GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

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GRIGORE T. POPA UNIVERSITY OF MEDICINE AND PHARMACY IASI

From basic care to emergency disease and future drama in neonates - an integrative approach

- HABILITATION THESIS -

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ABBREVIATIONS

3xTg-AD mice	triple transgenic mouse model of Alzheimer's disease
AD	Alzheimer's disease
AHI	apnea–hypopnea index
ALAT	alanine aminotransferase
ANC	neutrophil count
APPswe/PS1dE9 mice	APP/PS1 double transgenic mouse model of Alzheimer's disease over expressing amyloid precursor protein (APPswe), encoding the Swedish mutations at amino acids 595/596 and an exon-9-deleted human PS1 (PS1dE9)
APPV717I mice	transgenic mice containing human APP (isoform 695) with the London mutation as model for Alzheimer's disease and cerebral amyloid angiopathy
ASAT	aspartate aminotransferase
Aβ	amyloid-β protein
BBB	blood brain barrier
BiPAP	Bi-level positive airway pressure
BMI	body mass index
BP	blood pressure
CCHD	cyanotic congenital heart disease
CD	cognitive development
CMV	conventional mandatory ventilation
CNS	central nervous system
CONS	coagulase-negative staphylococci
CPAP	continuous positive airway pressure
CRP	C–reactive protein
CSF	cerebro-spinal fluid
CTG	cardiotocography
DHA	docosahexaenoic acid
DIC	disseminated intravascular coagulation
DISE	drug-induced sleep endoscopy
ECC	early childhood caries
ECLS	extracorporeal life support
EL	expressive language
ELBW	extremely low birth weight
EOS	early onset sepsis
ESM-1	endothelial cell specific molecule-1
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
FHR	fetal heart rate
FiO2	inspirated fraction of oxygen
FIZZ3	adipocyte-specific secretory factor
FM	fresh milk
fMLP	formyl-methionyl-leucyl-phenylalanine
GA	gestational age
GBS	group B streptococcus
GSE	grape seed extract

HFNC	high flow nasal canula
HIE	hypoxic-ischemic encephalopathy
HM	human milk
HSS	hematologic screening score
IDE	insulin-degrading enzyme
IFN	interferon-gamma
ILBW	incredibly low birth weight
IUGR	intrauterine growth restriction
IVH	intraventricular hemorrhage
LCPUFA	long-chain polyunsaturated fatty acids
LDH	lactat dehidrogenase
LOS	late onset sepsis
LP	lumbar punction
LPC	lowest previous systemic creatinine
MAP	median arterial pressure
MPV	mean platelet volume
NEC	necrotizing enterocolitis
NICU	neonatal intensive care unit
NIPPV	nasal intermittent positive pressure ventilation
NIRS	near infrared regional spectroscopy
NIV	non-invasive ventilation
NPO	nil per os
NS	neonatal sepsis
nSOFA	neonatal-specific sequential organ failure assessment score
OI	oxygenation index
OSA	obstructive sleep apnea
PaCO2	carbon dioxide arterial pressure
PaO2	oxygen arterial pressure
PCR	polymerase chain reaction
PCT	Procalcitonin
PDA	patent ductus arteriosus
PLT	Platelets
PMN	Polymophonucleares
PoCUS	point-of-care ultrasound
PPHN	persistent pulmonary hypertension of the newborn
PPV	positive pressure ventilation
PSG	Polysomnography
RBC	red-cell blood count
REM	rapid eye movement
RL	receptive language
ROS	reactive oxygen species
SAA	serum amyloid A
SAMP8 mice	a mouse model of sporadic AD
SaO_2	oxygen saturation
SD	standard deviation
SDB	sleep-disordered breathing
SGLT-2	sodium-glucose cotransporter 2 inhibitors
sICAM-1	soluble intercellular adhesion molecule 1
SIMV	synchronized intermittent mechanical ventilation
SIRS	systemic inflammatory response syndrome

SMA	spinal muscular atrophy
TAS	total antioxidant status
TLRs	toll-like receptors
TNF	tumor necrosis factor
TOF	tetrallogy of Fallot
TPN	total parenteral nutrition
TTN	transient tachypnea of the newborn
UNICEF	United Nations Children's Fund
UOP	urine output
VLBW	very low birth weight
WBC	white blood count
WHO	World Health Organization

ABSTRACT

The habilitation thesis entitled "**From basic care to emergency disease and future drama in neonates - an integrative approach",** reflects study and research activities from a period of over 25 years of my career representing the synthesis of some directions of postdoctoral scientific research.

Section I, after detailing my achievements in the medical, academic and scientific research activity, presents the main study directions to which I contributed to and the synthesis of the most important articles published in journals indexed in both Thomson ISI Web of Science Core Collection, as well as in international databases. The personal scientific contributions followed an integrative approach, referring to nutrition, neonatal sepsis, neonatal hypoxia with its long-term consequences, ethical aspects and dilemmas.

Chapter I.1 presents the results of studies on breastfeeding, human milk composition, variations of macronutrients content in different stages of lactation and for different gestational ages. The methods of storage were studied and compared with respect to preservation of protein, lipid, carbohydrates and energy content, and their benefits were explored. A particular attention was focused on total antioxidant capacity of human milk. Finally, the benefits of breastfeeding on preventing dental caries in childhood were reviewed. This preoccupation on human milk was in part sustained by an internal grant from University of Medicine and Pharmacy "Grigore T. Popa" (no. 30881/30.12.2014) entitled "Optimal storage practices to preserve macronutrients, energy and total antioxidants in human milk from mothers of term and preterm newborns". This chapter brings together the results of 2 ISI quoted articles, 1 ISI indexed article and 1 indexed article in international databases. Our studies are among the very first published so far on this topic in România.

Chapter I.2 includes the results of the most important research related to one of the most challenging pathology in neonates, sepsis. It comprises 3 ISI quoted articles, 1 ISI indexed article and 1 article indexed in international databases. The subject is still of actuality, concerns the scientific community of neonatologists and pediatricians, as it represents one of the most emergent challenge for early diagnosis in neonates, especially in tiny premature infants with immature immune defense. Early neonatal sepsis is a life-threatening pathology for this category of patients and early diagnosis must be established in order to initiate the proper antibiotic treatment with real chances of success but avoiding unnecessary or prolonged administration that can lead to selection of bacterial strains with high level of antibiotic resistance. After an extensive review of current state of the art on current biomarkers in use for neonatal sepsis, we present the perspectives for future methods of identification of more reliable biomarkers for early detection of neonatal sepsis. Our focus is on endocan, known to be a valuable diagnostic and prognostic marker in adult sepsis, but not explored enough in neonates yet. We established baseline values for this biomarker in uninfected newborns and further studied the value of endocan as a tool for diagnosis. This preoccupation was in part sustained by an internal grant from University of Medicine and Pharmacy "Grigore T. Popa" (no. 30882/30.12.2014), entitled "Endocan - a possible marker for diagnosis and outcome prediction of neonatal sepsis".

Chapter I.3 brings together different aspects of neonatal hypoxia and some ethical challenges in decision making. Hypoxia represents for our fragile patients the origin of many impairments at all organ levels, but the brain might be mostly affected, sometimes with irreversible and severe consequences, not only on short but also on long term. After a brief concentrated presentation of multiorgan disfunction generated by hypoxia, we underlined the short-term consequences on the brain and gut, mostly regarding hypoxic-ischemic

encephalopathy and necrotizing enterocolitis. The later was also highlighted by a complex clinical case as an example of the challenges in managing combined neonatal pathologies at small gestational ages. A special subchapter was dedicated to research of some therapeutic methods in preventing severe consequences on hypoxia and asphyxia in term newborns, comparing the use of high-dose phenobarbital to erythropoietin. Based on a large-scale of epidemiological, clinical, and preclinical studies that have suggested a central role of gestational factors in promoting cognitive impairments that accelerate the onset and evolution of Alzheimer`s disease-like pathology later in life, the "*fetal origins of adult disease*" hypothesis was raised. The adverse prenatal environment can alter the developmental trajectory of organs/tissues in early life and may increase the risk of disorders later in life, including neurobehavioral conditions, heart, and metabolic disorders. Prenatal hypoxia is a common form of fetal stress, which leads to fetal growth restriction (reduced birth weight) and in the most important periods of brain formation results in essential changes in the development of cognitive functions in different stages of postnatal life, which correlates with morphological changes in the cerebral structures involved in learning and memory. An extensive overview on factors related and involved in later onset of dementia is presented in a large subchapter. Hypoxia may also generate obstructive sleep apnea, thus we focused on studying it in a special category of children, those with genetic disorders, enhancing on the special need for early multidisciplinary diagnosis and management. Last but not the least important, was the subject of ethical dilemmas in neonatal practice, with old and new concerns, with specific situations exemplified mostly when severe hypoxic status occurred. This problem is still current, as the legal frame for such cases is still missing in our country and the practitioner is faced with his/her own drama, the patient's, the care takers' and the societies'. This chapter presents the synthesis of 7 ISI quoted articles, 1 ISI indexed article and 1 article indexed in international databases.

Section II presents my future career plans and projects, in professional, academic, and scientific field. Mainly, I am planning to continue the research on human milk, but focusing on different factors that affect human milk composition and some drugs transmitted through the milk. Intranasal use of human milk in preventing severe intraventricular hemorrhage in very preterm newborns has recently been reported and represents another personal area of interest. Research on risk factors for congenital deafness or iatrogenic induced deafness is another future direction of my future studies. The role of maternal isotretionin treatment for acne as a risk factor for congenital malformations in newborns is also a subject that I started to research, as part of an international collaboration. A particular attention from my part will be directed to the impact of premature rupture of membranes on neonatal infection and possible implementation in current practice of a specific panel of laboratory tests, including new valuable markers for the risk of infection in the first days of life.

Section III includes the list of references consulted for the elaboration of this thesis and the articles included in this synthesis.

REZUMAT

Teza de abilitare cu titlul "**De la îngrijiri de bază la patologii de urgență și drame ulterioare la nou-născut – o abordare integrativă**" reflectă activitatea de studiu și cercetare pe o perioadă de 25 de ani din cariera personală și reprezintă o sinteză a principalelor direcții de cercetare postdoctorală personală.

Secțiunea I, după expunerea unei selecții a realizărilor mele în activitatea profesională medicală, în cea academică și în cercetarea științifică, prezintă principalele direcții de studiu la care am contribuit și sinteza celor mai importante articole publicate în jurnale de specialitate indexate atât în Thomson ISI Web of Science Core Collection, cât și în baze de date internaționale. Contribuțiile personale științifice au urmărit o abordare integrativă, referindu-se la nutriția nou-născutului, la sepsisul neonatal, la hipoxia neonatală și consecințele pe termen lung, aspect și dileme etice în practica neonatală.

Capitolul I.1 prezintă rezultate studiilor cu privire la alimentația naturală, compoziția laptelui matern, variabilitatea conținutului în macronutrienți în diferite stadii ale lactației și pentru diferite vârste de gestație. Au fost studiate și comparate modalitățile de conservare și păstrare a laptelui uman pentru interval variate de timp, urmărind influența acestora asupra conținutului în proteine, lipide, carbohidrați și energie, focusat pe beneficiile potențiale. O atenție particulară s-a acordat variabilității capacității antioxidante totale a laptelui matern. În final este prezentată o trecere în revistă a beneficiilor alăptării în prevenirea cariilor dentare în copilărie. Aceste preocupări cu privire la laptele uman au fost în parte posibile și susținute printr-un grant intern al Universității de Medicină și Farmacie "Grigore T. Popa" din Iași (nr. 30881/30.12.2014) intitulat "Optimal storage practices to preserve macronutrients, energy and total antioxidants in human milk from mothers of term and preterm newborns". Capitolul reunește rezultatele publicate în 2 articole publicate în reviste indexate ISI, un articol în revistă cotată ISI și un articol publicat în baze de date internaționale. Aceste studii sunt printre primele publicate până la acel moment pe acest subiect, în România.

Capitolul I.2 include rezultate ale cercetărilor cu privire la una din cele mai severe și provocatoare patologii neonatale, sepsisul. Sunt cuprinse date din 3 articole cotate ISI, un articol indexat ISI și un articol publicat în baze de date internaționale. Subiectul este în continuă actualitate, preocupând comunitatea științifică a specialiștilor neonatologi și pediatri și reprezintă una dintre cele mai dificile urgențe de diagnostic precoce la nou-născut, mai ales la cei mai fragili prematuri, cu apărare imună imatură. Sepsisul precoce neonatal este o patologie cu risc vital, pentru această categorie de pacienți diagnosticarea fiind necesară a se face cât mai rapid, în vederea inițierii unui tratament antibiotic țintit cât mai adecvat și cu reale șanse de succes, în același timp urmărindu-se evitarea administrării prelungite inutile a medicației ce ar putea duce la selectarea unor specii bacteriene cu nivel crescut de antibiorezistență. După o detaliată trecere în revistă a noțiunilor actuale cu privire la biomarkerii utilizați curent în practică în diagnosticarea sepsisului neonatal, sunt prezentate perspectivele unor metode viitoare potențiale de identificare a unor biomarkeri mai precișiși în detectarea precoce a acestei afecțiuni. Sunt prezentate rezultatele studiilor originale cu privire la endocan, cunoscut ca fiind un marker de diagnostic și prognostic al sepsisului la adult, dar insuficient explorat la nou-născut. Principalele rezultate prezintă valorile normale ale acestui biomarker și valorile semnificative pentru diagnosticul sepsisului precoce la nou-născut. Aceste cercetări au fost susținute printr-un grant intern al Universității de Medicină și Farmacie "Grigore T. Popa" din Iași (nr. 30882/30.12.2014) intitulat "Endocan - a possible marker for diagnosis and outcome prediction of neonatal sepsis".

Capitolul I.3 reunește diferite aspect ale hipoxiei neonatale prezentând și unele provocări de ordin etic în luarea deciziilor medicale. Hipoxia reprezintă pentru fragilul pacient originea unor multiple deficite potențiale la nivelul tuturor organelor, dar creierul pare a fi cel mai mult afectat, uneori consecințele fiind severe și ireversibile, nu doar imediat pe termen scurt, dar și ulterior, pe termen lung și foarte lung. După o prezentare concentrată și succintă a disfuncțiilor multiorganice generate de hipoxie, ne-am axat pe prezentarea efectelor pe termen scurt asupra creierului și intestinului, în speță cu privire la encefalopatia hypoxic-ischemică și enterocolita ulcero-necrotică. Aceasta din urmă a fost evidențiată și prin prezentarea unui caz clinic complex ce a constituit exemplificarea dificultăților de diagnostic și tratament a unor patologii combinate neonatale la vârste de gestație foarte mici. Un subcapitol special a fost dedicat cercetărilor cu privire la potențiale metode și terapii utile în prevenirea sechelelor severe ale hipoxiei și asfixiei la nou-născutul la termen, comparând administrarea eritropoietinei cu dozele mari fenobarbital. Pornind de la o largă varietate de studii epidemiologice, clinice și preclinice publicate în literatura de specialitate ce sugerează rolul central al factorilor gestaționali în generarea afectării cognitive cu accelerarea debutului și evoluției patologiilor ulterioare Alzheimer`s-like, s-a născut ipoteza „originii fetale a bolii adultului”. Afectarea statusului prenatal poate altera traiectoria dezvoltării organelor și țesuturilor din primele etape de viață și poate crește riscul tulburărilor ulterioare din viața adultă, inclusiv afectări neurocomportamentale, afectări cardiace și metabolice. Hipoxia prenatală este o cauză frecventă de suferință fetală ce poate genera restricția creșterii intrauterine cu scăderea greutății la naștere într-o perioadă esențială pentru formarea și dezvoltarea neuronală, având drept consecință modificări cruciale în dezvoltarea funcțiilor cognitive pe parcursul diferitelor etape de evoluție ulterioară postnatală. Aceste modificări se corelează cu afectări morfologice ale structurilor cerebrale implicate în procesele de învățare și memorare. Într-un subcapitol extins sunt prezentați pe larg factori implicați și determinanți în debutul ulterior al demenței. O altă patologie secundară hipoxiei cu efecte pe termen lung este apneea obstructivă de somn, astfel încât ne-am aplecat atenția în studierea acestei afectări la o categorie specială de copii, cei cu afecțiuni genetice, subliniind necesitatea diagnosticului și managementului precoce și multidisciplinar. În final, dar nu mai puțin important, sunt abordate aspecte și dileme etice ale practicii neonatale în contextul hipoxiei, probleme vechi și noi, cu exemplificări ale unor situații practice specifice sau particulare, mai ales complicate cu stadii severe de hipoxie. Această problematică este încă de actualitate având în vedere că în țara noastră există un vid legislativ și un cadru legal imprecis și incomplet, iar neonatologul practician este confruntat nu doar cu o situație profesională dramatică, dar și cu drama pacientului însuși, a personalului de îngrijire și chiar a societății. Acest capitol se bazează pe sinteza a 7 articole indexate ISI, un articol cotat ISI și un articol publicat în baze de date internaționale.

Secțiunea II cuprinde proiectele și planurile pentru dezvoltare pe viitor a carierei profesionale, academice și nu în ultimul rând, științifice. În principal intenționez să continui cercetarea asupra laptelui uman, de data aceasta focusându-mă asupra diferiților factori ce pot afecta compoziția, mai ales transmiterea anumitor medicamente în laptele uman. O altă arie de interes o reprezintă ideea rolului benefic al laptelui uman administrat minimal intranazal la prematurii cu vârstă mică de gestație, cu rol preventiv în agravarea hemoragiilor intraventriculare și evoluția spre stadii avansate de boală, aspect recent raportat în literatură. Surditatea congenitală sau iatrogen indusă și factorii de risc asociați vor constitui o altă direcție de cercetare. Ca parte implicată într-o colaborare internațională interdisciplinară și în continuarea unui studiu deja început, voi contribui la precizarea rolului terapiei materne a acneei cu izotretionină în apariția malformațiilor fetale. În continuarea cercetărilor axate pe detectarea precoce a infecției neonatale, o atenție mai mare va fi acordată impactului rupturii premature de membrane asupra riscului de infectare a nou-născutului și potențiala

implementare în practica profesională curentă a unui set specific de teste de laborator, incluzând noi markeri dovediți a fi utili în confirmarea sepsisului din primele zile de viață.

Secțiunea III cuprinde lista referințelor consultate în elaborarea acestei teze și a articolelor incluse în prezenta sinteză.

SECTION I. ACADEMIC, PROFESSIONAL AND SCIENTIFIC ACHIEVEMENTS

Brief overview of the academic and professional career

Teaching in the medical field represents one of the most valuable achievement of a society. Universities are now days challenged to find the best ways to blend the practical and online theoretical tools in order to assure the mastering in formation the future high performing physicians.

Ultimately, both research and teaching should combine in a high value and standard medical academics. Moreover, for the clinical field, the practical professional performances should assure the foundation of a complete university teaching staff. In my opinion, one cannot be the proper teacher in a clinical specialty without being a high professional in the same field.

The academic didactic career in the medical field is complementary in most fields and specialties with the medical professional activity and with the research activity. Managing this trio of excellence is a challenge for anyone who wants such a career. Being a very good doctor, a dedicated teacher and an ambitious researcher with recognized results requires learning, involvement and dedication, time, passion, effort, sometimes sacrifice, team spirit, empathy, and excellence - all being mandatory for a successful professional, academic and scientific successful path.

As for my personal professional and academic career, it started after completion of my 6 year university studies in medicine and one year of clinical training. In 1994, after passing the national exam for entering the residency I chose Neonatology as my future specialty, and I started my formation at "Cuza-Voda" Clinical Hospital of Obstetrics and Gynecology in Iasi, which is the largest maternity of our region. In my last year of residency (1999) I passed the exam for becoming a staff neonatologist. It was a great decision and a huge chance, as Neonatology was at that time the youngest specialty, founded just a year before, so I was the second generation of future neonatologists. It is important to mention that until that time, in all maternities over the country there were only the pediatricians that provided neonatal care, and there was no Neonatology Department in any Romanian university, the formation in neonatal care for medical students was performed by pediatricians, as part of pediatric courses, and the practical activities took place in the maternities, supervised by staff pediatricians, neither of them affiliated to the university department. As a volunteer, I involved myself in the work with 6th year students, during all my residency. After passing the exam for becoming specialist in neonatology, the same year, the Discipline of Neonatology, affiliated to Obstetrics and Gynecology was founded. It was composed by a lecturer position and an assistant professor position, so in June 2000 I passed another exam, thus becoming the first assistant professor in Neonatology in the country. My mission was to teach practical activities to medical students, together with the next generation of residents, in a pioneering new specialty that developed in the fastest manner and progressed in the years to come.

Beside the challenge of becoming a good professional at the bedside, I was continuously preoccupied in achieving and mastering my teaching skills. So, in 1997 I enrolled myself in a 2 year course in mastering teaching at University "Al. I. Cuza", a formation that comprised Teaching, Scholar Psychology, Logics, Educational Sociology, Medical Methodic and Teaching Practice.

An important role in professional development and teaching performances came from many opportunities that emerged at that time, and the years that came, from which I will list some of them:

- **2021,** April: "A Practical Approach to Interpretation of Preterm aEEG" – Neonatal Care Academy
- **2021,** March: "Near Infrared Spectroscopy monitoring for sick infants workshop I 2021" – POCUSNEO Canada (online)
- **2021,** March: "Perinatal Sepsis" – European Association of Perinatal Medicine
- **2020,** October: Course "Good Clinical Practice" – NIDA Clinical Trials Network
- **2020,** October – December: "Ecografia Sistemului Nervos Central (rolul ultrasonografiei cerebrale în evaluarea efectului nutriției asupra neurotroficității)" – "Titu Maiorescu" University Bucharest (6 modules online)
- **2019**: Post Graduate Program in Pediatric Nutrition – Boston University School of Medicine (7 modules online)
- **2018**: International Program on Preterm Nutrition – The University of Western Australia (6 modules online)
- **2014,** March: Neonatal Cranial Ultrasound Course – Imperial College London, UK
- **2014,** June: Course "Simularea urgenței neonatale", Iași
- **2013,** June: Course continuous medical education: "Simularea în educația medicală continuă, aspecte etice în patologia malformativă neonatală", Văratec.
- **2012,** June: Cours "Noi strategii ventilatorii în tratamentul detresei neonatale – ventilația cu frecvență înaltă", Tg. Mureș.
- **2012,** May: Workshop "Elaborarea protocoalelor specific neonatale", Dubrovnik, Croația.
- **2012,** April - September: Course "Ultrasonografie generală – nivelele I și II", Iași
- **2011,** February: Cours de Reanimation Avancee Neonatale et Pediatrique –European Pediatric Live Support (RANP-EPLS), European Resuscitation Council, Poitiers, France
- **2009:** Cours "Etica cercetării", Iași
- **2009**: Workshop Instruirea formatorilor asupra curiculei din cadrul proiectului "Programul de reforma a Ministerului Sănătății – Proiectul de formare a personalului din secțiile de obstetrică-ginecologie și nou-născuți", Sinaia.
- **2007**: Course "Sisteme de Management al Calității – Principii Fundamentale și vocabular" SR EN ISO 9000:2001 și " Sisteme de Management al Calității – Cerințe" SR EN ISO 9000:2001, Iași
- **2006**: NRP and S.T.A.B.L.E. Program Lead Instructor course – International relief Teams, Iași.
- **2003:** January 18th – April 18th: "Training on Applied Techniques in Neonatology", HUG Geneva, Switzerland, as part of intergouvernmental romanian-swiss RoNeoNat program.
- **2003:** Workshop in „Reanimation neonatale", Hopital Cantonal Universitaire de Geneve, Switzerland**.**
- **2002**, April 3rd – May 1st, Louisville, Kentucky, USA: Training in neonatology (neonatal intensive care).
- **2002**, March 25th – April 2nd, Course "Modern Techniques of Neonatal Intensive Care", Iowa City, Iowa, USA**.**
- **1999 – 2001**: Annual Course in modern neonatology, high risk newborn care – Newstart III, IOMC „Alessandrescu-Rusescu", Project Concern Internaţional, Bucharest.
- **1999,** October: "Mortalitatea Perinatală", Ministerul Sănătății, Institutul de Perfecționare Postuniversitară a Medicilor și Farmaciștilor, București
- **1999,** August: Intensive course in Neonatology "Neoprep" San Antonio, Texas**,** USA.

