of mine as I coordinate every year a course of Continuing Medical Education with interdisciplinary themes where my guests are professors at other paraclinical and clinical subjects that are known as specialists in Romania and abroad.

My mission as a professor and laboratory specialist is to guide young graduates to discover the magic of biochemical processes in all their complexity, to guide them to research directions by coordinating their bachelor's degree papers or within the guidance commissions of their doctoral theses.

I want to reveal to them that the patient is not in front of them only by the given laboratory result, but they contribute to his diagnosis, therapy and healing, whereas laboratory medicine is a branch of interdisciplinarity, teamwork and of the future.

CONCLUSION

Biochemistry is probably the only basic science that has a gamut of ramifications. The major changes produced in the last decades at the level of fundamental research but also of clinical research offer, in my opinion, to biochemistry a central place as a binder and bridge between the two, being a key piece in the puzzle of interdisciplinarity. The increase in laboratory tests, detection techniques and methods, but also in the complex healthcare environment have made clinical information to be integrated with laboratory data.

Research must have a major impact at the level of the clinical laboratory, requiring the integration of clinicians' preferences with valid and up-to-date clinical research evidence. Advances in science and technology will increase the complexity of the medical system, education and research along with medical services.

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