- **1999,** May: ”Frontiers in Diagnosis and Management of Congenital Heart Diseases”, Newport, Rhode Island, including 3 weeks of practical stages and workshops in Boston Children’s Hospital, MA, USA**.**

One of the most valuable experiences in my professional path consisted in the early opportunity to be visiting physician in large and famous neonatal intensive care units from Birmingham, Alabama, Boston, Massachusetts, Iowa City, Iowa, Louisville, Kentucky in USA, between 1998 and 2002, which allowed access to updated practical medical information, procedures, and techniques, that could be adapted and adopted in our local practice at the bedside. Programs like Newstart II and Newstart III, together with Humana Foundation Collaboration Program for Neonatal Development included me initially as a translator and later as a full participant, offering me the double learning possibility: to hear directly from the “source”, to process, translate and present it to the audience and so, to double understand and express it.

In 2003 my implication in RoNeoNat swiss-romanin intergovernmental program consisted in 3 months training in Hospital Universitaire de Geneve, where I was also initiated and immersed in local neonatal NICU and the process of elaborating specific protocols for practice, followed by a 4 year period of teaching neonatology in Iasi and the region of Moldavia. Thus, I contributed to the training and professional development of more than 300 neonatologists and neonatal nurses from regional maternities.

Continuous medical education providing process was completed by teaching Neonatal Resuscitation and Stabilization, as an Instructor in “The Stable Program” since 2006 until present, doubled by ”Cours de Reanimation Avancee Neonatale et Pediatrique” – European Pediatric Live Support (RANP-EPLS), European Resuscitation Council, Poitiers, France in 2011.

The course of my career in university education so far continued with the following steps:

- 2006: University Lecturer in Neonatology
- 2016 – present time: Associate Professor in Neonatology.

Teaching was for my career one of the most successful activities. As so, lately my abilities in presenting, explaining and synthesizing the information, together with team building and leadership traits were used in large, well-received and appreciated projects in which I was one of the lecturers in neonatology:

- Expert – course ”Asistența medicală pentru nou-născuți în perioada neonatală” în cadrul Proiectului POCU/91/4/8/109586 „Spital – Comunitate, Flux de îngrijire continuă a nou-născutului și a sugarului cu risc crescut de îmbolnăvire și deces”, 2017-2018, implemented by INSMC ”Alessandrescu-Rusescu”, Bucharest
- Scientific research-development managing director for the project POSDRU/179/3.2/151363 „Formarea specialiștilor în domeniul cardiologiei pediatrice pentru un act medical de calitate cu scopul îmbunătățirii calității vieții”, 2015, U.M.F. Iași. (awarded 2nd prize at Structural Funds Gala, by European Union on December 18 2017).
- Member in POSDRU/81/3.2/S/59587 Project (2011-2012): “Formarea profesională în domeniul neonatologiei și promovarea utilizării noilor tehnologii pentru personalul din sectorul sănătății” - U.M.F. Iași
- Supervisor in POSDRU/81/3.2/S/59587 Project (mars 2013): “Formarea profesională în domeniul neonatologiei și promovarea utilizării noilor tehnologii pentru personalul din sectorul sănătății” - U.M.F. Iași
- Member in project ”Medicalis” 2010-2013 “Management educațional și învățământ de calitate în societatea informațională” POSDRU/86/1.2/S/62594 U.M.F Cluj and U.M.F. Iași.

Academic and teaching role also implies the ability for evaluation, not only students and residents, but also in committees for examination for awarding specialist or consultant in neonatology, or staff neonatologist in different maternity hospitals in Romania, either as a member committee, or, in the last 3 years, as a president of such committees.

I was also member of the commission for competitions for teaching positions, member of doctoral admission commissions or doctoral study guidance commissions. I have repeatedly participated in the organization of admission exams at the "Grigore T. Popa" University of Medicine and Pharmacy Iași and residency competitions.

I coordinated bachelor's theses at the Faculty of Medicine, with neonatal subjects.

Since 2018, I am the coordinator of the residency program in Neonatology and modules of Neonatology of connected specialties: general pediatrics, emergency medicine, pediatric surgery, genetics, pediatric gastroenterology, pediatric nephrology, pediatric pulmonology, pediatric oncology.

At present, I am a member of professional organizations: since 1997 – member of *Romanian Neonatology Association* and since 1999 - member of The Society of Physicians and Naturalists of Iaşi.

Academic and professional functions:

- Member in Neonatology Specialty Commission of the Ministry of Health (MH Order no. OMS nr 100/25.01.2019)
- Elected member in Mother and Child Medicine Department - ”Grigore T. Popa” UMF Iași from 2016 to 2019.

Professional Aspects

I completed the residency in Neonatology in 1999 and passed the exam for specialist doctor. In the same year, as I had been already employed as staff neonatologist by exam in “Cuza-Voda” Clinical Hospital of Obstetrics and Gynecology in Iasi, I entered the academic community of “Grigore T. Popa” University of Medicine and Pharmacy. As requested at that time, I had to resign from my full position at the hospital and continue to practice for a half part of the same position, including regularly monthly on-calls. Between 2002-2007 I practiced also monthly on-calls at another level II neonatal clinic, “Elena-Doamna” Clinical Hospital of Obstetrics and Gynecology in Iasi. In 2003 I passed the exam for competence in neonatology, as consultant.

In my opinion, a good pediatrician must have good achievements in neonatal and perinatal medicine, as the good neonatologist has to have a larger perspective of his patient and his quality of life, so he/she also has to have proper achievements in pediatric field. Because of my constant interest in pediatrics and because in Romania, Neonatology is a separate specialty with a different curriculum, I decided to do Pediatrics residency as a second specialty. Thus, in 2009 I passed the specialty exam for accreditation as specialist in Pediatrics.

A remarkable chapter of my professional career was a one year (July 2010 - June 2011) private practice, as a pediatrician, at Clinique “Fief de Grimoire”, Poitiers, France where I activated as neonatologist in a level II neonatal unit and both pediatrician in private office. Along with the medical activity I was implicated in organizational field of the Clinic, participating at the elaboration of specific neonatal local protocols and enrolling in local community of physicians with private practice.

It was a large perspective, a huge experience accumulated by practicing in neonatology units with various levels of competence, as I had to adapt to different places, teams, languages, regulations, politics, and situations. But more than this, I was able to learn and exchange experience with many of the physicians from Centre Hospitalier Universitaire of Poitiers, Nantes and Bordeaux, from connected specialties. This involved a close

collaboration regarding complex cases, knowing specific local medical policies, and keep up to date with the most recent and actual protocols from a developed medical system as the French system, which is the most similar to ours, in Romania. For me, such an experience was for real benefit not only from financial point of view, but also professionally and socially, as I got remarkable recognition from colleagues, patients, and administrative staff. I convinced myself that such an experience would be beneficial for any physician. This is the reason for which I recommend and encourage students, residents, doctors to take any opportunity and chance for a training, experience exchange or internship abroad or in another medical center than the one where they graduated.

After returning to Romania, my integrated practice included a lot of practical demonstrations and workshops with residents, annually, not only in neonatology, but also in other connected specialties like pediatrics, emergency medicine, pediatric surgery or genetics.

I constantly updated my medical professional knowledge and acquired new skills by attending training courses in the country and abroad (over 40 courses).

In 2019 I was designated member in National Committee in Neonatology, a position that in my opinion was a recognition of my professional achievements and of my human quality traits. Some of my preoccupations were to review the curricula for Neonatology residency, for specialty accreditation, to assess the revision process of national guidelines and to actively work at new guidelines.

Scientific activity

My first contribution to scientific activity started immediately after graduating the Faculty of Medicine and Pharmacy and consisted in an article published in *The Medical-Surgical Journal (Revista Medico-Chirurgicala a Societatii Medicilor si Naturalistilor)* the single medical journal affiliated to our university at that time. The subject was related with my license thesis, “The X Metabolic Syndrome in obese patients”.

During the residency years, my research field of interest started with basic sciences (anatomy and physiology) and step by step combined clinical research in neonatology. The largest research from that period was centered on a very important aspect in neonatal practice: “Research using antenatal therapy with corticosteroids for preventing complications of prematurity”. This became the subject of my doctoral thesis, which was completed and presented in 2004 at "Grigore T. Popa" University of Medicine and Pharmacy in Iași, under scientific coordination of Acad. Prof. Dr. Antranic Negura. In 2005 I obtained the doctoral degree in medicine, field of obstetrics-gynecology-neonatology, confirmed by Ministry of Education and Scientific Research Order no. 3956/25.04.2005, with the distinction "Magna cum laude".

Later on, my scientific area of interest centered on neonatology, but also on connected specialties as pediatrics, ENT, genetics, dermatology, but also basic sciences and research, with high area of interest, such as antioxidants, Alzheimer’s disease and multiorgan disfunction, pharmacological implications in antioxidants activity of some drugs and their effect in experimental animal models.

I am a member of the editorial staff of the specialty publication *Perinatologia* indexed in international databases (Index Copernicus) and I had been invited to perform peer-review analyzes for the following publications:

1. *Nigerian Journal of Clinical Practice*
2. *Clinics and Practice*
3. *Experimental and Therapeutic Medicine*
4. *Journal of Integrative Neuroscience*
5. *Neural Regeneration Research*
6. *Journal of Pediatric Infectious Diseases*

7. *Medical Science Monitor*
8. *Journal of Pediatrics Genetics*
9. *Life*
10. *Children*
11. *International Journal of environmental Research and Public Health*

The scientific and research activity has been recognized by awards for two articles in which I was lead author and by distinctions:

CNCSIS prize for:

- Rusu MC, Poalelungi CV, Vrapciu AD, Păduraru L, Didilescu AC, Stan CI: Anoctamin 1 Positive Esophageal Interstitial Cajal Cells in Late Stage Human Embryos. The Anatomical Record 2014, 297 (2):301-30

PRECISI prize PN-III-P1-1.1- PRECISI-20 for:

- Stanciu GD, Bild V, Ababei DC, Rusu RN, Cobzaru A, Paduraru L, Bulea D. Link Between Diabetes and Alzheimer's Disease due to the Shared Amyloid Aggregation and Deposition Involving both Neurodegenerative Changes and Neurovascular Damages. J Clin Med. 2020 Jun 3;9(6):1713. doi: 10.3390/jcm9061713.
  PRECISI list 2, position 276, pg 114-115.

In summary, my scientific contribution is represented by a total of**:**

- Indexed publications – Thomson ISI: 22 articles (14 articles as lead author, 5 as co-author in quoted journals; 3 ISI indexed journals and proceedings - 2 as lead author and 1 as co-author)
- BDI publications: 28 articles (15 as lead author and 13 as coauthor)
- Books and chapters in books – 18 (coauthor)
- H-Index – **6** in ISI Web of Science, having 85 citations and h index Google Scholar 7, having 123 citations

Lately, after achieving the NIDA certification, I involved myself in clinical studies as an investigator, as part of the neonatology team.

As mentioned before, the main area of interest is represented by clinical and experimental neonatology, therefore more references upon the most important aspects of my scientific activity and research being highlighted further.

CHAPTER I.1. INSIGHTS ON HUMAN MILK AND BREASTFEEDING

Neonatology is a specific and special branch of Pediatrics dealing with the most sensitive patient, the neonate, from 0 to 28 days of life. The most challenge patient is the premature neonate, that might be even from 24 weeks gestation, so the neonatologist may deal with a very immature function from very small and undeveloped anatomical organs. For them, the neonatal period takes not only 1 month after birth, but as long it takes to reach term and maturity that is supposed to be gained at 40 weeks corrected age. For all categories of patients, after checking, stabilizing the vital signs, nutrition is one of the first goal.

Breastfeeding is recommended as optimal all over the world, although actual Covid - 19 pandemic times generated a challenge in implementing this practice safely (1), but continuing as being one of the most effective ways to ensure child health and survival. Nearly 2 out of 3 infants are not exclusively breastfed for the recommended 6 months and this rate has not improved in 2 decades.

Breast milk is the ideal food for infants. It is safe and clean, cheap and contains antibodies which help protect against many common childhood illnesses. Human milk provides all the energy and nutrients that the infant needs for the first months of life. It continues to provide more than half of a child's nutritional needs during the second half of the first year, and up to one third during the second year of life. Even so, 3 in 5 neonates are not exclusively breastfed in the first hour of life (2,3).

Breastfed children perform better on intelligence tests later in life. They are less likely to be overweight, obese and less prone to diabetes as adults (4).

I.1.1. Research regarding the macronutrients composition of human breastmilk

Human milk has been proven by multiple studies to be the most complete and perfectly adapted food for the growth and development of both, full-term and preterm newborn. The composition in macronutrients, trace elements, immunological factors and vitamins makes breast milk the ideal food in the first 6 months postnatal. These findings have been the basis of numerous studies that have justified the WHO (2011) recommendations on the optimal timing of diversification of the infant nutrition. However, in some parts of the world, the rate of breastfeeding is still low (5). A study in the United States shows that only 33% of infants are breastfed at 3 months of age and less than 14% at 6 months of age (6). Due to the immediate postnatal discomfort, cesarean delivery has been shown to be a limiting factor in natural nutrition. Studies in preterm infants have shown that although its composition is richer in protein, free amino acids, fats, immune factors and sodium than the milk of the mother who gave birth at term, this content becomes insufficient in calories and protein after the first weeks of life and requires supplementation and strengthening protein to ensure the special growth the preterm infant needs (7). When compared to formulas for preterm infants, human milk, even if it comes from the so-called "milk banks", is more indicated in the diet of the preterm newborn due to the multiple advantages on postnatal evolution: it decreases the incidence of infections and especially enterocolitis (8,9), decreases the incidence of the retinopathy of prematurity (10), of subsequent hospitalizations in the first year of life (11), ensures better somatic growth and neurobehavioral development (12,13).

Published paper:

- **Paduraru L**, Avasiloaiei A, Patriciu M, Zaboloteanu C, Moscalu M, Stamatin M. Natural Nutrition – Present and Future, *Buletin de Perinatologie*, 2014, 2(62): 123-133

Objective

The study aimed to quantitatively analyze the main nutritional principles and energy value in the milk of mothers who gave birth to term / premature infants, to evaluate the timing of breastfeeding, its influence on the subsequent duration of natural nutrition during infancy, in the context of different modes of delivery (natural or cesarean section), gestational age and other significant parameters.

Material and methods

In order to achieve the proposed goals, 403 samples of breast milk were studied. These were collected from 222 mothers who gave birth at term or prematurely at ”Cuza-Vodă” Hospital of Obstetrics and Gynecology, Iași. The composition in macronutrients and energy content of the milk samples collected by pumping was determined using the Medela electric milking pump, 2.5 ml each, analyzed in most cases in the first 5 hours after harvest or exceptionally in the first 24 hours (stored in the refrigerator at +4 degrees Celsius until analysis). The determinations were performed by spectrophotometric method (infrared transmission), with the Miris Human Milk Analyzer, at a temperature of 40 degrees Celsius. The second part of the study used the questionnaire method, which involved questioning 1098 mothers who gave birth in the same maternity ward and who were asked about the type of diet, time of initiation, duration of natural nutrition, time of diversification, influence of birth, age and the environment or maternal pathology on the incidence of natural nutrition.

Results

The gestational age of the newborns included in the study varied between 27 and 42 weeks, 72% (n = 160) being full-term newborns and 28% (n = 62) being preterm (Fig. 1).

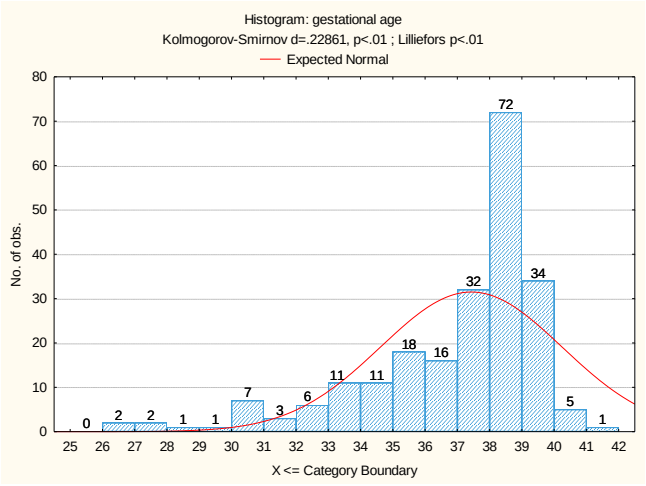

Fig. 1. Gestational age histogram

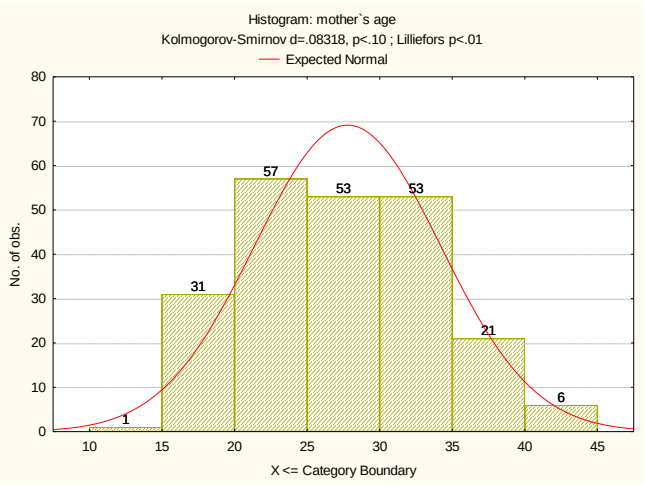

Fig. 2. Mother`s age histogram

The mean value of the mother's age was 27.8 years (± 6.4 SD), with minimum values of 14 years and maximum values of 45 years (Fig. 2).

The mean value of energy for the breast milk registers minimum values in the first 2 days postnatal in the case of newborns with GA less than 29 weeks (46 kcal/100 ml ± 8.5 SD) and maximum values (75 kcal/100 ml ±19.9 SD) in the same category during the second week of life (Tables1 and 2).

Table 1. Statistical indicators of energy value (Kcal / 100ml)

	Newborn's Age [days]	Mean Energy	Mean		DS	Min	Max	Q25	Median	Q75
			-95%	*+ 95%*						
<29 GA	1-2 days	46.0	-30.2	122.2	.5	0.0	2.0	0.0	6.0	2.0
	4 days	49.5	-96.6	195.6	6.3	8.0	1.0	8.0	9.5	1.0
	7 days	52.0	35.7	68.3	.6	6.0	9.0	6.0	1.0	9.0
	8-14 days	75.0	43.3	106.7	9.9	4.0	3.0	8.0	6.5	2.0
	in 14 days	64.6	54.3	74.9	2.3	1.0	1.0	8.5	1.0	8.0
30-33 GA	1-2 days	68.8	64.9	72.7	.1	4.0	2.0	8.0	9.0	1.0
	4 days	58.7	13.9	103.5	8.0	0.0	6.0	0.0	0.0	6.0
	7 days	68.7	54.1	83.2	.9	2.0	3.0	2.0	1.0	3.0
	8-14 days	60.4	50.5	70.2	4.7	5.0	5.0	6.0	6.0	5.0
	in 14 days	57.8	53.0	62.5	0.2	7.0	0.0	8.5	8.0	6.0
34-36 GA	1-2 days	63.5	19.0	108.0	.9	0.0	7.0	0.0	3.5	7.0
	4 days	54.1	49.0	59.3	.6	9.0	2.0	7.0	3.5	1.0
	7 days	60.6	55.9	65.4	0.8	2.0	3.0	2.0	1.5	7.0
	8-14 days	61.9	55.9	67.9	1.7	5.0	1.0	7.0	3.0	7.0
	in 14 days	58.8	48.7	68.9	5.0	3.0	3.0	5.0	4.0	0.0
≥ 37 GA	1-2 days	59.8	54.1	65.4	7.5	4.0	18.0	0.0	6.0	3.0
	4 days	59.3	57.4	61.1	2.7	8.0	19.0	1.0	7.0	6.0
	7 days	58.4	54.4	62.3	1.3	0.0	5.0	0.0	8.5	4.0

Regarding the GA between 30-33 weeks, there is a progressive decrease in energy value after 2 weeks, from 68.8 to 57.8 kcal/100 ml, which is why it is necessary to strengthen breast milk over time.

Table 2. Statistical indicators of lipids in HM

	Newborn's age [days]	Mean Lipids	Mean		DS	Min	Max	Q25	Median	Q75
			-95%	*+ 95%*						
<29 GA	1-2 days	1.3	1.2	8.2	0.8	0.7	1.8	0.7	1.3	1.8
	4 days	1.4	1.1	5.8	0.5	1.0	1.7	1.0	1.4	1.7
	7 days	1.7	0.1	3.3	0.7	1.1	2.4	1.1	1.7	2.4
	8-14 days	4.7	1.1	8.4	2.3	2.2	6.8	2.8	5.0	6.7
	in 14 days	3.7	2.3	5.2	1.7	1.8	6.3	2.5	3.1	5.3
30-33 GA	1-2 days	1.7	0.9	2.6	0.7	1.2	2.9	1.3	1.4	1.8
	4 days	2.8	1.2	6.8	1.6	1.3	4.5	1.3	2.7	4.5
	7 days	2.9	1.7	7.5	1.8	0.8	4.0	0.8	4.0	4.0
	8-14 days	3.1	2.0	4.2	1.7	1.3	6.7	1.8	2.5	4.3
	in 14 days	2.4	1.8	2.9	1.2	0.9	5.4	1.4	2.5	3.0
34-36 GA	1-2 days	2.9	0.4	5.4	0.3	2.7	3.1	2.7	2.9	3.1
	4 days	2.3	1.8	2.8	1.0	0.8	4.1	1.5	2.3	2.9
	7 days	2.6	2.1	3.1	1.1	1.1	5.2	1.6	2.7	3.1
	8-14 days	2.9	2.2	3.6	1.3	1.0	5.4	2.2	2.9	3.6
	in 14 days	2.4	1.6	3.3	1.3	1.0	4.5	1.5	2.0	3.3
≥ 37 GA	1-2 days	2.2	1.8	2.7	1.3	0.4	6.4	1.4	2.0	2.7
	4 days	2.6	2.5	2.8	1.2	0.2	7.9	1.8	2.5	3.4
	7 days	2.6	2.2	3.0	1.2	0.3	5.5	1.7	2.6	3.3

On the total analyzed group, the energy value was statistically significantly correlated with gestational age and postnatal age (F = 1.91 p = 0.016, 95% CI).

The lipid content of the analyzed breast milk showed minimum values in the first days of lactation in infants with GA <29 weeks (1.3 g/100 ml ± 0.8 SD) and maximum values in the samples collected in second week, in the same category (4.7 g/100 ml ± 2.3 DS) (Table I.2). In the full-term newborn, the values are significantly lower, between 2.2 g/100 ml in the first 2 days and 2.6 g/100 ml in 7 days. The large differences between preterm and full-term newborns may be due to the fact that in the case of premature birth, milk was collected by milking using an electric pump, until breast emptying and homogenized before analysis and administration. In the case of full-term newborn mothers, the milk was collected before the newborn was breastfed, so it was the first milk. On the total analyzed group, the lipid content was statistically significantly correlated with gestational age and postnatal age (F = 2.02, p << 0.01, 95% CI).

The carbohydrate content showed minimum values in the first days regarding the mothers who gave birth between 30-33 weeks (4.9 g/100 kcal ± 0.5 SD) and maximum values of 6.8 g/100 ml, and in the full-term newborn the highest amount of carbohydrates was 6.4 g/100 ml, but after the first week. In all categories of newborns, the carbohydrate content showed an upward trend, to the detriment of the protein content of milk (Table 3). On the total analyzed group, the carbohydrate content was statistically significantly correlated with gestational age and postnatal age (F = 3.97, p << 0.01, 95% CI).

***Table 3**. Statistical indicators of carbohydrates in HM*

	Newborn's age [days]	Mean Carbo-hydrates	Mean		DS	Min	Max	Q25	Median	Q75
			-95%	*+ 95%*						
<29 GA	1-2 days	6.0	-1.0	12.9	0.8	5.4	6.5	5.4	6.0	6.5
	4 days	6.0	-24.5	36.5	3.4	3.6	8.4	3.6	6.0	8.4
	7 days	6.7	6.2	7.2	0.2	6.5	6.9	6.5	6.7	6.9
	8-14 days	5.5	2.2	8.7	2.0	2.4	6.7	4.3	6.3	6.6
	in 14 days	6.8	6.3	7.3	0.6	5.5	7.4	6.7	7.1	7.2
30-33 GA	1-2 days	4.9	4.3	5.6	0.5	4.5	5.7	4.6	4.7	5.2
	4 days	6.8	6.0	7.6	0.3	6.6	7.2	6.6	6.7	7.2
	7 days	6.8	6.0	7.6	0.3	6.6	7.2	6.6	6.7	7.2
	8-14 days	6.1	5.3	6.9	1.2	2.9	7.4	5.7	6.6	6.8
	in 14 days	6.8	6.4	7.1	0.8	4.2	8.1	6.5	6.8	7.2
34-36 GA	1-2 days	6.5	4.5	8.4	0.2	6.3	6.6	6.3	6.5	6.6
	4 days	6.1	5.6	6.5	0.8	4.7	7.7	5.4	6.3	6.5
	7 days	6.8	6.5	7.1	0.7	5.5	8.7	6.4	6.7	7.1
	8-14 days	6.6	6.1	7.1	1.0	3.7	8.5	6.5	6.7	7.0
	in 14 days	6.4	5.9	7.0	0.8	4.4	7.2	6.2	6.6	7.0
≥ 37 GA	1-2 days	5.5	5.1	5.9	1.4	1.1	7.1	4.8	6.1	6.5
	4 days	6.2	6.0	6.3	0.8	1.5	7.9	5.9	6.3	6.5
	7 days	6.4	6.1	6.8	1.1	1.9	8.7	6.4	6.6	6.8

The protein content varies in milk samples collected from mothers of preterm infants under 33 weeks at 4 and 7 days, from 1.0 g/100 ml ± 0.7 SD to 1.6 g/100 ml ± 0.5 SD in full-term mothers of newborns after 4-7 days postnatally (Table 4).

We note that at less than 29 weeks, in the first 4 days, the amount of protein in breast milk registers the highest values (2.3 g/100 ml ± 0.5 SD), but does not cover the protein needs of the preterm. In all categories of gestational age and especially in preterm neonates, protein has a declining trend over time, which is an additional argument for strengthening premature

breast milk, which, from the second week of life no longer meets the minimum protein required for weight gain of the preterm.

***Table 4**. Statistical indicators of proteins in HM*

	Newborn's age [days]	Mean proteins	Mean -95%	Mean + 95%	DS	Min	Max	Q25	Median	Q75
<29 GA	1-2 days	2.0	-3.8	7.7	0.6	1.5	2.4	1.5	2.0	2.4
	4 days	2.3	-2.2	6.7	0.5	1.9	2.6	1.9	2.3	2.6
	7 days	1.6	1.3	2.0	0.2	1.5	1.8	1.5	1.6	1.8
	8-14 days	1.3	0.4	2.2	0.6	0.9	2.1	0.9	1.1	1.7
	in 14 days	1.0	0.7	1.4	0.4	0.4	1.8	0.9	0.9	1.2
30-33 GA	1-2 days	1.7	1.6	1.9	0.1	1.6	1.9	1.6	1.7	1.8
	4 days	1.0	-0.8	2.8	0.7	0.2	1.5	0.2	1.4	1.5
	7 days	1.0	-1.0	3.0	0.8	0.1	1.6	0.1	1.3	1.6
	8-14 days	1.3	1.0	1.7	0.6	0.1	2.1	1.1	1.2	1.9
	in 14 days	1.2	0.9	1.5	0.7	0.1	2.9	0.8	1.1	1.7
34-36 GA	1-2 days	1.9	-1.3	5.0	0.4	1.6	2.1	1.6	1.9	2.1
	4 days	1.7	1.5	1.9	0.4	1.0	2.3	1.5	1.8	1.9
	7 days	1.6	1.4	1.8	0.5	0.6	2.9	1.3	1.6	1.9
	8-14 days	1.4	1.1	1.7	0.6	0.7	2.9	1.1	1.3	1.9
	in 14 days	1.2	1.0	1.4	0.3	0.8	1.9	1.0	1.2	1.3
≥ 37 GA	1-2 days	1.7	1.4	1.9	0.7	0.1	2.9	1.3	1.7	2.2
	4 days	1.6	1.5	1.7	0.5	0.1	2.9	1.4	1.6	1.8
	7 days	1.6	1.4	1.7	0.5	0.1	2.6	1.3	1.6	1.7

On the total analyzed group, the protein content was statistically significantly correlated with gestational age and postnatal age (F = 2.42, p << 0.01, 95% CI).

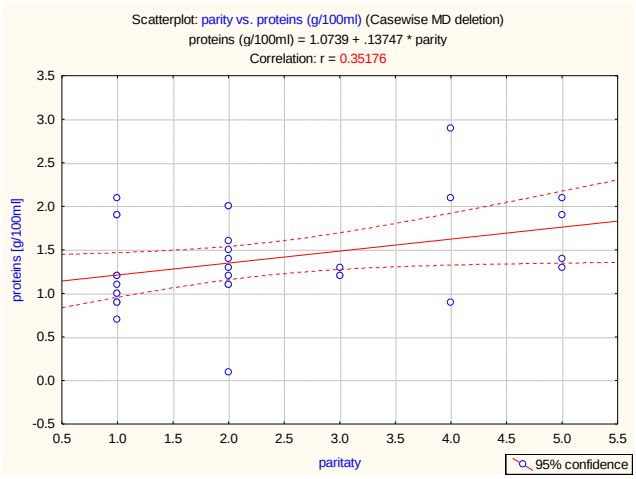

Fig. 3. Parity vs. protein content of HM

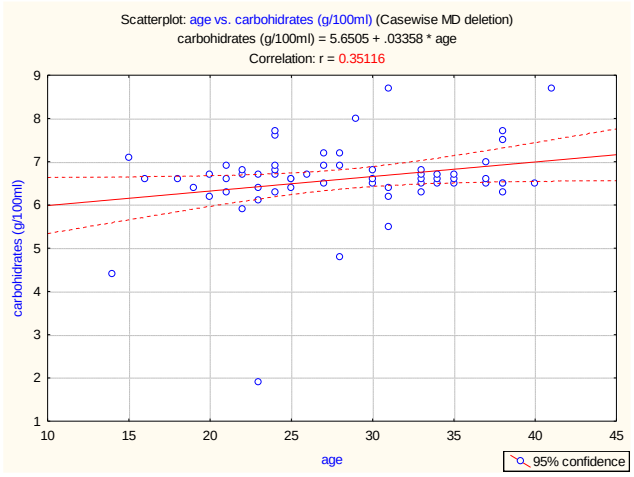

Fig. 4. Mother`s age vs. carbohydrates content of HM

The milk composition has a weak positive correlation with parity in terms of protein content (r = 0.35, p = 0.048, 95% CI) (Fig. 3). Parity does not correlate with lipid content (r = 0.02, p = 0.88, 95% CI), carbohydrates (r = -0.04, p = 0.79, 95% CI), energy value (r = 0.09, p = 0.62, 95% Cl). The composition of milk correlates with the age of the mother only in terms of carbohydrate content (r = 0.35, p = 0.04, 95% CI) (Fig. 4).

There is a negative correlation between the time of initiation of lactation and the protein content of breast milk (r = -0.32, p = 0.04, 95% CI) (Fig. 5). There are no correlations between the time of initiation of lactation and other macronutrients.

Apart from the protein content that seems to be influenced by previous breastfeeding (p = 0.001, 95% CI), none of the other macronutrients show statistically significant changes. Interestingly, mothers of full-term newborns who have previously breastfed have a lower protein content in milk compared to those who are breastfeeding for the first time. Referring to the preterms, if their mothers have breastfed in the past, the protein content of the milk seems to be higher (Fig. 6).

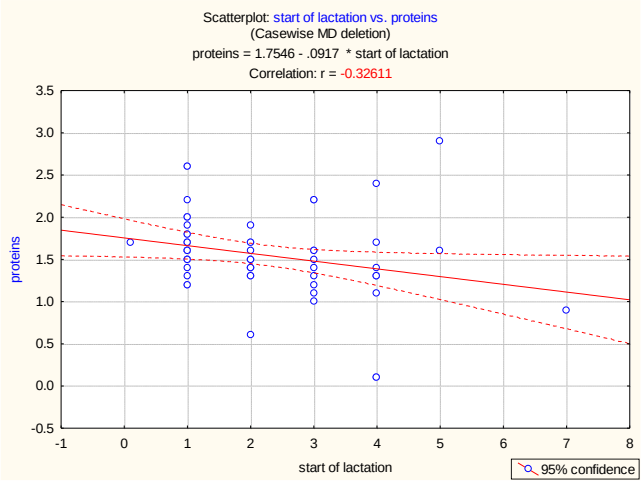

Fig. 5. Start of lactation vs. protein content of HM

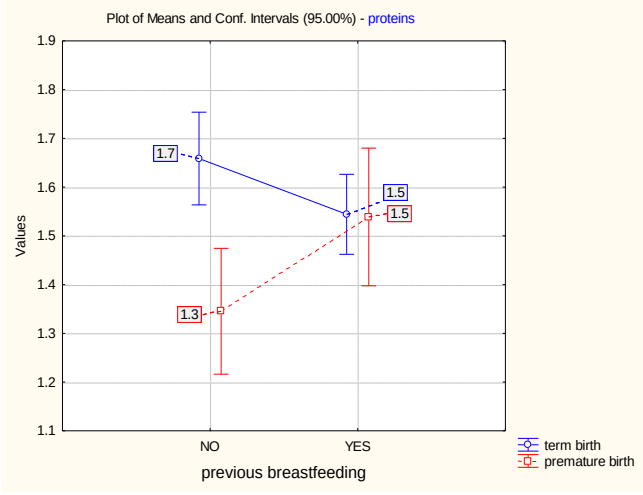

Fig. 6. Previous breastfeeding vs. protein content of HM

The birth method only influences the energy value of breast milk, regarding the mothers who gave birth by caesarean section, the energy value of milk is lower, regardless of gestational age (p = 0.02, 95% CI) (Fig. 7).

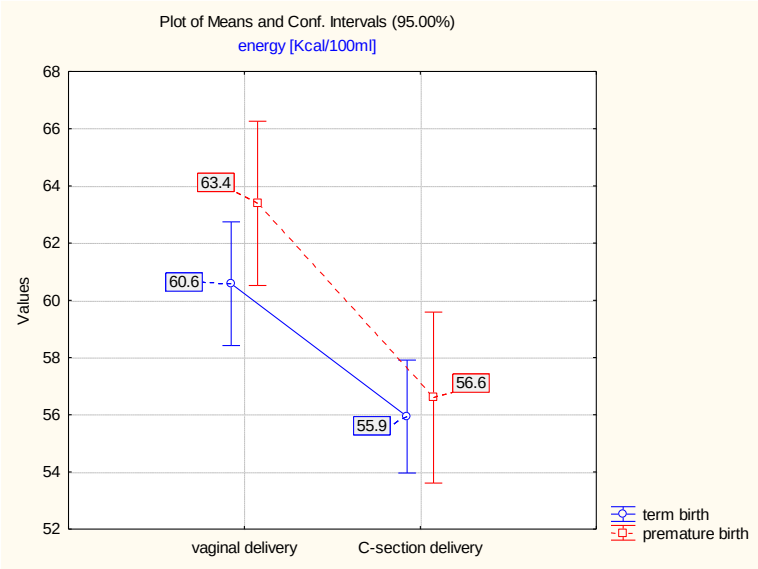

Fig. 7. Delivery mode vs. calories content of HM vs. gestational age

The correlations between the composition of breast milk and maternal diseases, and the treatments administered to the mother were also analyzed. None of these factors influenced the composition of the milk.

Using the *method of the questionnaire* data were evaluated from 1098 mothers who gave birth in the ”Cuza-Vodă” Hospital of Obstetrics and Gynecology, Iasi between July 1, 2012 and June 30, 2013 regarding the following aspects related to the mode of feeding in the

maternity ward and later at home: type of birth, place of origin/mother's education, gestational age, breastfeeding duration and formula introduction time, time of complementary feedings, correlation with infant pathology.

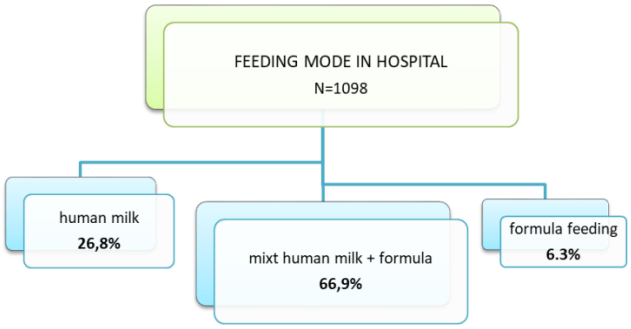

Fig. 8. Feeding mode in hospital

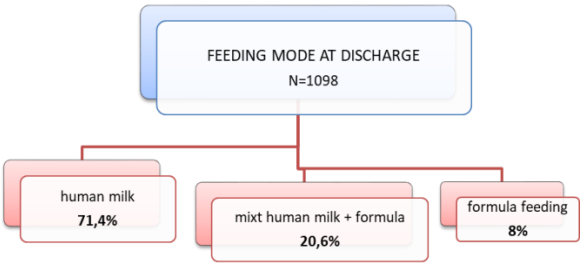

Fig. 9. Feeding mode at discharge

If on the first day, in the maternity ward, only 26.8% newborns were breastfed, and 66.9% received both HM and formula (Fig. 8), following breastfeeding counseling, at discharge 71.4% of newborns were exclusively breastfed and 20.6% received mixed feedings (Fig. 9).

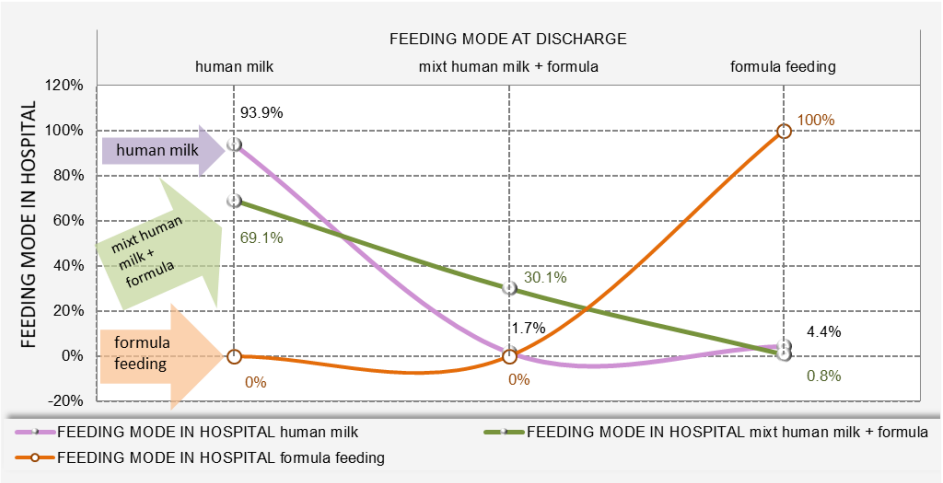

Fig. 10. Feeding mode in hospital vs. feeding mode at discharge

Regarding the newborns initially fed naturally in maternity, at discharge 93.9% remained exclusively breastfed, 1.7% received mixed feedings and 4.4% only formula (p = 0.0000) (Fig. 10).

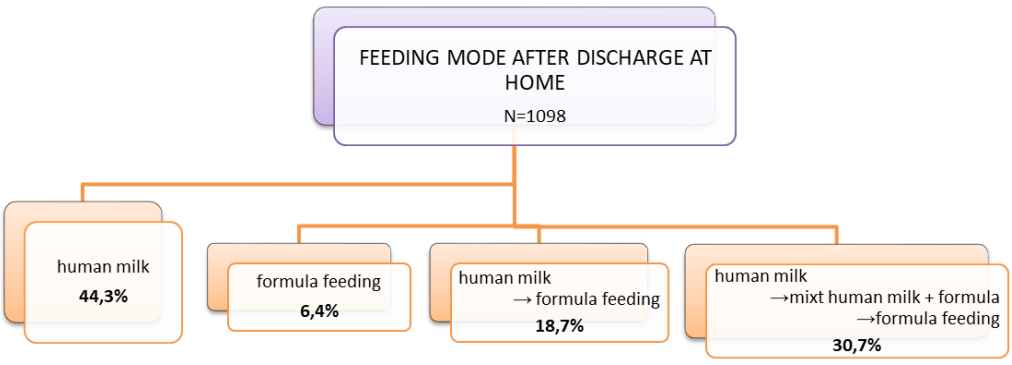

Fig. 11. Feeding mode after discharge at home

At home, the percentage of natural nutrition decreased significantly, from 71.4% at discharge to 44.3%, 18.7% being switched directly to milk formula and 30.7% went through phases with variable duration of mixed feeding (Fig. 11).

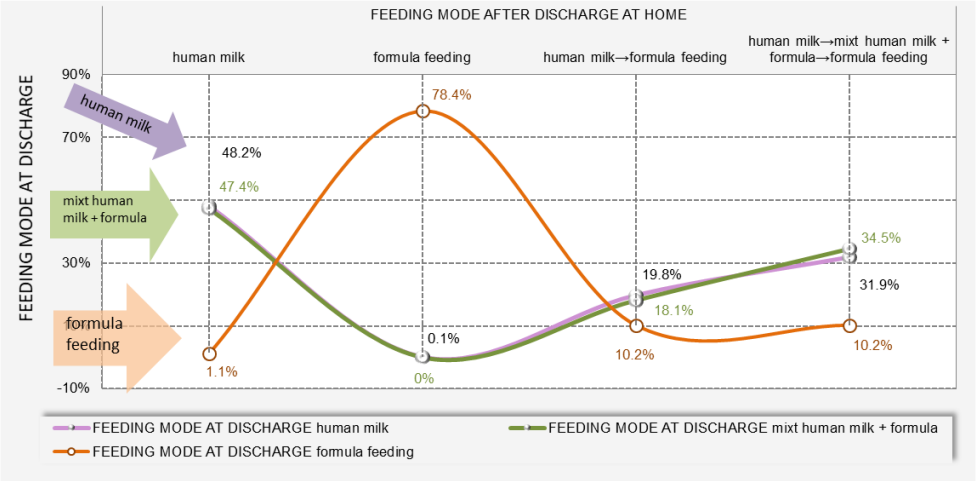

Fig. 12. Feeding mode at discharge vs. feeding mode after discharge at home

Concerning the newborns who had a natural diet at discharge for only 48.2% maintain this type of diet, 0.1% are switched to artificial feeding, 19.8% after a period receive only artificial feeding, and 31.9% after a period receive mixed feeding after which only artificial feeding is maintained ($p << 0.01$) (Fig. 12).

The period of natural feeding was significantly longer in infants who received only natural feeding (48.1 weeks ± 13.3DS). Newborns that after a period in which they received only natural food received mixed food, and then were switched only to formula, the period of natural food decreased to 24.2 weeks ± 15.7DS ($p << 0.01$, $F = 442$, 5.95% CI) (Fig. 13).

The initiation of the milk formula was not statistically correlated with the type of nutrition at home (Fig. 14). This study did not include newborns who received only formula because they had this type of feeding since birth.

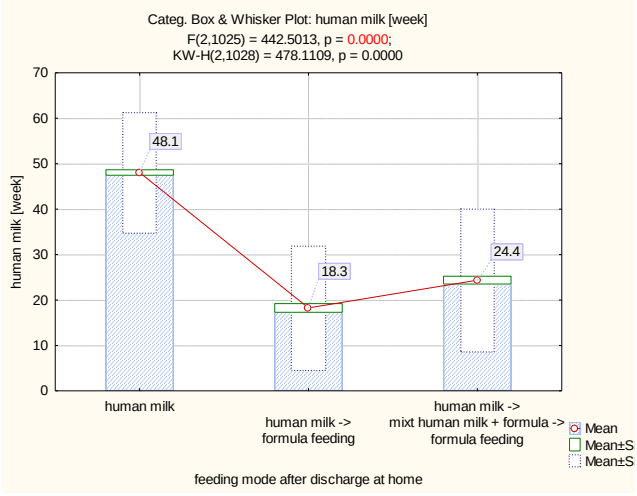

Fig. 13. Time of breastfeeding (weeks)

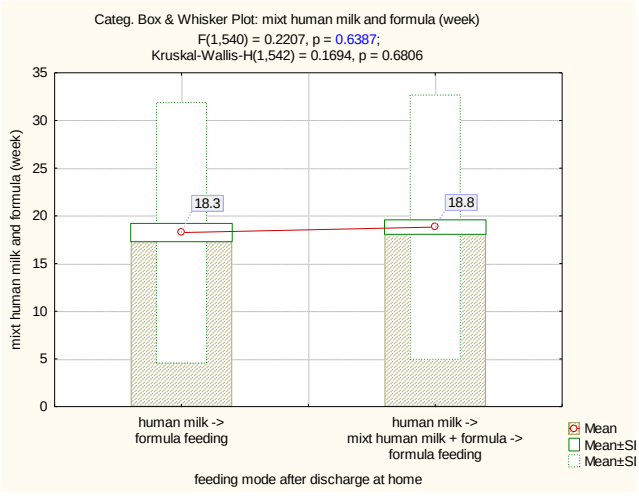

Fig. 14. Time of initiating formula (newborn`s age in weeks)

In the studied group, the mean time for complementary feedings introduction was 21.5 weeks. 75% of the newborns included in the study were on complementary feedings at 24 weeks (Fig. 15).

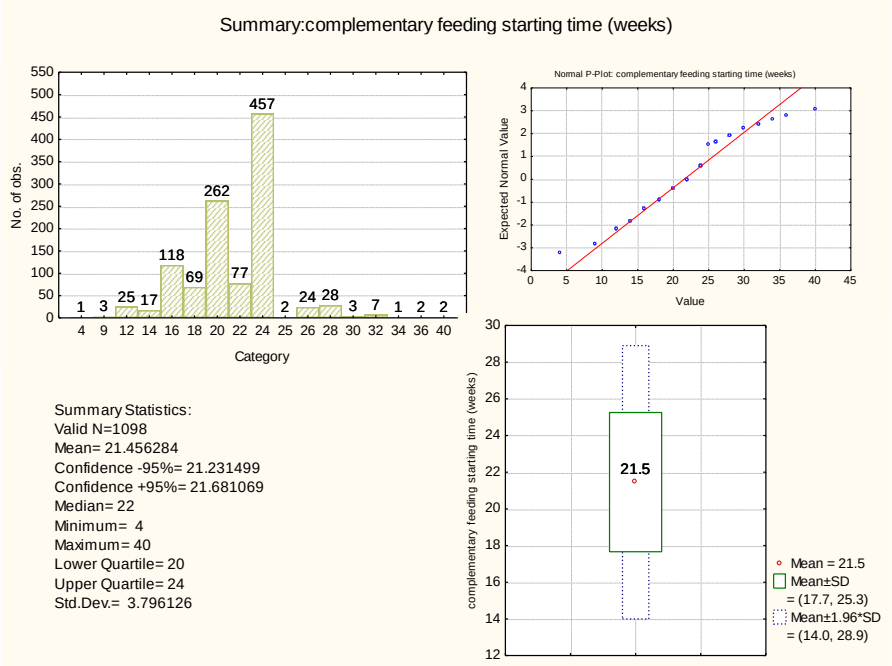

Fig. 15. Time of complementary feedings (weeks)

Mothers from rural areas fed a significantly higher percentage of natural food (54.2%) than those from urban areas (39.7%), and only 12.1% later switched directly to artificial feeding, compared to 21.7 % to those in urban areas (p = 0.00002) (Fig. 16).

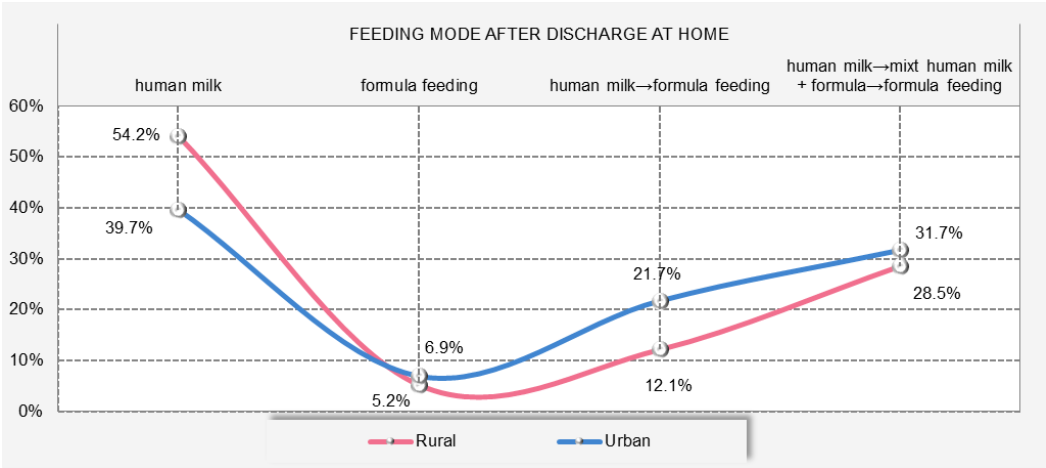

Fig. 16. Feeding mode after discharge at home vs. the environment of origin

Mothers who did not attend any school and those with primary education, breastfeed less than those with secondary or higher education (χ^2= 40.54, p = 0.00001, 95% CI) (Fig. 17). This suggests insufficient promotion of breastfeeding, and the influence of unhealthy prejudices on the benefits of breastfeeding.

Exclusive breastfeeding after discharge predominated among mothers who gave birth naturally (51.8%), and only 27.8% of those that delivered by cesarean section continued breastfeeding at home. The percentages were similar for those that breastfed initially and later switched to formula (18.9% vs. 18.3%) (χ2 = 108.04, p << 0.01, 95% CI) (Fig. 18).

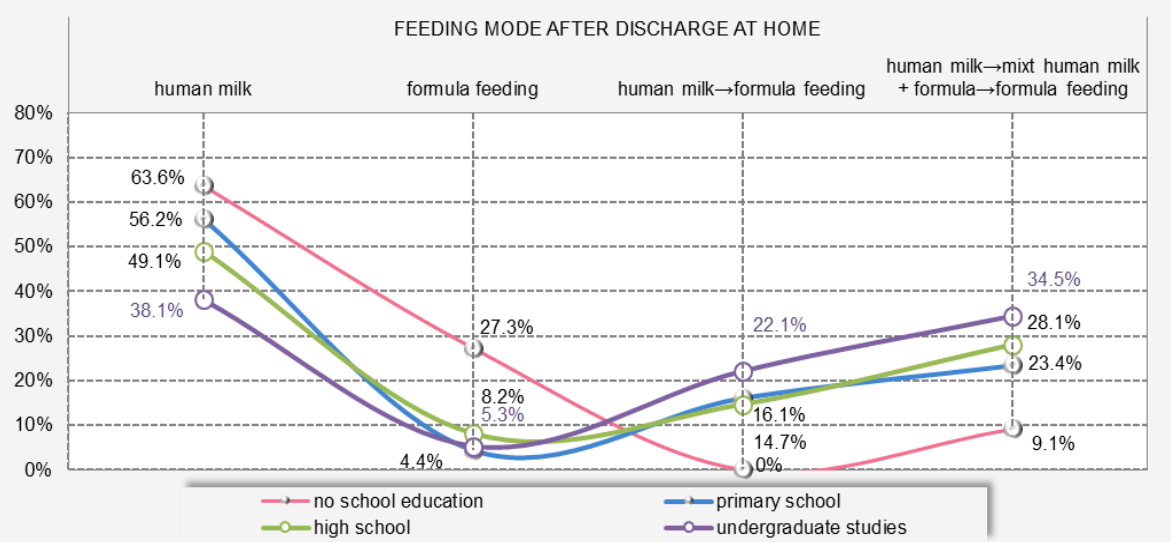

Fig. 17. Education status vs. feeding mode after discharge at home

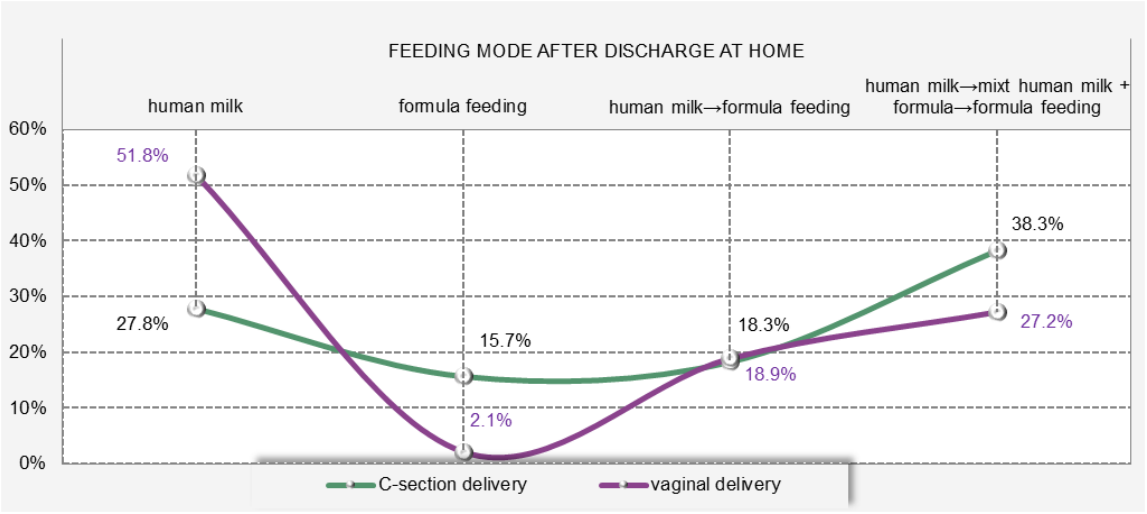

Fig. 18. Mode of delivery vs. feeding mode after discharge at home

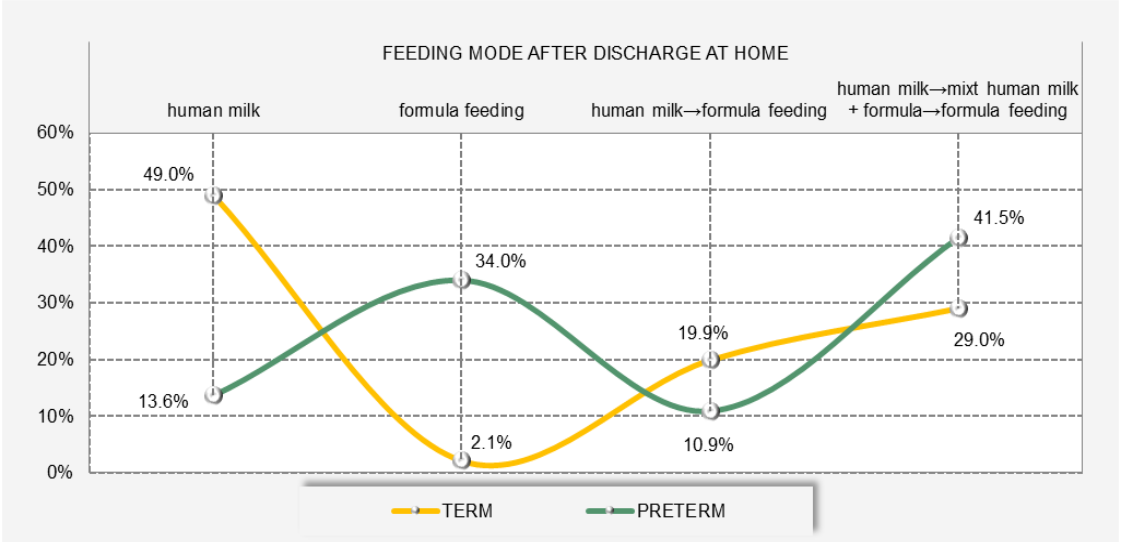

Fig. 19. Gestational age vs. feeding mode after discharge at home

There is a significant association between nutrition and prematurity (χ^2= 251.36, p << 0.01, 95% CI). Only 13.6% of the preterm newborns were breastfed at home (Fig. 19). Natural nutrition is more difficult and less available for preterm infants, due to neonatal pathology that involve prolonged hospitalization, only possible for the newborn, maternal issues that interfere with milk pumping, or the transfer of the newborn to another hospital.

When introducing milk formula into the diet, the rate of cases with associated pathology increases significantly, from 18.7%, in the case of natural nutrition, to 34.6% in the case of newborns who switched from natural to exclusively formula (Fig. 20) (χ2 = 26.14, p = 0.00001).

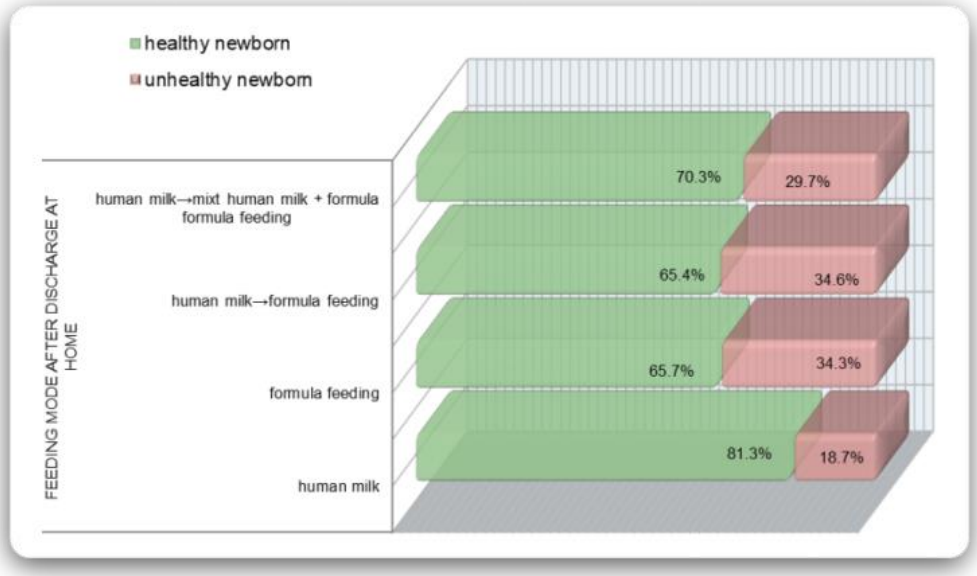

Fig. 20. Feeding mode at home vs. infant`s morbidity

Discussions

One of the most important problems of prematurity is postnatal nutrition. As preterm infants have a degree of fetal growth restriction, recovery becomes essential. Preterm infants admitted in neonatal intensive care unit present the greatest difficulties in initiating and continuing enteral feeding. ELBW infants have the highest risk of postnatal growth restriction. Insufficient nutritional support in the first week of life is the first and most important cause of postnatal growth restriction, but in practice this period may be longer, leading to a cumulative major protein and energy deficit and to malnutrition.

Postnatal growth restriction in VLBW infants can range from 50-100% on discharge from the neonatal intensive care unit (14). In addition, it can also have a negative impact on neurological development with long-term consequences (which can manifest as adult diseases). Taking into consideration that the preterm infant is a “fetus” forced to live outside the uterine environment, it is logical that the ideal postnatal growth rate should be similar to fetal growth rate. This rate differs depending on the degree of immaturity and/or intrauterine growth restriction. The protein and energy needs estimated by ESPGHAN are a valuable nutritional recommendation in order to achieve a postnatal growth rate similar to fetal growth. In practice, however, this desideratum is more difficult to achieve due to the digestive immaturity of the preterm infant. Despite this, most references propose a progressive increase in nutritional intake immediately after birth (15). Former study on a lot of 117 VLBW preterm infants suggested initiating feeding from the first day of life with a minimum of 40 kcal/kg/day and 2g of protein/kg/day, reaching 120 kcal/kg/day and 3.8g of protein/kg/day at the end of the first week. This aggressive early nutrition contributed to the increase in caloric

and protein intake and to the reduction of the cumulative caloric and protein deficit on day 28 of life (16). This strategy could expose the infants to major metabolic risks such as: hyperglycemia, uremia, metabolic acidosis and hyperammonemia, but the greatest risk for ELBW preterm infants is to develop necrotizing enterocolitis. For these reasons, in order to use this strategy, a permanent monitoring of the biochemical parameters of the preterm infants is necessary. In current practice, for fear of complications, the progressive method (conventional method) is preferred.

There are currently several types of fortifications, each with its own advantages and disadvantages. Each country and neonatology center can opt for one of these strategies depending on the technical possibilities, the material resource and the specialized human resource it has.

Standard fortification is the addition of fortifier in quantities per volume. Due to the fact that an empirical dose of nutrients is added to the mother's milk, this type of strategy does not always match the preterm infant's nutritional needs (17). Also, in this type of strategy, the energy intake will be sufficient at a volume of 150 ml/kg/day, but it will exceed the recommendations at higher volumes. Instead, the protein intake will be adequate only at volumes of 180 ml/kg/day or even higher, volumes difficult or even impossible to administer for very small preterm infants. On the other hand, the problem of supplying too much energy in relation to the amount of proteins required, the ratio of protein to energy recommended by ESPGHAN must be taken into account, but this ratio is not achieved with this type of fortification. In conclusion, the standard fortification of milk from mothers who gave birth prematurely fails to meet the recommended protein intake (3.5/4.0 g/kg/day at volumes of 150 ml/kg/day). A 2004 Cochrane meta-analysis recommended an assessment of both short-term and long-term outcomes in an attempt to discover an "optimal" composition of fortifiers (18).

Super fortification means adding a higher amount of fortifier, for example adding the standard dose to a smaller volume of milk than recommended. This strategy is relatively simple, does not involve additional costs or staff for the analysis of milk samples. It is possible to consume more proteins, but energy and micronutrients are also provided. Therefore, this strategy cannot change the protein/energy ratio enough to promote the gain of lean tissue mass. The risk of hypercalcemia cannot be neglected either, therefore, if this strategy is chosen, it is necessary to monitor serum calcium and phosphorus. A study conducted in 2012 reported two levels of blind fortification (respectively moderate and aggressive), compared to standard fortification, in a single group of preterm ELBW and VLBW with a gestational age around 28 weeks (19). Moderate and aggressive fortification led to insignificant increases in weight and length, but to significant increases in head circumference. The lack of significant increase in weight and length can be explained by estimating the protein intake of only 3.3-3.6g of protein/kg/day in the intervention groups; this intake is not suitable for preterm infants with a birth weight ≤ 1000g (20).

Individualized-adjustable fortification refers to a more particular method in which the newborn's metabolic response is used to guide the gradual addition of protein supplement. The values of urea nitrogen in the blood (blood urea), which has been shown to correlate closely with the enteral intake of protein, guide the amount of additional proteins required (21). Studies using this type of strategy have reported significant increases in the main anthropometric indices (length, weight and head circumference). These results were obtained without adjusting the ration volume or energy consumption. The average value of the daily volume administered was 140 ml/kg/body/day, which makes this type of strategy appropriate for ELBW infants who are required to limit fluids, at least in the initial period (22).

The individualized-target fortification is adapted to the individual needs of the preterm infant and is performed by analyzing the breast milk before fortification. Own and/or donated breast milk is analyzed providing quantitative information on the composition in

macronutrients, its energy and protein-energy ratio. The next step involves adding supplements to reach the target levels for macronutrients defined by ESPGHAN (23). Weight gain in the individual fortification group is similar to the group that received standard fortification, but the difference is that in the standard group the required volumes were higher (> 150 ml/kg/day), which favor individualized-target fortification (24).

A linear relationship between milk consumption and weight gain was demonstrated only in the individualized (personalized) fortification group. Compared to other types, this type of personalized fortification requires material resources and important human resources, but offers greater security in the short and long term regarding the quality of recovery.

The MIRIS HMA presents some limits, because using old or conserved human milk can influence the results, thus only freshly expressed milk can be analyzed with full confidence.

The composition in macronutrients varied depending on the time of harvest and the type of collection, in the sense that the harvest was not homogeneous, from 24-hour milk and probably the composition varied depending on the socio-economic status of the mother.

Significant correlations of milk composition with gestational age and postnatal age demonstrate that the variability of breast milk gives it inimitable and unmatched qualities.

The initiation of breastfeeding in the delivery room had an incidence of 26%, partly explained by the high rate of cesarean section (52%), of which only 21.8% fed naturally. Subsequently, at discharge, 71% of newborns were fed exclusively naturally. This demonstrates the need to continue efforts to initiate and promote breastfeeding. Mothers who fed naturally continued breastfeeding until an average of 48.1 weeks postnatal. The timing of diversification averaged 21.8 weeks.

Conclusions

The analysis of breast milk in macronutrients is necessary to be done in large number of cases and by collecting milk for 24 hours, being appropriate multicenter studies to analyze the variability of human milk content in different regions.

All efforts aimed at preterm nutrition are currently focused on finding the best means of optimizing the nutritional intake of proteins and energy, thereby reducing the cumulative nutritional deficit and reducing the incidence of protein/energy malnutrition.

On the long run, the milk of mothers who gave birth prematurely does not provide sufficient proteins and energy support to ensure optimal postnatal growth during hospitalization and nutritional recovery of preterm infants. It is a sufficient argument to impose the need to fortify human milk for preterm infants fed exclusively on their own mother's milk or from donor’s milk.

The future of natural nutrition depends on the promotion of breastfeeding in maternity hospitals and family doctors, this being the best and cheapest prophylaxis of neonatal pathology and the infant.

I.1.2. Freezing and refrigeration of human milk and its effect on macronutrients and energy content in early lactation

Exclusive breastfeeding for the first 6 months, continued after introduction of complementary feeding for 1 to 2 years represents one of the gold standards of pediatric nutrition (4). This is not possible for all preterm infants, or for neonates that are in respiratory distress or other medical condition that affect their capacity of suckling, coordination between suckling, swallowing and breathing, or their digestive capabilities, as it is the case for patients in the Neonatal Intensive Care Unit (NICU). After initial parenteral nutrition and respiratory recovery, enteral feeds are progressively introduced, according to their feeding tolerance. Understanding human milk composition provides an important tool for the management of

infant feeding, particularly that of fragile, high risk infants, and for understanding the potential impact of storage and pasteurization upon milk components.

With the progress of lactation, the concentration of lactose, lipids, calories, and phosphate increases significantly, the changes being more pronounced in preterm than in term milk (25,26).

Because of the major biological advantages of human milk and the inability of small preterm infants to breastfeed, freezing expressed breastmilk for future use is a common practice in the NICU (27) as this guarantees its microbiological safety and hinders the growth of microorganisms. However, the enzyme activity inherent to breastmilk may remain at this temperature (28,29), but it also affects its composition.

Protein and lactose levels are increased after thawing, probably due to the loss of water during the freezing and thawing process (volatilization), and sublimation. Furthermore, thawing of human milk may cause aggregation of the protein micelles, resulting in a variation of the protein content (30,31).

The fat content decreases significantly following storage, freezing, and thawing processes. The process of freezing and thawing can change the physical and chemical properties of breast milk by favoring the formation of micelles, which can adhere to plastic, facilitating the loss of fat (32,33) especially when administered by continuous infusion. Fat loss is generally attributed to its adherence to the container, to lipolysis, or to lipid oxidation (34).

Most neonatal units freeze expressed breast milk exclusively at -20˚C. According to the international guidelines, the maximum period for freezing time at < -20˚C recommended for both mother`s own milk and donor milk (both pre- and post-pasteurization) is highly variable, ranging between 1 and 12 months (35,36).

Published paper:

- **Paduraru 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* 2019; 24(4):250–257

Objective

(a) to better clarify if fresh mother's milk really differs between term and preterm delivery;

(b) to point out the moment during lactation when the macronutrients provide maximum energy for the neonate, as international consensus has not been reached;

(c) to evaluate the effect of refrigeration and freezing in household condition on macronutrient and energy content of human milk in early stages of lactation.

Material and methods

The study involved mothers with infants born in a level III maternity facility from the eastern area in Romania, who needed neonatal intensive care at the Regional Level III NICU from "Cuza-Vodă" Hospital of Obstetrics and Gynecology, Iasi. This area is characterized by the highest natality rate in the country combined with a low economic level.

The mothers were assigned to the following predefined subject groups: group 1 – 60 mothers who delivered prematurely (gestational age < 37 weeks); group 2 – 30 mothers who delivered at term (gestational age ≥ 37 weeks).

Inclusion criteria were, as follows: mothers of premature or term infants whose lactation started in the first 3 ± 1 day postpartum, aged 16 – 40 years, with normal recovery

after delivery. Exclusion criteria: acute illnesses, treatment with drugs that potentially pass in human milk, chronic treatment for epilepsy, cancer, thyroid conditions, arterial hypertension, localized infections of the breasts.

Subjects were assigned to one of the two groups, according to the gestational age (term or preterm), and the groups were homogenous regarding: maternal age (< 25 or ≥ 25 years), type of delivery (vaginal vs. C-section), parity (primipara or multipara), body mass index (< 25 kg/m^2 or > 25 kg/m^2), previous breastfeeding experience, social status, multiple vs. singleton birth.

We assessed macronutrients (true proteins, carbohydrates, lipids) and energy content, at different postpartum intervals (days): 3 ± 1 (colostrum), 7 ± 1 (transitional milk), 14 ± 1, 21 ± 1, 30 ± 1 (mature milk), and 60 ± 1. Samples of breastmilk were collected by pumping it in the same conditions, between 8:00 and 11:30 a.m., using the electric breast pump (Medela® Symphony Plus, McHenry, IL, USA) available in the unit, and plastic sterile bottles.

Each sample was divided into several aliquots of 2 ml plastic Eppendorf tubes: fresh milk, refrigerated at 4°C and frozen at -20°C. Fresh milk was analyzed the same day, within 2 hours after expression. Refrigerated milk was analyzed within the next 24, 48 and 72 hours, after rewarming at room temperature. Samples of frozen milk were thawed at room temperature: 1, 2, 4, 8, and 12 weeks from sampling, the same way as for feeding the infant in our NICU. For each patient, 54 aliquots were analyzed.

For determination of energy and macronutrient content (true proteins, carbohydrates, lipids) we used the Miris AB® Human Milk Analyser (Uppsala, Sweden). First the sample was brought to room temperature, transferred to a sterile capillary plastic tube, shaken for homogenization, and finally introduced into the device. After warming at 40°C, the sample was analyzed by spectrophotometry and the results were electronically displayed within 2 minutes.

Data were collected in a computer database, together with information about the neonate (gender, gestational age) and the mother (age, body mass index – as marker for nutritional status, parity, social status, financial status, educational level, age).

Statistical analysis used the SPSS V.20.1 (SPSS, Chicago, IL, USA), MATLAB (MathWorks, Natick, MA, USA) software programs. Data were expressed as means ± standard deviation (SD). A paired-samples *t* test was used for comparison of means at different times. Statistical comparisons between all groups were made by analysis of variance (ANOVA). The correlation between parameters was determined by Pearson correlation analysis. Statistical significance was defined as $p < 0.05$.

In order to identify the socio-demographic and clinical factors which can influence the level of macronutrients and energy, we performed a multivariate analysis based on linear regression. Mother's age, parity, social status, financial status, nutritional status (BMI) and child's gender were considered independent variables. The multivariate analysis allowed the development of a model which defines the significant predictive factors that influence the levels of macronutrients and energy. In our study we applied the "ENTER" method, in which independent factors were included in one step. Informed consent was obtained from each subject and the study was approved by the "Grigore T. Popa" University's Ethical Committee.

Results

Analyzing clinical and demographic structure of the two groups, we observed a significant correlation for mother`s age, social status and financial status ($p = 0.03253$, $p = 0.0091$, $p = 0.03110$ respectively). Median GA for term deliveries was 38 weeks vs. 32.5 weeks in premature delivering mothers (Table 5).

Table 5. Clinical and demographic characteristics

Clinical and demographic characteristics	**Group 2** GA ≥ 37 weeks N=30	**Group 1** GA <37 weeks N=60	**95%CI**
Age			
< 25 years	13 (43.3%)	13 (21.7%)	**p= 0.03253***
≥ 25 years	17 (56.7%)	47 (78.3%)	
Gestational age (GA)	Median(IQR) 38 (37-39)	Median(IQR) 32.5 (30.5-33.5)	
Delivery mode			
Vaginal	16 (53.3%)	27 (45%)	p= 0.45561
C-section	14 (46.7%)	33 (55%)	
Newborn gender			
Male	20 (66.7%)	33 (55%)	p= 0.28579
Female	10 (33.3%)	27 (45%)	
Parity			
Primipara	19 (63.3%)	37 (61.7%)	p=0.87782
Multipara	11 (36.7%	23 (38.3%)	
BMI (body mass index)			
<25 Kg/m^2	9 (30%)	26 (43.3%)	p=0.22127
>25 Kg/m^2	21 (70%)	34 (56.7%)	
Residence			
Urban	17 (56.7%)	37 (61.7%)	p=0.64808
Rural	13 (43.3%)	23 (38.3%)	
Educational level			
Primary school	4 (13.3%)	10 (16.7%)	
High school	10 (33.3%)	30 (50%)	p=0.18334
Graduates	16 (53.3%)	20 (33.3%)	
Social status			
Single	3 (10%)	0 (0%)	**p=0.00911***
Married	27 (90%)	60 (100%)	
Financial level**			
Low income	14 (46.7%)	12 (20%)	
Medium income	10 (33.3%)	29 (48.3%)	**p=0.03110***
Good income	6 (20%)	19 (31.7%)	

95% CI: 95% confidence interval;
**p-value <0.05 was considered to be statistically significant;*
***low income: < 100 €/family member/month; medium income: 100-200 €/family member/month; good income: > 200 €/family member/month.*
IQR – interquartile range

Proteins (g/dl)

In fresh milk true proteins constantly decreases during lactation, with a significant difference between the two groups starting with 3 weeks lactation. Preterm milk of day 21 and day 30 has significantly lower proteins than term milk (1.27 vs. 1.43, p = 0.014787 and 1.13 vs. 1.28, p = 0.000867). After day 60 proteins continue to decrease, but with no difference between term and preterm (1.04 vs. 1.10, p = 0.1286) (Tables 6 and 7).

Refrigeration for 72 hours decreased protein content less severely than freezing, although not statistically significant, assuming that in practice human milk can be used after being refrigerated for 3 days, without significant loss of proteins.

Analyzing protein content of each lactation moment separately, we observed that milk samples from D3 (colostrum) significantly lack in protein after 12 weeks of being frozen, those from D7 after 8 weeks, D14 after 4 weeks, D21 after 8 weeks, D30 and D60 after 4 weeks.

This observation may have good practical use: colostrum may be kept frozen for 3 months, whereas milk from D7 - D21 only 2 months, and mature milk expressed after 30 days of lactation can only be kept for 1 month. Analysis of protein content variation with GA in fresh milk revealed positive significant correlations only for colostrum of day 3 (p = 0.001) and mature milk of day 60 (p = 0.039).

Table 6. Mean values, (SD) and confidence intervals for true protein in fresh, refrigerated, and frozen milk in term and preterm human milk

True protein milk (g/dl) Mean (SD)		FM	R24	R48	R72	F1	F2	F4	F8	F12
Term	**d3**	2.03(0.5)	2.12(0.7)	2.03(0.4)	2.02(0.4)	2.01(0.5)	1.91(0.6)	1.83(0.6)	1.89(0.6)	1.84(0.7)
	95%CI	1.9-2.12	1.86-3.28	1.86-2.21	1.85-2.2	1.8-2.22	1.69-2.13	1.61-2.05	1.65-2.13	1.65-2.13
	d7	1.68(0.3)	1.71(0.3)	1.66(0.3)	1.79(0.3)	1.69(0.3)	1.7(0.3)	1.72(0.4)	1.63(0.3)	1.6(0.3)
	95%CI	1.63-1.73	1.59-1.83	1.55-1.76	1.68-1.9	1.59-1.79	1.59-1.81	1.56-1.88	1.51-1.76	1.51-1.76
	d14	1.39(0.2)	1.54(0.4)	1.61(0.6)	1.37(0.3)	1.34(0.2)	1.28(0.2)	1.17(0.2)	1.19(0.1)	1.02(0.1)
	95%CI	1.36-1.42	1.39-1.70	1.39-1.84	1.26-1.48	1.28-1.41	1.22-1.34	1.08-1.26	1.16-1.22	1.16-1.22
	d21	1.43(0.4)	1.47(0.5)	1.39(0.4)	1.33(0.4)	1.41(0.4)	1.38(0.3)	1.26(0.2)	1.13(0.2)	0.91(0.3)
	95%CI	1.37-1.50	1.29-1.65	1.24-1.54	1.18-1.49	1.25-1.58	1.26-1.5	1.2-1.31	1.05-1.22	1.05-1.22
	d30	1.28(0.2)	1.26(0.3)	1.31(0.2)	1.44(0.8)	1.27(0.2)	1.26(0.3)	1.21(0.2)	1.06(0.3)	1.03(0.3)
	95%CI	1.24-1.32	1.16-1.35	1.22-1.4	1.13-1.76	1.18-1.36	1.15-1.36	1.13-1.29	0.94-1.17	0.94-1.17
	d60	1.1(0.2)	1.1(0.2)	1.12(0.2)	1(0.2)	1.07(0.2)	1(0.3)	1.09(0.3)	0.89(0.2)	0.86(0.2)
	95%CI	1.07-1.13	1.02-1.18	1.05-1.2	0.92-1.08	0.99-1.14	0.88-1.12	1.13-1.29	0.81-0.97	0.81-0.97
Preterm	**d3**	1.97(0.4)	1.89(0.4)	1.84(0.4)	1.88(0.4)	1.77(0.4)	1.81(0.4)	1.75(0.4)	1.73(0.4)	1.55(0.5)
	95%CI	1.94-2.00	1.82-1.96	1.77-1.91	1.81-1.95	1.7-1.84	1.73-1.88	1.13-1.29	1.66-1.8	1.66-1.8
	d7	1.6(0.3)	1.62(0.3)	1.61(0.3)	1.61(0.3)	1.58(0.3)	1.58(0.3)	1.55(0.3)	1.48(0.3)	1.44(0.3)
	95%CI	1.58-1.63	1.57-1.68	1.56-1.67	1.55-1.67	1.52-1.63	1.52-1.63	1.13-1.29	1.41-1.54	1.41-1.54
	d14	1.35(0.3)	1.50(0.5)	1.43(0.4)	1.41(0.3)	1.31(0.3)	1.24(0.3)	1.23(0.3)	1.21(0.4)	1.23(0.3)
	95%CI	1.33-1.38	1.40-1.60	1.35-1.5	1.35-1.46	1.24-1.37	1.18-1.3	1.13-1.29	1.14-1.27	1.14-1.27
	d21	1.27(0.3)	1.24(0.3)	1.28(0.2)	1.3(0.3)	1.16(0.3)	1.14(0.2)	1.16(0.3)	1.14(0.3)	1.01(0.3)
	95%CI	1.25-1.30	1.19-1.29	1.23-1.32	1.26-1.35	1.11-1.22	1.1-1.18	1.13-1.29	1.08-1.2	1.08-1.2
	d30	1.13(0.2)	1.14(0.2)	1.16(0.2)	1.26(0.5)	1.12(0.2)	1.08(0.2)	1.05(0.2)	0.96(0.2)	0.92(0.3)
	95%CI	1.11-1.14	1.10-1.18	1.13-1.2	1.17-1.35	1.08-1.16	1.04-1.12	1.13-1.29	0.92-1	0.92-1
	d60	1.04(0.2)	1.05(0.2)	1.00(0.2)	0.93(0.2)	1.01(0.2)	0.94(0.3)	0.96(0.2)	0.83(0.2)	0.71(0.1)
	95%CI	1.03-1.06	1.02-1.09	0.96-1.04	0.9-0.97	0.98-1.05	0.89-0.98	0.91-1	0.79-0.87	0.79-0.87

95%CI=95% Confidence interval; SD=Standard deviation; *Data given as mean (SD)/ 95% Confidence interval for means*
FM: fresh milk; R24, R48, R72: refrigerated for 24, 48, 72 hours; F1, F2, F4, F8, F12: frozen for 1, 2, 4, 8, 12 weeks

Table 7. Significance level (p) in comparison of protein value of breastmilk (preterm vs. term)

Time	p (FM)	p (R24)	p (R48)	p (R72)	p (F1)	p (F2)	p (F4)	p (F8)	p (F12)
	Protein preterm vs. term								
d3	0.452486	0.037064	0.017540	0.081049	0.007787	0.275037	0.399205	0.082363	0.009316
d7	0.234136	0.184957	0.471338	0.009800	0.084454	0.058739	0.016470	0.031604	0.022643
d14	0.552973	0.698839	0.035738	0.514982	0.546276	0.595492	0.390477	0.809993	0.002571
d21	0.014787	0.001013	0.035663	0.615721	0.000629	0.000014	0.071069	0.908406	0.118902
d30	0.000867	0.010839	0.001100	0.119555	0.001620	0.000265	0.000666	0.071860	0.042907
d60	0.128630	0.275248	0.010697	0.081694	0.178838	0.266274	0.011205	0.147801	0.000409

**p-value <0.05 was considered to be statistically significant;*

Carbohydrates (g/dl)

Carbohydrates in fresh milk increased constantly over first two months of lactation. They were significantly higher in preterm than term milk, especially in D7, D21 and D30 (Tables 8 and 9). Milk from D3 doesn`t significantly vary in terms of sugar content, by means of conservation studied, so it can be stored either refrigerated or frozen, with similar carbohydrate content. Only milk from D7 loses carbohydrates if frozen for 3 months, and D60 milk preserves sugar content only for 48 hours when refrigerated and 2 months when frozen.

Table 8. Mean values, (SD) and confidence interval of carbohydrates in fresh, refrigerated and frozen milk in term and preterm human milk

Carbohydrates in milk Mean (SD)		FM	R24	R48	R72	F1	F2	F4	F8	F12
Term	**d3**	5.93(0.9)	6(0.7)	5.97(0.6)	6.13(0.5)	6.13(0.6)	6.21(0.5)	6.12(0.6)	6.27(0.5)	6.21(0.7)
	95%CI	5.6-6.27	5.71-6.29	5.73-6.2	5.94-6.33	5.89-6.38	6-6.42	5.88-6.36	6.05-6.48	5.99-6.43
	d7	6.53(0.6)	6.53(0.6)	6.86(0.4)	6.71(0.3)	6.74(0.5)	6.64(0.4)	6.69(0.6)	6.79(0.5)	6.83(0.6)
	95%CI	6.29-6.78	6.3-6.77	6.7-7.02	6.58-6.84	6.53-6.96	6.48-6.81	6.47-6.91	6.58-6.99	6.62-7.05
	d14	6.72(0.5)	6.6(0.7)	6.36(0.9)	6.37(1.5)	6.8(0.7)	6.84(0.8)	6.76(0.8)	6.68(0.7)	6.68(0.8)
	95%CI	6.52-6.92	6.31-6.89	5.98-6.73	5.78-6.95	6.53-7.07	6.54-7.15	6.45-7.06	6.38-6.97	6.37-6.98
	d21	6.82(0.9)	6.61(0.7)	6.59(1.5)	6.64(1.5)	6.61(1.6)	6.64(1.6)	6.67(1.3)	6.58(1.4)	6.67(1.2)
	95%CI	6.45-7.19	5.95-7.27	5.98-7.2	6.06-7.23	5.98-7.24	6.02-7.27	6.15-7.18	6.02-7.14	6.18-7.16
	d30	6.91(0.7)	6.92(0.7)	6.87(0.8)	6.97(0.8)	6.94(0.8)	6.91(0.8)	6.94(0.8)	6.8(0.9)	6.79(0.8)
	95%CI	6.62-7.2	6.62-7.22	6.57-7.17	6.65-7.28	6.63-7.26	6.61-7.22	6.63-7.26	6.47-7.13	6.48-7.1
	d60	7.16(0.6)	7.14(0.7)	7.1(0.7)	7.08(0.7)	7.12(0.8)	7.09(0.8)	6.94(0.8)	7.03(0.9)	7.02(0.9)
	95%CI	6.91-7.4	6.88-7.41	6.82-7.38	6.81-7.35	6.81-7.43	6.77-7.41	6.62-7.27	6.69-7.38	6.67-7.37
Preterm	**d3**	6.09(0.8)	6.19(0.7)	6.24(0.7)	6.24(0.8)	6.36(0.8)	6.3(0.8)	6.31(0.8)	6.36(0.9)	6.44(0.9)
	95%CI	5.94-6.24	6.05-6.33	6.11-6.38	6.1-6.38	6.22-6.5	6.15-6.45	6.16-6.46	6.2-6.52	6.27-6.61
	d7	6.81(0.6)	6.85(0.4)	6.86(0.4)	6.84(0.4)	6.89(0.4)	6.95(0.4)	6.98(0.3)	7.02(0.4)	7.1(0.5)
	95%CI	6.7-6.91	6.78-6.92	6.79-6.94	6.77-6.91	6.82-6.96	6.87-7.02	6.92-7.04	6.95-7.09	7-7.2
	d14	6.76(0.8)	6.79(0.8)	6.84(0.8)	6.96(0.7)	7(0.6)	6.97(0.7)	6.95(0.8)	7.1(0.7)	7.11(0.6)
	95%CI	6.61-6.92	6.64-6.94	6.7-6.98	6.84-7.08	6.88-7.12	6.84-7.11	6.8-7.09	6.97-7.24	6.99-7.23
	d21	7.11(0.3)	7.15)0.3)	7.1(0.3)	7.07(0.3)	7.18(0.3)	7.23(0.3)	7.27(0.3)	7.23(0.4)	7.32(0.4)
	95%CI	7.05-7.17	7.09-7.21	7.04-7.16	7.01-7.13	7.13-7.24	7.17-7.28	7.22-7.33	7.16-7.3	7.24-7.39
	d30	7.16(0.3)	7.16(0.3)	7.14(0.3)	7.1(0.5)	7.25(0.3)	7.23(0.3)	7.21(0.3)	7.27(0.4)	7.18(0.4)
	95%CI	7.11-7.22	7.1-7.22	7.07-7.21	7.01-7.19	7.2-7.31	7.18-7.29	7.15-7.27	7.2-7.33	7.09-7.26
	d60	7.3(0.3)	7.28(0.3)	7.24(0.2)	7.13(0.4)	7.32(0.3)	7.28(0.3)	7.23(0.3)	7.21(0.4)	7.09(0.5)
	95%CI	7.24-7.35	7.23-7.33	7.2-7.28	7.06-7.19	7.27-7.37	7.22-7.34	7.17-7.29	7.14-7.28	7-7.18

95%CI=95% Confidence interval; SD=Standard deviation; *Data given as mean (SD)/ 95% Confidence interval for means*

FM : fresh milk; R24, R48, R72: refrigerated for 24, 48, 72 hours; F1, F2, F4, F8, F12: frozen for 1, 2, 4, 8, 12 weeks.

Table 9. Significance level (p) in comparison of carbohydrates value of breast milk

Time	p (FM)	p (R24)	p (R48)	p (R72)	p (F1)	p (F2)	p (F4)	p (F8)	p (F12)
	Carbohydrates: preterm vs. term								
d3	0.373007	0.226753	0.065425	0.483000	0.155768	0.587496	0.245837	0.584149	0.206447
d7	0.027896	0.000689	0.938911	0.095511	0.101419	0.000603	0.000341	0.007384	0.023508
d14	0.792469	0.272725	0.005203	0.001911	0.141204	0.408060	0.260003	0.007522	0.003220
d21	0.007829	0.001736	0.001392	0.006185	0.000514	0.000372	0.000014	0.000027	0.000010
d30	0.005298	0.010702	0.008667	0.282157	0.001049	0.000626	0.006429	0.000017	0.000813
d60	0.077992	0.095418	0.079975	0.598849	0.031291	0.043011	0.003673	0.010783	0.592633

**p-value <0.05 was considered to be statistically significant;*

Lipids (g/dl)

In fresh milk from term mothers, the lipid content increases constantly during the first month, followed by a slight decrease over the following 30 days to a value similar to that on day 21 of lactation (Table 10).

Table 10. Mean values (SD) and confidence interval for lipid values (preterm vs. term)

Lipids in milk Mean (SD)		FM	R24	R48	R72	F1	F2	F4	F8	F12
Term	**d3**	2.39(0.8)	2.53(0.7)	2.28(0.7)	2.37(0.9)	2.37(0.7)	2.22(0.8)	2.23(0.6)	2.13(0.6)	2.2(0.8)
	95%CI	2.06-2.71	2.26-2.8	1.99-2.56	2.03-2.7	2.07-2.66	1.9-2.54	1.98-2.49	1.89-2.37	1.87-2.53
	d7	3.22(0.7)	3.22(0.6)	3.32(0.7)	3.23(0.9)	3.08(0.5)	3.32(0.6)	3.12(0.5)	3.13(0.7)	3.16(0.5)
	95%CI	2.96-3.49	2.98-3.46	3.04-3.61	2.87-3.59	2.87-3.28	3.08-3.57	2.93-3.32	2.86-3.41	2.97-3.35
	d14	3.32(1)	3.17(1.3)	3.9(1)	3.46(0.8)	3.4(0.8)	3.24(0.9)	3.24(0.7)	3.16(0.7)	2.99(0.6)
	95%CI	2.93-3.72	2.67-3.67	3.49-4.31	3.15-3.76	3.08-3.72	2.91-3.58	2.95-3.54	2.88-3.43	2.75-3.23
	d21	3.64(1.1)	3.56(1.2)	3.46(1.1)	3.52(1)	3.47(1.2)	3.4(1.2)	3.24(0.8)	3.21(0.9)	3.06(0.8)
	95%CI	3.2-4.09	3.08-4.03	3.01-3.9	3.14-3.9	3-3.93	2.94-3.86	2.92-3.57	2.83-3.59	2.75-3.36
	d30	3.89(0.7)	3.83(0.8)	3.44(0.9)	3.77(0.7)	3.83(0.7)	3.79(0.6)	3.67(0.7)	3.33(0.7)	2.96(1)
	95%CI	3.62-4.16	3.53-4.13	3.08-3.8	3.49-4.05	3.55-4.12	3.55-4.03	3.4-3.93	3.07-3.59	2.55-3.38
	d60	3.68(0.9)	3.66(0.9)	3.61(0.9)	3.68(0.7)	3.5(0.8)	3.44(0.8)	3.26(0.9)	3.13(0.8)	2.94(0.7)
	95%CI	3.33-4.02	3.3-4.01	3.27-3.95	3.38-3.97	3.17-3.83	3.13-3.76	2.91-3.6	2.81-3.46	2.68-3.21
Preterm	**d3**	2.56(1)	2.56(0.9)	2.42(0.8)	2.45(0.8)	2.47(0.8)	2.47(0.8)	2.38(0.9)	2.38(0.8)	2.29(0.8)
	95%CI	2.38-2.75	2.39-2.73	2.26-2.57	2.3-2.6	2.32-2.63	2.32-2.63	2.21-2.54	2.23-2.53	2.15-2.44
	d7	3.23(0.9)	3.24(0.9)	3.15(0.9)	3.13(0.9)	3.1(0.8)	3.11(0.8)	3.02(0.8)	3.06(0.9)	2.96(0.9)
	95%CI	3.06-3.41	3.07-3.42	2.98-3.33	2.97-3.3	2.94-3.25	2.96-3.27	2.87-3.18	2.89-3.22	2.78-3.14
	d14	3.48(1.3)	3.37(1.4)	3.43(1.2)	3.24(1.2)	3.23(1.1)	3.17(1.2)	3.15(1.1)	3(1)	2.71(0.9)
	95%CI	3.24-3.72	3.11-3.63	3.2-3.66	3.02-3.46	3.02-3.44	2.95-3.39	2.95-3.36	2.81-3.2	2.55-2.87
	d21	3.47(1.1)	3.32(1)	3.31(1)	3.19(1)	3.14(0.9)	3.11(1)	3.03(1.4)	2.87(0.8)	2.71(0.8)
	95%CI	3.27-3.67	3.14-3.51	3.11-3.5	3.01-3.37	2.96-3.31	2.93-3.29	2.78-3.29	2.72-3.01	2.56-2.85
	d30	3.36(1.2)	3.3(1.2)3	3.27(1.2)	3.27(1.2)	3.21(1.3)	3.16(1.2)	3.12(1.2)	2.86(1)	2.86(1)
	95%CI	3.14-3.59	.06-3.54	3.05-3.5	3.04-3.51	2.97-3.45	2.93-3.38	2.88-3.35	2.66-3.05	2.67-3.06
	d60	3.57(1)	3.55(1)	3.39(1)	3.2(0.8)	3.44(0.9)	3.34(0.9)	3.29(0.9)	3.18(0.8)	3.01(0.8)
	95%CI	3.38-3.76	3.37-3.73	3.2-3.57	3.05-3.35	3.26-3.61	3.17-3.5	3.12-3.46	3.02-3.34	2.86-3.15

95%CI=95% Confidence interval; SD=Standard deviation; *Data given as mean (SD)/ 95% Confidence interval for means*
FM : fresh milk; R24, R48, R72: refrigerated for 24, 48, 72 hours; F1, F2, F4, F8, F12: frozen for 1, 2, 4, 8, 12 weeks.

For preterm milk the lipid content is more variable, with a significant increase during the first two-three weeks, followed by a slight decrease at the end of the first month and another significant increase during the second month. Also, the data shows that preterm milk has a constantly higher fat content compared to term milk, the maximum concentration being reached on day 60 for preterm and on day 30 for term.

Mature milk shows significant differences in term of lipid content between term and preterm and this remains true regardless of the preservation method (Table 11).

Refrigeration of term milk for up to 72 hours doesn't significantly change the fat content for colostrum, transitional or mature milk. For preterm milk however, only colostrum maintains an adequate lipid content after refrigeration for 72 hours, but transitional and mature milk stored in the fridge for over 24 hours no longer has an appropriate lipid content. Freezing for more than 2 weeks generates a significant loss in lipids.

Table 11. Significance level (p) in comparison of lipids value of breast milk (preterm vs. term)

Time	p (FM)	p (R24)	p (R48)	p (R72)	p (F1)	p (F2)	p (F4)	p (F8)	p (F12)
	Lipids: preterm vs. term								
d3	0.389291	0.886684	0.430085	0.643270	0.551146	0.159565	0.418425	0.133901	0.577663
d7	0.956793	0.911401	0.372520	0.595416	0.906629	0.215133	0.555568	0.683510	0.296787
d14	0.542944	0.478570	0.068477	0.353896	0.459080	0.766445	0.684568	0.471562	0.107909
d21	0.440491	0.289786	0.512302	0.106979	0.121409	0.183550	0.441732	0.057842	0.034889
d30	0.029723	0.035316	0.482742	0.048154	0.014584	0.009648	0.027606	0.025297	0.652112
d60	0.606927	0.594700	0.273032	0.004623	0.749445	0.555660	0.863894	0.801191	0.707189

**p-value <0.05 was considered to be statistically significant;*

Energy content (cal/100 ml)

The caloric content is mainly linked to the fat level and the other nutrients, with variable weight factor, complete and balance the energy value.

The energy content of breast milk increases steadily during lactation, but there were no statistically significant differences between term and preterm (Tables 12 and 13).

There was no statistical difference concerning the energy value, regardless of the preservation method between term and preterm milk.

Refrigeration for up to 72 hours did not change significantly the energy value of colostrum or transitional milk, either term or preterm, but for term deliveries, mature milk maintains appropriate energy levels when refrigerated for a maximum of 48 hours.

Also, the data showed that for term deliveries, either colostrum, transitional and term milk can be preserved frozen for 2 weeks, but loses significantly its energy value when frozen for 4, 8 and 12 weeks respectively. There are no important differences between term and preterm deliveries in term of caloric values of human milk in early lactation.

Predictive factors

Mother's age and financial status are important factors which influence the level of all macronutrients in breast milk. Young mothers' milk had more protein ($\beta = -0.103$, $p = 0.008$), fat ($\beta = -0.435$, $p = 0.028$) and calories ($\beta = -0.486$, $p = 0.025$) compared to those over 25 years of age, but lower levels of carbohydrates ($\beta = 0.315$, $p = 0.022$) (Table 14).

Also, mothers with a better financial status had a higher protein content ($\beta = 0.049$, $p = 0.017$), fat ($\beta = 0.175$, $p = 0.001$) and energy ($\beta = 1.370$, $p = 0.005$), but less carbohydrates ($\beta = -0.074$, $p = 0.038$) in their milk.

Marital status was statistically correlated only with the carbohydrate content of the milk - single mothers had more carbohydrates than those who were married ($\beta = -0.363$, $p = 0.047$).

Table 12. Mean values (SD) and confidence interval for energy values (preterm vs. term)

Energy milk Mean (SD)		FM	R24	R48	R72	F1	F2	F4	F8	F12
Term	**d3**	59.44 (7.7)	59.33 (6.6)	56.44 (7.4)	57 (8.1)	57.11 (7.4)	56.56 (9)	56.22 (8.5)	55 (6.5)	53.67 (6.9)
	95% CI	56.42-62.47	56.73-61.94	53.5-59.39	53.81-60.19	54.18-60.04	52.99-60.12	52.86-59.59	52.45-57.55	50.93-56.41
	d7	65.11 (7.2)	65.22 (6)	67.33 (7.8)	66.89 (7.2)	64.44 (5.7)	66.44 (6.3)	65 (4.5)	65.11 (7.1)	65.33 (4.8)
	95% CI	62.28-67.94	62.84-67.61	64.24-70.43	64.04-69.74	60.09-68.79	63.94-68.94	63.21-66.79	62.31-67.91	63.44-67.22
	d14	64.67 (8.8)	63.78 (9.4)	70.11 (9.4)	64.78 (6.2)	65.44 (7.1)	64 (7)	63.78 (6.7)	62.56 (5.7)	60.67 (4.2)
	95% CI	61.18-68.15	60.07-67.49	66.39-73.83	62.33-67.22	60.01-70.88	61.23-66.77	61.15-66.41	60.29-64.82	59.01-62.32
	d21	67.44 (7.7)	66.89 (8.3)	65.78 (7.7)	65.56 (6.7)	65.44 (9.2)	65.33 (8.6)	64 (7.9)	63.44 (6.9)	61.11 (7)
	95% CI	64.41-70.48	63.62-70.16	62.71-68.84	62.89-68.22	58.35-72.54	61.95-68.72	60.87-67.13	60.74-66.15	58.33-63.89
	d30	70.11 (6.1)	70 (6.6)	65.78 (8.1)	65.89 (8.7)	69.44 (6.2)	68.89 (5.6)	67.44 (6)	64.44 (5.8)	64 (4.5)
	95% CI	67.7-72.52	67.41-72.59	62.58-68.97	62.47-69.31	64.67-74.22	66.69-71.09	65.05-69.83	62.14-66.75	62.21-65.79
	d60	68.67 (7.3)	68.56 (7.2)	68.11 (6.9)	64.78 (6)	66.22 (7.2)	65.22 (6.9)	63.22 (7.2)	62.22 (6.2)	60.67 (5)
	95% CI	65.8-71.54	65.72-71.39	65.39-70.83	62.41-67.15	60.7-71.75	62.51-67.93	60.37-66.08	59.76-64.68	58.71-62.62
Preterm	**d3**	60.84 (9.4)	59.89 (9)	58.46 (8.3)	58.7 (8.1)	58.86 (8.5)	59.11 (8.1)	58.14 (8.3)	57.92 (7.7)	57.05 (7.7)
	95% CI	59.07-62.6	58.21-61.58	56.9-60.01	57.17-60.23	57.27-60.46	57.58-60.64	56.58-59.69	56.47-59.37	55.6-58.51
	d7	66.3 (8.6)	66.14 (8.9)	65.54 (8.8)	65.19 (8.5)	64.49 (8.4)	65.16 (8)	64.43 (7.9)	64.46 (8.3)	64.3 (8.9)
	95% CI	64.68-67.92	64.47-67.8	63.89-67.2	63.6-66.78	61.7-67.27	63.66-66.66	62.94-65.92	62.89-66.03	62.62-65.98
	d14	66.73 (11.5)	66.46 (13.3)	66.51 (10.4)	65.46 (10.8)	64.89 (10.6)	64.11 (11.2)	63.14 (10)	62.27 (9.9)	61.84 (7.9)
	95% CI	64.56-68.9	63.95-68.96	64.57-68.46	63.44-67.48	61.37-68.41	62-66.22	61.25-65.02	60.42-64.12	60.36-63.32
	d21	67.59 (9.8)	66.14 (9.1)	66.03 (9.4)	65.03 (8.7)	64.05 (9.1)	63.65 (7.8)	64.7 (12.1)	61.89 (7)	60.86 (7.9)
	95% CI	65.76-69.43	64.43-67.84	64.27-67.79	63.38-66.67	61.04-67.07	62.18-65.12	62.43-66.98	60.58-63.21	59.37-62.36
	d30	66.35 (11.5)	66.05 (11.7)	65.49 (10.7)	65.03 (12.1)	65 (11.7)	64.51 (11.6)	64 (11.3)	62.41 (10.2)	60.86 (8.8)
	95% CI	64.2-68.51	63.86-68.25	63.48-67.49	62.75-67.3	61.11-68.89	62.33-66.69	61.87-66.13	60.49-64.32	59.21-62.52
	d60	69.46 (9.9)	69.62 (9.7)	67.3 (10)	64.43 (8.5)	68.32 (9.7)	67.24 (9.4)	66.03 (9.3)	64.27 (8.1)	60.43 (7.2)
	95% CI	67.59-71.33	67.79-71.45	65.43-69.17	62.83-66.03	65.11-71.54	65.48-69.01	64.29-67.77	62.76-65.78	59.08-61.79

95%CI=95% Confidence interval; SD=Standard deviation; *Data given as mean (SD)/ 95% Confidence interval for means*

FM : fresh milk; R24, R48, R72: refrigerated for 24, 48, 72 hours; F1, F2, F4, F8, F12: frozen for 1, 2, 4, 8, 12 weeks.

Table 13. Significance level (p) in comparison of energy value of breast milk (preterm vs. term)

Time	p (FM)	p (R24)	p (R48)	p (R72)	p (F1)	p (F2)	p (F4)	p (F8)	p (F12)
	Energy: preterm vs.term								
d3	0.475729	0.761241	0.249240	0.330420	0.324687	0.154414	0.286456	0.071090	0.038912
d7	0.508803	0.613632	0.334203	0.337534	0.980046	0.438671	0.721251	0.708669	0.561900
d14	0.386051	0.325226	0.101819	0.752151	0.794651	0.961906	0.751898	0.885379	0.457000
d21	0.940767	0.693713	0.898357	0.769678	0.470258	0.326243	0.774802	0.301030	0.882770
d30	0.102027	0.093277	0.894459	0.727580	0.055534	0.058760	0.129266	0.320133	0.076069
d60	0.697107	0.593700	0.688454	0.842340	0.284220	0.294849	0.144221	0.219411	0.873388

**p-value <0.05 was considered to be statistically significant;*

The protein levels in the breast milk was statistically correlated with the nutritional status of the mother ($\beta = 0.140$, $p = 0.001$), higher for overweight and obese mothers (BMI < 25 Kg/m^2), fact that confirm data from other studies (37–40). Carbohydrates, lipids and energy levels were not influenced by the nutritional status of the mother.

Fat content and energy were positively correlated with the number of pregnancies ($\beta = 0.360$, $p < 0.001$ and $\beta = 3.717$, $p = 0.001$, respectively), but parity had no statistically significant influence on protein and carbohydrate levels in breast milk.

The infant's gender did not influence the carbohydrate, fat and energy content, but our data showed that mothers who gave birth to male infants had significantly more protein in their milk than those who had female infants ($\beta = -0.092$, $p = 0.003$). These data are consistent with recent studies (41). What happens with human milk protein content from mothers who deliver twins/triplets (both males and females) and where is situated their protein level are issues to be further studied.

Discussions

Although some initial studies considered human milk as relatively homogenous (42), lately, in many countries several studies revealed large variations in macronutrients (43–45) within the same feed, over lactation, diurnally, and between mothers and different populations. Also the expression and methods for storage the milk, together with other maternal factors like socioeconomic status, previous breastfeeding, age and nutritional status may influence milk composition; especially macronutrient composition differs between preterm and term milk, with preterm milk tending to be higher in protein and fat early in lactation (46). According to other studies protein levels decrease in human milk over the first 4 to 6 weeks or more of life, regardless of the timing of delivery (47).

We studied a period of refrigeration of 72 hours and found that overall macronutrients integrity and energy were preserved with minimal changes, in order to be used in the NICU and at home, similar with Slutzah (48) who studied a 96 hours period and recommended to use fresh milk refrigerated for 4 days at 4°C.

Preterm milk of day 21 and day 30 has significantly lower proteins than term milk. This finding is inconsistent with other data (46) and may be influenced by specific demographic aspects, ethnic differences, nutritional intake or nutritional status.

The energy content of human milk is mostly dependent on the overall fat content (49). There are many studies on breastmilk fatty acids composition, but the results are not consistent. Aydin (50) found increased alpha-linolenic acid from lipids levels in the preterm group suggesting that breastmilk, and especially early infancy period colostrum, may have an important role for preterm nutrition. We found an increased content of lipids in fresh milk during the first month of lactation that adds more benefits for the breastfed infants. Moreover, this content does not change significantly during preservation for term infants, while mothers of premature infants should keep colostrum for only 24 hours in the refrigerator.

Table 14. Multiple linear regression method regarding the factors that influence the level of macronutrients and energy in HM

Protein Model[a] Predictors	Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	T	p-Sig. level
Mothers' age	-.103	.039	-.104	-2.650	.008
Social status	.131	.106	.045	1.233	.218
Financial status	.049	.021	.087	2.393	.017
Parity	-.018	.031	-.021	-.581	.561
BMI	.140	.032	.163	4.379	.000
Infants' gender	-.092	.031	-.107	-2.979	.003
a. Dependent Variable: protein					
$R=0.786$, $R^2=0.618$, for $p<0.00000001$ we noted $p<<0.001$					
Carbohydrates Model[a] Predictors	Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	T	p-Sig. level
Mothers' age	.315	.067	.009	.225	.022
Social status	-.363	.183	-.074	-1.991	.047
Financial status	-.074	.035	-.077	-2.077	.038
Parity	-.040	.052	-.027	-.755	.451
BMI	-.086	.055	-.060	-1.565	.118
Infants' gender	.011	.053	.008	.217	.828
a. Dependent Variable: carbohydrates					
$R=0.560$, $R^2=0.314$, for $p<0.00000001$ we noted $p<<0.001$					
Lipids Model[a] Predictors	Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	T	p-Sig. level
Mothers' age	-.435	.101	-.014	-.348	.028
Social status	.024	.276	.003	.086	.932
Financial status	.175	.054	.119	3.259	.001
Parity	.360	.079	.163	4.542	.000
BMI	.065	.083	.029	.786	.432
Infants' gender	-.122	.080	-.055	-1.524	.128
a. Dependent Variable: lipid					
$R=0.726$, $R^2=0.528$, for $p<0.00000001$ we noted $p<<0.001$					
Energy Model[a] Predictors	Unstandardized Coefficients B	Std. Error	Standardized Coefficients Beta	T	p-Sig. level
Mothers' age	-.486	.926	-.312	-3.309	.025
Social status	-.636	2.524	-.009	-.252	.801
Financial status	1.370	.490	.102	2.797	.005
Parity	3.717	.725	.184	5.129	.000
BMI	1.084	.758	.054	1.430	.153
Infants' gender	-1.094	.731	-.054	-1.498	.135
a. Dependent Variable: energy					
$R=0.709$, $R^2=0.504$, for $p<0.00000001$ we noted $p<<0.001$					

Bitman (51) did not find any difference when comparing fatty acids composition of breast milk on day 42 of lactation in women giving birth to various degrees of premature infants. Paul (1997) reported a significant increase in lipid concentration with the progression

of lactation, but no significant difference between term and preterm milk. Genczel-Boroviczeny (1997) did not find any differences on similar days (5, 10, 20, and 30) of lactation in mothers of preterm as compared to term infants. Controversies started when, contrary to all of them, Luukkainen (52) reported significantly higher contributions of C20:4 and C22:6 to the FA composition of breast milk in mothers of preterm rather than term infants, and Kovacs (53) reported significant differences in long chain PUFA (LCPUFA), C20:4, and C22:6 between term and preterm breast milk. Recently Jackson (54) used dried spot milk samples to analyze DHA (an important component of fatty acids) in human milk, with proven benefits for neurodevelopmental outcome of the infants and found no variation in composition compared with fresh liquid milk samples.

There is a large variation in term of macronutrients in human milk and the literature is abundant. Many factors may influence this, but almost all authors affirms that population type, geographical area and nutritional habits generates wide differences between studies. Method used for determinations, type of containers (plastic or glass), thawing process contributes also. Other measurable conditions like BMI, age, financial status, parity may influence in various degrees. We think that a larger multicentric randomized trial based on a similar population in terms of race, habits, traditions from our geographic area, should be performed with results that can better clarify our findings.

Limitations

The major limitation of our study is the lack of information about the nutritional intake of the mothers; it would have been useful to examine how the mothers' intake of macronutrients and energy affected the levels of these nutrients in their milk. Human milk protein content from mothers who deliver twins or triplets (both males and females) was not addressed by our study but is worthy of further evaluation.

Conclusions

Protein content in fresh milk varies from term to preterm mothers in an inconsistent manner, influenced by multiple factors and conditions, mainly BMI, mother`s age, financial status and infant's gender. Fresh milk is the best option if available. Refrigeration for up to 72 hours is more benefic than freezing longer than 2 weeks. Using frozen milk for 12 weeks at -20^{0}C should be avoided as it contains significantly less protein, lipids and energy. Human milk remains the best nutrient and milk bank would be much beneficial either for term and preterm neonates.

I.1.3. Research concerning total antioxidant status in fresh and stored human breastmilk

Free radicals are highly reactive molecules containing one or more unpaired electrons. They donate or gain electrons from other molecules in an attempt to pair their electrons and generate a more stable species (55). Free radicals are normally produced in living organisms. Animal studies proved that, when produced in physiological concentrations, reactive oxigen species behave as important mediators of almost all cell functions. On the other hand, when excessively produced, they induce oxidative stress, which is responsible for cell and tissue injury (56). Free radical reactions may cause alterations of macromolecules, such as polyunsaturated fatty acids and proteins (57). Under physiological circumstances, free radicals are kept under control by an adequate antioxidant system whose activation depends upon the entity of the oxidative injury itself. Both in human and veterinary medicine, oxidative stress may be a cofactor in the development of many neonatal dysfunctions, leading to serious systemic effects and impairing vitality (57,58).

Breast milk has been proven to have important and essential antioxidant composition to prevent and protect against diseases in infancy.

Many components of milk change with storage, including immune cells, which get inactivated by freezing (59). Lipid peroxides formation in human milk stored at low temperatures was documented, probably caused by an increased presence of free fatty acids due to lipoprotein lipase activity during storage (60). This could be ascribed to the higher susceptibility of human milk to degradation, process that is not demonstrated in formula milk (61).

Miranda *et al* found an increase of malondialdehyde, a marker of oxidative stress, in refrigerated milk, but not in frozen samples and a decrease of glutathione peroxidase activity in both refrigerated and frozen samples of human milk (62).

Published paper:

- **Paduraru L**, Dimitriu DC, Avasiloaiei AL, Moscalu M, Zonda GI, Stamatin M. Total antioxidant status in fresh and stored human milk from mothers of term and preterm neonates. *Pediatrics & Neonatology* 2018; 59(6):600-605

Objective

The main goal was to determine the total antioxidant status (TAS) of human milk and evaluate the differences between premature milk and term milk at different moments of lactation (colostrum, transitional milk and mature milk). A second objective was to evaluate how TAS varies whether the human milk is refrigerated (+4°C) or frozen in domestic conditions (-20°C), as is in our routine practice, in the absence of a milk bank.

Material and methods

The study involved 2 groups of lactating mothers admitted to a level III maternity hospital from eastern Romania, area with the highest natality rate, but with low income and poor economic status: group 1 enrolled 60 mothers who gave birth prematurely (< 37 weeks gestation) and group 2 included 30 mothers who delivered term infants (≥ 37 weeks gestation).

There were large inclusion criteria: mother of premature or term infants whose lactation started in the first 3 ±1 days postpartum, aged 16-40 years, with normal recovery after delivery. Cases with any acute illness, treatment with antibiotics or any drugs that potentially pass in human milk, chronic treatment for epilepsy, cancer, thyroid conditions, arterial hypertension, breast infections, were excluded.

Human milk from days 3 ±1 (colostrum), 7 ±1 (transitional milk), and 30 ±1 (mature milk) was pumped in the same conditions, between 8:00 and 11:30 a.m., using the electric breast pump (Medela® Symphony Plus, McHenry, IL, USA) and was divided in 5 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 2 hours.

TAS was measured from the following milk types: fresh (labelled FM), refrigerated for 24 h (labelled R24) and 72 h (R72) and frozen for 1 week (F1) and 12 weeks (F12). For each patient a total of 15 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 only few samples were retested, as there were very few values less than 2.5 mmol/L.

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

Statistical analysis used SPSS V.20.1 (SPSS, Chicago, IL, USA), MATLAB (MathWorks, Natick, MA, USA) software programs. The data were expressed as means ± standard deviation (SD). A paired-samples *t* test was used for comparison of means at different times. Statistical comparisons between all groups were made by analysis of variance (ANOVA). The correlation between parameters was determined by Pearson correlation analysis. Statistical significance was defined as $p < 0.05$.

For the multivariate analysis, we used „ENTER" method, where independent factors were included in a single step. The effect of independent variables on TAS values that was considered dependent variable was described by unstandardized coefficients B, standardized coefficient Beta and p value. The association between independent variables and TAS values was expressed in regression model described, by determination coefficient (R^2).

Results

The main characteristics of the 2 groups are briefly presented in Table 15.

Table 15. Description of the study groups

Characteristics	Group 1 N=60	Group 2 N=30	95%CI
Mother`s age			
< 25 y	13 (21.7%)	13 (43.3%)	**p= 0.03253**
≥ 25 y	47 (78.3%)	17 (56.7%)	
Gestational age Median (IQR)	32.5 (30.5-33.5)	38 (37-39)	
Delivery route			
Vaginal	27 (45%)	16 (53.3%)	p= 0.45561
C – section	33 (55%)	14 (46.7%)	
Gender of newborn			
Male	33 (55%)	20 (66.7%)	p= 0.28579
Female	27 (45%)	10 (33.3%)	
Parity			
Primipara	37 (61.7%)	19 (63.3%)	p=0.87782
Multipara	23 (38.3%)	11 (36.7%	
Body Mass Index (BMI)			
<25 Kg/m^2	26 (43.3%)	9 (30%)	p=0.22127
>25 Kg/m^2	34 (56.7%)	21 (70%)	
Residence			
Urban	37 (61.7%)	17 (56.7%)	p=0.64808
Rural	23 (38.3%)	13 (43.3%)	
Educational level			
Primary	10 (16.7%)	4 (13.3%)	p=0.18334
High school	30 (50%)	10 (33.3%)	
Graduate level	20 (33.3%)	16 (53.3%)	
Marital status			
Single	0 (0%)	3 (10%)	**p=0.00911**
Couple/married	60 (100%)	27 (90%)	
Financial level*			
Low	12 (20%)	14 (46.7%)	**p=0.03110**
Medium	29 (48.3%)	10 (33.3%)	
Good	19 (31.7%)	6 (20%)	

*low: < 100 €/family member/month; medium: 100-200 €/family member/month; good: > 200 €/family member/month.
IQR – interquartile range

Mother`s age, marital and financial status were significantly different between the two groups (p = 0.03253, p =0.00911, p =0.03110, respectively). The groups were homogenous regarding delivery route, gender of the neonate, parity, BMI, residence, educational level.

The mean values of TAS in the two groups increased from day 3 to day 30 (Table 16).

Table 16. Mean values, (SD) and CI for TAS (Premature vs. term mothers)

TAS in human milk Mean (SD) 95%CI		**FM**	**R24h**	**R72h**	**F1**	**F12**
Term	**d3**	1.39(0.3)	1.36(0.3)	1.33(0.4)	0.93(0.2)	0.74(0.2)
	95%CI	1.28-1.51	1.24-1.48	1.17-1.48	0.86-0.99	0.86-0.99
	d7	2.1(1.7)	2.21(1.3)	2.38(2.1)	0.97(0.6)	0.68(0.3)
	95%CI	1.43-2.77	1.68-2.73	1.54-3.22	0.72-1.22	0.72-1.22
	d30	2.55(1.7)	2.39(1.6)	2.3(2)	1.51(0.7)	0.9(0.4)
	95%CI	1.86-3.23	1.74-3.03	1.52-3.08	1.25-1.77	1.25-1.77
Preterm	**d3**	1.27(0.3)	1.24(0.3)	1.23(0.3)	1.06(0.4)	0.81(0.3)
	95%CI	1.2-1.33	1.18-1.3	1.17-1.29	0.99-1.13	0.99-1.13
	d7	2.03(1.8)	1.9(1.3)	1.78(1.4)	0.99(0.4)	0.79(0.3)
	95%CI	1.7-2.36	1.65-2.15	1.52-2.05	0.93-1.06	0.93-1.06
	d30	1.95(1.2)	1.79(1.2)	1.74(1.2)	1.16(0.6)	0.8(0.4)
	95%CI	1.73-2.18	1.56-2.01	1.52-1.96	1.06-1.27	1.06-1.27

95% CI=95% Confidence interval; SD=Standard deviation; *Data given as mean (SD)/ 95% Confidence interval for means*

FM: fresh milk; R24, R72: milk refrigerated for 24, 72 hours; F1,F12: milk frozen for 1, 12 weeks.

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. 17).

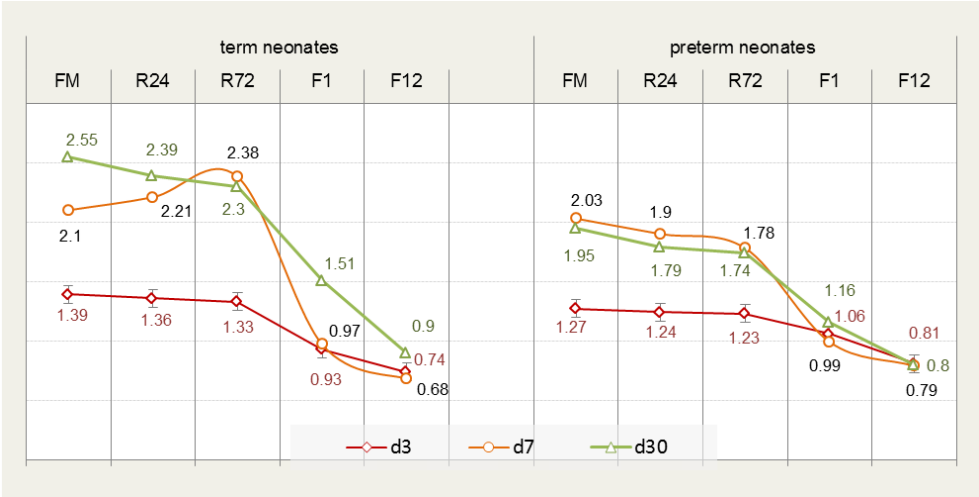

Fig. 17. TAS dynamics depending on storage method of different types of human milk

Term (group 2) FM has 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 = 0.038978). A similar pattern was maintained if the milk was refrigerated 24 hours (p = 0.033047) or frozen 1 week (p = 005407). For R72 the differences were maintained, but were not statistically significant (Table 17).

Table 17. Level of significance (p) in comparison of TAS values in HM from term and preterm mothers

Day of lactation	p* (fresh milk)	p* (R 24h)	p* (R 72h)	p* (F1)	p* (F12)
		TAS: preterm vs. term			
D3	0.079330	0.081682	0.180936	0.055115	0.201615
D7	0.860016	0.285988	0.078413	0.771669	0.095554
D30	0.038978*	0.033047*	0.056289	0.005407*	0.235300

**p-value <0.05 was considered to be statistically significant;*

The multivariate analysis showed that TAS was influenced by mother`s age and parity: mothers > 25 years had a significant higher TAS than those < 25 years (ß = 0.517, p = 0.013) and multipara higher than primipara (ß = -0.226, p < 0.00001) (Table 18). These findings need to be confirmed by further studies in order to be better explained and understood. Neither social, nor financial status had any influence on TAS.

Table 18. Multiple linear regression method concerning factors that influence TAS values

TAS **Model[a]**	**Unstandardized Coefficients**		**Standardized Coefficients**	**t**	**p-Sig. level**
Predictors	**B**	**Std. Error**	**Beta**		
Mother`s age	**.408**	**.164**	**.517**	**5.046**	**.013**
Social status	.539	.460	.059	1.171	.242
Financial status	-.086	.090	-.048	-.953	.341
Parity	**-.607**	**.135**	**-.226**	**-4.500**	**.000**

a. Dependent Variable: TAS

$R=0.671$, $R^2=0.451$, for $p<0.00000001$ we used $p<<0.001$

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 (Fig. 17). These data suggest that 24 h refrigeration is better than 72 hours, but 72 h of refrigeration for human milk remains a reasonable time in order to avoid severe decreases in TAS concentration.

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 D3 and D7 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 significant 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).

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 (knowing that in premature infants excess oxygen can secondarily generate retinopathy of prematurity or chronic lung disease) – so oxygen is a required treatment but also a potential dangerous ”drug”. Parenteral nutrition, blood transfusions, necrotizing enterocolitis, intraventricular/periventricular hemorrhage, retinopathy of prematurity are all thought to be consequences of this imbalance between

antioxidant capacity and oxidative stress added to an already demonstrated reduced antioxidant capacity (63). Also, for severely asphyxiated infants, either preterm or term, total antioxidant status is reduced, together with different fractions of radical scavenging activity in plasma. So, to counteract this high risk, enteral nutrition, when possible, should provide maximum protection. Zarbaran in a quite extensive study on milk samples (colostrum, transitional and mature milk) collected from 115 healthy women only with full term neonates, demonstrates that TAS was obviously higher in colostrum than transitional and mature milk (64). These data suggest that using colostrum, during the first days of life is vital, due to its high antioxidant potential. Quiles 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 decreases through lactation in mothers delivering full-term infants (65). The major downside of this study is enrolling only 30 cases.

Ezaki conducted a study on 56 cases of premature delivering Japanese mothers and found that TAS tends to decrease in time, when studying milk`s composition up to 150 days, with TAS values homogenously distributed during the first month of lactation (66). Their TAS values are remarkably higher compared to other studies, which is probably due to the method used or ethnic or geographical particularities. They used the biological antioxidant potential (BAP) test for measuring of TAS which is very similar as principle to the FRAP assay, used by Zarbaran *et al*., Fidanza reported a high, but not significant antioxidant capacity in colostrum, using ORAC (oxygen radical absorbent capacity) assay on only 30 samples (67). Abuhandan *et al*, found that the oxidants and antioxidants in the milk of mothers of premature infants were significantly higher than those of full-term birth mothers (68). Although the study groups enrolled similar number of subjects, the prematurity focused on 30-32 weeks gestation, the method used differed in terms of technique (Erel method) and units, so absolute values can`t be compared with ours. Moreover, the samples were collected on day 5 and preserved for an unspecified interval of time at 80°C, so a comparison with our data is rather inappropriate.

In a recent study on 15 subjects in different stages of lactation, Mehta and Petrova showed the identified enhancement of the antioxidant capacity of human milk by bio-active proteins that are lacking in commercial formula, and supports consideration of breast milk as the ideal nutrition for preterm-born neonates (69).

Many studies were conducted in this field, but only one measured the antioxidant activity of fresh, refrigerated and frozen milk at the same temperature as in our study (70). They only studied 16 subjects and used different techniques. We chose to use the ABTS® technique because according to the latest studies and meta-analysis, it is the best reliable method *in vivo* and *in vitro* (71,72).

Endogenous antioxidants in breast milk such as catalase, glutathione peroxydase, and superoxide dismutase were thought to increase with the passage of days after birth (73,74). This explains our results, as TAS in fresh breast milk increases 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. 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 rather probable that a woman who gave birth prematurely will not synthetize a higher TAS concentrated milk as if she would when delivering at term. We also consider that plausible reasons for these results are stress, prolonged hospitalization, concerns about infant`s health and frustrations generated by separation from the child and limited access to his care during the initial intensive care time, which contribute to a lower level of antioxidants at this category of mothers. In preterm milk,

TAS doesn`t increase after 7 days, suggesting that premature infants benefit best 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 is better than freezing human milk at -20˚C.

In a study of Xavier performed on 20 subjects – term and preterm delivering mothers – the highest TAS was found in colostrum and decreased over time, more through freezing (-80˚C) than refrigeration, with no difference between term and preterm milk, emphasizing the need of awareness and curtailment of the practice of storing and later use of human milk in medical practice and home care (75).

In term of mother`s age, Matos *et al.* found no statistically significant differences in TAS contents, when comparing < 30 years with > 30 years. By decreasing the limit at 25 years of age, we found significant differences (76).

According to the Breastfeeding Committee of the Spanish Pediatric Association, the study of different ways to preserve the antioxidant capacity of breastmilk throughout lactation and of factors which may improve the antioxidant status of both mother and infant, constitute important fields of research (77). However, many questions are still to be answered. More research on methods for extraction and storage of expressed breast milk is needed to best preserve antioxidant properties and constituents of breast milk.

This is the first such study performed in our country, exploring antioxidant activity in human milk. A larger, multicenter prospective study on Romanian population, including endogenous antioxidants components such as catalase, glutathione peroxidase, and superoxide dismutase, along with specific oligo elements (Cu, Zn, Mg and Se) would be a real benefit, for better confirm and explain the present results.

Conclusions

Breastfeeding remains an essential tool to help protection against free radicals, oxygen reactive species and oxidative stress. Fresh human milk has the highest antioxidant capacity. When fresh milk is not available, preserving milk at the refrigerator for a short time up to 72 hours is a better option than freezing, even for 1 week. Regardless of the type of preservation, human breastmilk remains more beneficial than formula in terms of providing antioxidant protection.

I.1.4. Recent opinions concerning breastfeeding and dental caries in children

Introduction and benefits of breastfeeding

Breastfeeding is the best method of providing infants with the all the nutrients they need for healthy growth and development. WHO recommends that oral feeding should be initiated within the first hour after birth followed by exclusive breastfeeding up to 6 months of life. Breastfeeding can be continued along with appropriate complementary foods up to two years of age or beyond (4).

Critically ill preterm infants who cannot be breastfeed can still benefit from the advantages of their mothers' milk preserved by refrigeration or freezing to be administered later. These guarantee microbiological safety, however refrigeration for more than 72 hours or long-term freezing decreases protein and fat content (78).

In addition to being an ideal nutritional source for infants, exclusive breastfeeding is associated with a lower rate of mortality from gastrointestinal infections and acute respiratory infections, urinary, middle ear infections, and childhood caries, as well as atopic diseases and strengthens the infant immune system (79,80). It also protects against chronic diseases such as obesity and diabetes but depending on race and ethnicity coupled with healthy feeding practices in infancy and early childhood (81).

A specific and concerning issue for our country is the birth rate for teenage mothers which is extremely high. In some cases, teen birth is the consequence of child abuse, another concern in Romania. Teen mothers have lower rates of breastfeeding duration and exclusivity than older mothers.

However, UNICEF recently communicated that many countries continue to underestimate the benefits of breastfeeding even when the evidence supports its short- and long-term effects. Improving breastfeeding rates around the world could save the lives of more than 820,000 children under age 5 every year, as well as preventing an additional 20,000 maternal deaths from breast cancer (82–84).

Improved neurocognitive development, intelligence, memory performance, early language, and motor skills at 14 and 18 months have also been described in the case of breastfed children (85,86). New evidence in a 16-year follow-up study of a large, randomized trial of 13 557 participants showed little evidence on beneficial effect of breastfeeding on overall neurocognitive function at age 16 years, suggesting limited but persistent benefit only on verbal ability. These benefits were small in magnitude compared to other family and birth factors and appeared to decrease with age from childhood to adolescence (87).

Published paper:

- Hincu MA, Besliu G, Rosu OM, Zonda GI, Diaconescu S, Anistoroaei D, **Paduraru L.** Breastfeeding and dental caries in children – a review. *Romanian Journal of Oral Rehabilitation* 2020; 12(3):175-180 (ESCI)

Dental caries (tooth decay) is a major public health problem affecting 60–90% of school aged children, with higher prevalence in children with low socio-economic status. It is caused by multifactorial and complex interactions between cariogenic bacteria in the mouth and dietary carbohydrates that lead to the demineralization of the teeth. The pain and infection caused by dental caries can be extremely distressing and produce an impact on the quality of life and ability to function, leading to loss of productivity and involve high health care costs including general anesthesia for treatment of severe cases. This accounts for one of the most common causes of child hospitalization in industrialized countries and is among the most common causes of avoidable child hospitalizations. Early loss of deciduous dentition can lead to ongoing dental problems in the permanent dentition.

Human milk with a rich microbiome helps establish optimal oral and intestinal flora and may mediate protection from early childhood caries. A 2016 *Lancet* global collaboration gathered information from 28 systematic reviews and meta-analyses and analyzed the implications of breastfeeding in oral health. While a role was suggested for breastfeeding in preventing malocclusion, caries was the only included disease condition unfavorably associated with breastfeeding (88).

The French Society of Pediatrics conducted in 2019 a review of publications and meta-analyses dating from the past 10 years regarding early childhood caries and breastfeeding and concluded that extended breastfeeding is a protective factor for childhood caries before 1 year of age. By contrast, breastfeeding beyond the age of 12 months has an increased risk of caries in infants not taking into account factors such as eating habits of the mother or infant (feeding during the night, number of meals per day, eating sweet foods etc.), dental hygiene, or the socio-cultural context.

Most recent recommendations of pediatric and dental societies advise breastfeeding until the age of 2 years, accompanied by dental hygiene and better nutrition, reducing the frequency and consumption of sugary foods, aiming to help mothers into prolonged

breastfeeding (89). However, concern has been raised that breastfeeding and its duration may increase the risk of early childhood caries.

Risk factors for development of early childhood caries

Early childhood caries (ECC) represents a complex and multifactorial disease that is impacted by biomedical factors and unmet social needs.

The bacteria that cause dental caries is most often *Streptococcus mutans, that* strongly adheres to the teeth and produces acids as waste products of fermentable carbohydrate metabolism that demineralize tooth enamel, progressing into the dentin. Weakened enamel and dentin can result in cavitation. Left untreated, caries can extend to the pulp and destroy the entire tooth.

Early childhood caries are a risk factor not only for dental caries in primary teeth, but in permanent dentition as well (90).

However, not all children who carry *Streptococcus mutans* manifest caries, even with similar oral hygiene, diet, and other environmental factors. This suggests that host susceptibility plays a role in the development of dental caries. *IL32*, *GALK2*, and *CELF4* were identified as potentially plausible genes that may play a role in the development of dental caries and interact with *Streptococcus mutans* through their involvement in galactose and carbohydrate metabolism, and host immune response (91)

An initial protective effect of breastfeeding against early childhood caries may be related to breast milk's immunomodulatory factors and rich microbiome. Breast milk contains *Lactobacilli*, human casein and secretory IgA that inhibit growth and attachment of *Steptococcus mutans.*

It is believed that *Streptococcus* and *Actinomyces* acquired at delivery and after birth induce selective growth of other species (including more strictly anaerobic bacteria like *Veillonella* and *Fusobacteria*). Thus, as the baby grows microbial species evolve and increase in diversity, reaching adult-like stability around 2 years of age. Most evidence available today shows that the early oral environment is strongly shaped by the mother and maternal oral microbiota has been proposed to colonize the placenta where it could influence fetal immune tolerance towards the mother's microbiome. Further transition into a more mature and complex microbial ecosystem is mainly influenced by the external environment, as well as vertical transmission from the parents. Children's oral microbiome changes with the emergence of primary teeth and the density of bacteria increases significantly with age (92).

The natural sugars in human milk may become the substrate for cariogenic bacteria causing early childhood caries to develop and progress rapidly.

Vitamin D status may influence childhood dental health. Low maternal vitamin D levels are associated with early childhood caries and mothers with higher prenatal vitamin D intakes are more likely to report that their children were caries-free (93). Additionally, the children's vitamin D levels were found to influence the development of caries (94). The presence of caries was significantly associated with 25(OH) levels < 75 nmol/L and < 50 nmol/L along with lower household education and poor oral hygiene. Improving children's vitamin D status may be an additional preventive consideration to lower the risk for caries.

Exposure to carbohydrates which is the essential substrate for cariogenic bacteria is a key factor in early childhood caries development. Refined sugars contribute considerably to tooth decay. Frequency of feeding and feeding practices, such as prolonged nocturnal feeding (either breast or bottle) may increase early childhood caries risk. A number of studies reported significant correlations between breastfeeding during the night (95), on demand, or sleeping with the nipple in the mouth and increased prevalence of dental caries (96). One cohort study found an increased adjusted risk of dental caries with increased daily breastfeeding frequency including nocturnal feeding (97).

Prolonged (> 18-24 months) breastfeeding in preterm babies and in children who started tooth brushing after 1.5 years of age was associated with an odds ratio for severe childhood caries of 5.31 (CI 1.50, 18.79) and 0.41 (CI 0.18, 0.93), respectively (98). Sugar and fruit-juice consumption and lack of periodic dental examination, nocturnal bottle feeding and nocturnal breastfeeding also affected early childhood caries formation significantly (99).

Nocturnal breastfeeding often used to comfort infants leads to prolonged exposure of the teeth surfaces to the cariogenic bacteria, hence increasing the risk of dental caries. Thus, oral hygiene practices to remove bacterial plaque are paramount as more teeth erupt.

Breastfeeding and early childhood caries

Although studies report that the carcinogenicity of human milk alone is low, plaque from solid foods together with frequent and prolonged exposure to human milk complicates the analysis of caries risk (100).

All breastfeeding mothers could benefit from individualized oral hygiene instruction, especially once teeth begin to erupt around 7 months of life. Mothers who breastfeed children with teeth throughout the night need to know how important it is to start out with a "clean slate" at bedtime.

Nighttime brushing routine is effective to remove plaque from every surface of the teeth and mothers should be instructed to wipe the teeth off at the end of the feeding, rather than letting the milk residue sit on the teeth. A clean washcloth and water could be kept next to the bed and used to rub as many of the surfaces of the teeth as possible once the child finished feeding. Although not as thorough as brushing the teeth, this practice could help to eliminate excess milk residue from the teeth without completely disrupting the child (101).

Oral health recommendations

To optimize the benefits of breastfeeding and minimize early childhood caries, parents should follow recommendations for proper oral hygiene regarding their children, appropriate fluoride exposure, regular dental visits, and a healthy diet.

Parents should be advised to avoid saliva-sharing behaviors (eg, sharing utensils with their children or cleaning a pacifier with their mouth), as these may increase early colonization of *Streptococcus mutans* in infants and to seek regular preventive dental care and attend to caries, both for their children and themselves.

In addition, prenatal counseling should include a discussion of the importance of good maternal oral health and diet – including an adequate vitamin D intake.

An oral health risk assessment and evaluation of fluoride exposure should be performed by 6 months of age. Parents should be advised to establish a routine by the time the child is 12 months of age, to clean their children's mouths after feedings (before teeth arrive) with a clean, wet, soft cloth and to brush their children's teeth, once they erupt, twice daily using a soft toothbrush. Use of fluoridated toothpaste in small amounts provides the benefits of fluoride without increasing the risk of fluorosis, especially for children at risk for caries.

Also, parents should be advised to avoid giving their children sugar-containing snacks and drinks to reduce ECC risk.

The prevention of early childhood caries depends on multidisciplinary efforts, involving different healthcare professionals (dentists, pediatricians, nurses, primary healthcare workers, gynecologists) who could provide dental care information during pregnancy.

CHAPTER I.2. RESEARCH ON NEONATAL SEPSIS

I.2.1. Overview and burden of neonatal sepsis

Neonatal sepsis (NS) is defined as an infection occurring during the first month of life with clinical manifestations varying from subtle nonspecific signs to severe systemic disease, shock and multisystem organ failure (102–104).

Due to the high mortality and unfavorable outcome of the survivors, neonatal sepsis represents a health problem of global proportion. Even with appropriate antimicrobial therapy and supportive care, surviving newborns may face long term impairment. For this reason, early diagnosis and treatment are of great importance. Thus, a consensus definition for neonatal sepsis became of critical importance. Following the International Pediatric Sepsis Consensus held in 2002, Goldstein and co-workers suggested definitions for infection in pediatric patients, as well as for systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis, septic shock and organ dysfunction, which included term newborns (0-7 days and 1 week-1 month) (105). Later, Wynn and Wong proposed adjustments of existing definitions to include premature infants as well (Table 19), providing clinicians and researchers with a uniform base for study and diagnosis of sepsis in this highly vulnerable category of patients (106).

Infections may be transmitted from the mother to the fetus mainly in the last trimester of pregnancy, or acquired from the health care staff later, after birth. Depending on the time of onset, neonatal sepsis has been divided into early-onset sepsis (EOS) and late-onset sepsis (LOS). EOS is typically considered an infection that presents itself within the first 3 days of life (< 72 hours), however some researchers extend this limit up to the first week of life (107–111). LOS is defined as an infection occurring after the 4^{th} or 7^{th} day of life within the neonatal period (112–114). The timing of symptoms presentation depends on the transmission route and type of exposure. EOS is considered as a maternal-fetal infection and LOS is mainly considered as hospital acquired.

Peripartum acquisition by transplacental route, *in utero* or during labor and delivery can generate infections which usually present within 24 to 72 hours of life, like in the case of *Listeria monocytogenes*, group B streptococcus (GBS), enteric Gram-negative organisms, gonococci, Chlamydia or viral pathogens. The incidence of EOS is reported as 1–2‰ live births (115), lower in developed countries, down to 0.5–1‰ (116–118). However, there are studies that report an incidence for EOS as high as 9.8‰ (119,120). This variation may be due to different gestational ages (GA) included in statistics, as very low birth weight (VLBW) and extremely low birth weight (ELBW) neonates are at a greater risk for EOS, with rates up to 20‰ (112,120). The overall mortality rate is up to 24.4%, but can be as high as 54% in infants between 22 to 24 weeks of gestation and 30% between 25 to 28 weeks of gestation (121,122).

According to the current literature, acquisition of EOS has been associated with multiple factors that, if present, would indicate close clinical monitoring of the newborn and laboratory work-up for infection (102,103,123).The most important risk factors for EOS are listed in Table 20 (124).

Postpartum acquired infections are transmitted most often horizontally, from family members or caregivers (through breastfeeding or direct contact), but environmental exposure from hospital staff or contaminated equipment may also be involved, especially in the case of preterm infants with prolonged hospital stays (125). Pathogens most frequently reported to cause LOS are Gram-positive bacteria like CONS, S. aureus, GBS and Enterococcus species, out of which GBS has been associated with the highest mortality (21.9%), followed by S. aureus (17.2%) (126–128).

Table 19. *Definitions for sepsis in newborns (adapted from Goldstein, Giroir and Randolph, 2005, modified by Wynn and Wong, 2010)*

SIRS The presence of at least 2 of the following 4 criteria, 1 of which must be abnormal temperature or leukocyte count: • Core[a] temperature of >38.0°C[b] or <36°C • Tachycardia, defined as a mean heart rate >2SD more than normal for age in the absence of external stimulus, chronic drugs, or painful stimuli; **or** otherwise unexplained persistent increase in a 0.5 to 4 h time period **OR** • Bradycardia, defined as a mean heart rate <10th percentile for age in the absence of b-blocker drugs or congenital heart disease[c]; **or** otherwise unexplained persistent bradycardia[d] • Mean respiratory rate >2SD more than normal for age or mechanical ventilation for an acute process not related to underlying neuromuscular disease or the receipt of general anesthesia • Leukocyte count increased or decreased for age or >20% immature to total neutrophil ratio[e] **or** C-reactive protein >10 mg/dL
Infection A suspected or proven (by positive culture, tissue stain, or polymerase chain reaction test) infection caused by any pathogen **OR** a clinical syndrome associated with a high probability of infection. Evidence of infection includes positive findings on clinical examination, imaging, or laboratory tests (eg, white blood cells in a normally sterile body fluid, perforated viscus, chest radiograph consistent with pneumonia, petechial or purpuric rash, or purpura fulminans)
Sepsis **SIRS** *in the presence of* or as a result of *suspected* or *proven infection*
Severe sepsis **Sepsis** *plus* 1 of the following: cardiovascular organ dysfunction **OR** ARDS **OR** 2 or more other organ dysfunctions
Septic shock **Sepsis** *and* cardiovascular organ dysfunction
[a] Core temperature must be measured by rectal, bladder, oral, or central catheter probe. [b] Neonatal fever is considered greater than 38°C. [c] External vagal stimulus use is uncommon in preterm infants. [d] Infrequent self-resolving bradycardic episodes can be common in premature neonates in the absence of sepsis. [e] More commonly accepted ratio is greater than 20% immature to total ratio and chemotherapy-induced leukopenia is uncommon in premature infants.

Gram-negative pathogens have been involved in less than 1/5 of LOS, but with a higher overall mortality of 36% compared to 11.2% reported for Gram-positive organisms.

Table 20. *Risk factors for neonatal EOS* (124)

Maternal risk factors	**Neonatal risk factors**
Chorioamnionitis Premature rupture of membranes/Preterm pregnancy with gestational age of <37 weeks Prolonged rupture of membranes even at term (>18 hours) Intrapartum maternal fever ≥38ºC Maternal group B streptococcal colonization (GBS) Positive bacteriuria Inadequate intrapartum antibiotic prophylaxis A history of a previous infant with gram negative pathogens infection	Preterm newborn Low birth weight Fetal distress Low APGAR score Multiple pregnancies Intensive resuscitation of the newborn

Amongst Gram-negative bacteria, the predominant pathogens associated with LOS were species of Pseudomonas, E. coli, Klebsiella, Enterobacter and Serratia, with mortality rates of 74%, 34%, 22.6%, 26.8% and 35.9% respectively (126). Fungal infections were

responsible for 12.2% of LOS cases, with an overall mortality of 31.8% (125).

Table 21. Definitions for organ dysfunction in newborns (adapted from Goldstein, Giroir and Randolph, 2005, modified by Wynn and Wong, 2010)

Cardiovascular dysfunction

Despite administration of isotonic intravenous fluid bolus >40 mL/kg in 1 h (>10 ml/kg in infants <32 weeks)[a]:

- Decrease in BP (hypotension) <5th percentile for age or systolic BP >2SD less than normal for age or MAP <30 mm Hg with poor capillary refill time (>4 s)[b]

OR

- Need for vasoactive drug to maintain BP in normal range (dopamine >5 mg/kg/min or dobutamine, or epinephrine at any dose)[c]

OR

- Two of the following:
  - Unexplained metabolic acidosis: base deficit >5.0 mEq/L
  - Increased arterial lactate >2 times upper limit of normal
  - Oliguria: urine output <0.5 mL/kg/h
  - Prolonged capillary refill >4 s[d]
  - Simultaneous measurement of core and peripheral temperature not
  - common in premature neonates

Pulmonary[e]

- PaO_2/FIO_2 <300 in absence of cyanotic heart disease or preexisting lung disease
- Excessive oxygen should be limited to avoid complications including retinopathy of prematurity
- $PaCO_2$ >65 torr or 20 mmHg more than baseline $PaCO_2$

OR

- Proven need[f] for >50% FIO_2 to maintain saturation >92% (88% for <32 weeks)

OR

- Need for nonelective invasive or noninvasive mechanical ventilation[g]

Neurologic

- Acute change in mental status[h]

Hematologic

- Platelet count <80,000/mm3 or a decline of 50% in platelet count from highest value recorded in the past 3 days[i]

OR

- International normalized ratio >2

Renal

- Serum creatinine >2 times upper limit of normal for age or 2-fold increase in baseline creatinine

Hepatic

- ALT 2 times upper limit of normal for age[j] or 50% increase over patient's baseline[k]

Abbreviations: ALT, alanine transaminase; BP, blood pressure

[a] Rapid large volume expansion can be associated with intraventricular hemorrhage.

[b] 30 mm Hg suggested as minimum MAP.

[c] Norepinephrine not commonly used in premature neonates.

[d] Greater than 4 s may reflect a low systemic blood flow (129)

[e] ARDS must include a PaO2/FIO2 ratio %200 mm Hg, bilateral infiltrates, acute onset, and no evidence of left heart failure. Acute lung injury is defined identically except the PaO2/FIO2 ratio must be R300 mm Hg.

[f] Proven need assumes oxygen requirement was tested by decreasing flow with subsequent increase in flow if required.

[g] In postoperative patients, this requirement can be met if the patient has developed an acute inflammatory or infectious process in the lungs that prevents them from being extubated.

[h] Glasgow Coma Score not applicable to term or preterm neonates.

[i] Neonates not frequently chronic hematology-oncology patients.

[j] Indirect hyperbilirubinemia is common in newborns.

[k] Transaminases are commonly increased in preterm neonates on long-term intravenous hyperalimentation.

Factors associated with an increased risk of LOS are low gestational age, extremely low birth weight, extended hospitalization, prolonged respiratory support, prolonged parenteral nutrition, repeated blood sampling, persistent intravenous lines, urinary catheters, and shunts. Also prolonged or repeated courses of broad-spectrum antibiotics have been

established as major contributing factors to occurrence of LOS especially in very low birth weight infants (125,128,130,131).

Signs of organ dysfunction are indicative for severe disease that goes beyond an infection accompanied by inflammatory response and progression towards unfavorable outcome. Even though for newborns admission in the intensive care unit is not necessarily associated with a significant change in the clinical status from baseline, as in older children or adults, in the case of a suspected infection, outcomes like organ dysfunction and death should be included in a consensus definition for neonatal sepsis. However, a significant challenge to develop a such complex definition (Table 21), is represented by those neonates that require intensive care from the first minutes of life, due to the lack of a prior baseline for comparison of organ function and developmental changes during adaptation to extrauterine life. This is especially true for extremely preterm newborns, who most often need critical care support without the context of an infection.

Taking into consideration the unique characteristics of the neonatal population, Wynn and Polin made the first attempt to develop a neonatal-specific sequential organ failure assessment score (nSOFA) that incorporates common modifiers of risk in the NICU, including gestational age and birth weight (132).

Table 22. Hypothetical nSOFA proposed by Wynn and Polin (2018)

System	0	1	2	3
Respiratory	No support **or** OI <2, PaO_2/FiO_2 >330 (70/0.21)	CPAP/HFNC **or** OI=2-8 (max of 40% O_2, max MAP 14, paO_2 70) PaO_2/FiO_2=230–330 (70/0.3)	NIPPV **or** OI=8–14 (max 60% O_2, max MAP 16, paO_2 70) PaO_2/FiO_2=140–260 (70/0.5)	Intubated: CMV/HFV **or** OI=14–20 PaO_2/FiO_2 <140 (70/0.6) or any iNO Score of 4: ECMO (if eligible), OI >20 if ineligible
Cardiovascular	MAP > GA **and** capillary refill<3 s	Two measurements of: (i) SBP decrease >10 mmHg **or** (ii) capillary refill >3 s 1–6 h apart	Vasopressor requirement	Vasopressor refractory state (requirement for post-vasoactive meds, e.g., corticosteroids)
Platelets (10^3/μl)	≥ 100	<100	<50	<50 in ≤ 24 h after transfusion
ANC (cells/μl)	>1500	1001–1500	500–1000	<500
Renal (Cr-mg/dl, UOP ml/kg/h)	UOP >0.5 **and** no change in sCr **or** rise <0.3	UOP <0.5 for 6–12 h **and** sCr increase <0.3 in 48 h **or** >1.5–1.9 × LPC value within 7 days	UOP <0.5 for ≥ 12 h **and** ≥ 2.0-2.9 × LPC value	UOP <0.5 for ≥ 12 h **and** ≥3 × LPC value **or** sCr42.5 **or** dialysis
CNS	Baseline responsiveness	Any change in status	Lethargic **or** hypotonic	Unresponsive
CMV, conventional mechanical ventilation; CPAP, continuous positive airway pressure; ECLS, extracorporeal life support; GA, gestational age; HFNC, high-flow nasal cannula; HFV, high-frequency ventilation; LPC, lowest previous sCr; MAP, mean arterial pressure; NIPPV, non-invasive positive pressure ventilation; OI, oxygenation index; SBP, systolic blood pressure; sCr, serum creatinine; UOP, urine output.				

This hypothetical score (Table 22) remains to be validated in cohorts of patients in order to determine its generalizability, but first of all it requires a consensus definition for

neonatal sepsis rigorous enough to be unequivocally validated on retrospective view. Such a definition should be based first on the clinicians' subjective assessment that infection is highly likely, complemented by objective evidence of organ dysfunction consistent with sepsis and objective documentation of systemic inflammation reflected in an acute phase response consistent with sepsis (132).

Up to this point, the current definitions available for SIRS and sepsis in the pediatric population have shown insufficient correlation with culture-proven EOS, due to the fact that during the period that follows immediately after birth up to the first few days of life, septic neonates may present a great variety of signs and symptoms. In this context, the definition adopted by the pediatric consensus conference for sepsis has limited applicability. Combining perinatal risk factors with a wider range of clinical signs and laboratory parameters may prove more useful. However, sepsis screening panels should include besides common inflammation markers like CRP and procalcitonin, cytokines, cell surface markers and other molecules in order to improve the sensitivity and specificity. Unfortunately, many of these have limited availability, only in research facilities, restraining their use as sepsis definition criteria (133).

I.2.2. Particularities of the immune response in neonates

The incidence of sepsis increases as the gestational age decreases, which indicate that premature neonates have a significantly higher risk of infection. Birth weight (BW) also seems to play an important role, as 10% of VLBW neonates (very low birth weight, BW= 1000-1500 g), 35% of ELBW (extremely low birth weight, BW<1000 g) and 50% of ILBW (incredibly low birth weight, BW<750 g) are diagnosed with sepsis during the neonatal period. Due to the progress of modern medicine, the survival rate of VLBW newborns has increased. However, this category of infants has a mortality rate caused by sepsis 3 times higher than in those without sepsis (125). Understanding the particularities of the immune system development and response in an important step towards developing more efficient diagnostic methods to facilitate early treatment intervention.

A fully functional immune system could pose problems due to maternal-fetal antigenic incompatibility and it is also unnecessary to the fetus, which develops in a protective environment. However, at the time of birth, the neonate must be capable of defending himself against pathogens. Previously it was believed that as a consequence of such contradictory requirements, lack of previous antigenic experience on one hand and prevalence of suppression factors *in utero* on the other hand, the immune system is incompletely matured at birth (134). However, more recent research supports the idea that instead, the immunity of the newborn is a "vigilance complex system" rather than silent and immature, with the ability to initiate selective responses adapted to various circumstances (135). The balance between counter-inflammatory responses designed to protect the mother and the defense mechanisms necessary during the transition period is extremely important for the neonate's survival (136,137).

The immune system begins its development at 4.5 – 6 weeks of intrauterine life and throughout gestation both innate and adaptive immunity evolve gradually (138). At birth, the neonate's first line of defense against infections is represented by the innate immune system, capable of operating without previous exposure to antigens, together with maternal antibodies transferred transplacentally (139,140).

Innate immunity includes several components and mechanisms: skin and mucosal barriers, a network of cells comprised of neutrophils, monocytes, natural killer cells and antigen presenting cells, toll-like receptors (TLRs) and humoral factors like the complement. TLRs are major components of the innate immune system capable to recognize pathogen associated molecular patterns (PAMP) (140). Activation of TLRs induces production of cytokines, chemokines, complement, coagulation proteins and antimicrobial effector

mechanisms (141). Studies on monocytes from umbilical cord blood of term newborns and adult peripheral blood cells showed similar TLRs expression and distribution (142). However, the level of cytokines involved in innate immune response of preterm infants was shown to be lower in comparison to term neonates (143).

The adaptive immunity is dependent on antigen exposure and develops after birth. Its components have no previous antigenic memory and show both quantitative and qualitative deficiencies (144). Neonatal T cells response is characterized by a predominance of $CD4^+$ over $CD8^+$, reduced cytokine production and inadequate Th1-like response (140,145). As a result, newborns have decreased defensive mechanisms against pathogens, which puts them at high risk for infections caused by viral agents or intracellular microbial agents like *Listeria spp*., *Toxoplasma* or *Mycobacterium tuberculosis* (146–148).

The major components of the intestinal immunity are the innate defenses represented by gastric acid, protolithic enzymes, mucin, defensins and lectins, and active immunity consisting in T and B cells. B lymphocytes travel through the bloodstream to the lymph nodes in the mesentery, from where they enter the intestinal submucosa and produce IgA, which provide protection against bacteria, viral agents and parasites. In addition, IgA are capable of inducing effector immune responses, therefore maintaining the intestinal microflora and homeostasis (149,150). The work of Zasada and colleagues proved that IgA production also increases with postconceptional age (140). Deficiency of secretory IgA, along with disruption of gut microbiome due to bacterial colonization and formula feeding, hypoxic injury of the intestinal mucosa and the immaturity of the gut mucosal immunity in preterm infants have been shown to be associated with a high risk of necrotizing enterocolitis (NEC) (151,152).

Human milk provides protective components that are deficient in the neonate, such as secretory IgA, cytokines, chemokines, receptors, innate immunity factors, hormones and growth factors, enzymes, carrier proteins, nucleotides, as compensatory mechanisms to counteract the immune deficiency of the neonate (134).

I.2.3. Relevant biomarkers for the diagnostic of early onset sepsis

I.2.3.1. Overview of available tools of diagnosis in EOS

Despite the abundance of research in the field of neonatal infection in the latest decades, a precisely marker or test to guarantee a certain diagnostic for every case has not yet been developed. In neonates with risk factors and clinical suspicion of EOS, currently used biomarkers have insufficient predictive performance and confirmation of diagnosis by positive cultures is not always possible in a timely manner. Therefore, at present there is no international consensus concerning which biomarker or combination of tests is best to accurately diagnose neonates with EOS (153,154).

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The ideal marker for infection should be valuable for establishing the diagnosis, as well as for predicting the outcome and for evaluation of the response to treatment; concomitantly it should be easy to quantify and available for routine clinical use (155–157).

Until present time, several biomarkers (Fig. 18) were studied and used, many of them only for research purposes, as the techniques and devices needed are not available in every clinical facility in a timely manner (Table 23).

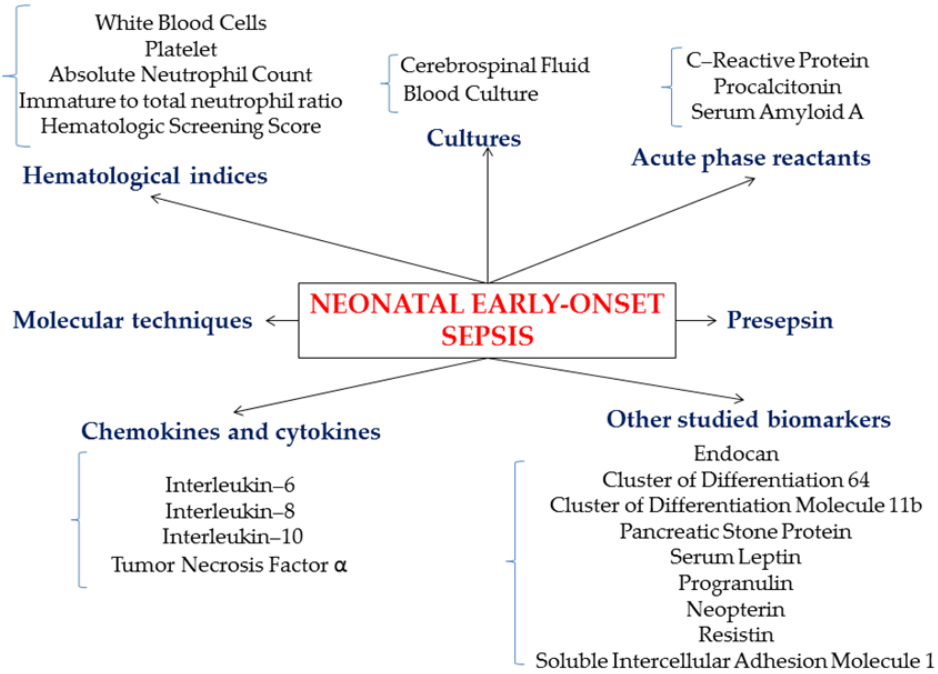

Fig. 18. Schematic representation of common and developing biomarkers for neonatal early-onset sepsis

Table 23. Currently used biomarkers for sepsis diagnosis in neonates

Marker	Cut-off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Ref
White Blood Cells	20000/mm^3 <5000/mm^3	59.5	79.6	52	86.1	(158)
CRP	10 mg/L	49	91	73	77	(159)
Procalcitonin	2.5 ng/mL	75	83	NA	NA	(160)
Interleukin-6	100 pg/ mL 181 pg/ mL 60 pg/mL 10-150 pg/mL 60 pg/mL	95.83 80.1 54 75-87 54	87.50 85.7 100 50-82 100	92 84.6 100 92 100	93.33 81.8 59 52 59	(161) (161) (159) (162) (163)
Interleukin-8	60 pg/mL 300 pg/mL 70 pg/mL 60-300 pg/mL	95 91 92 90	10 93 70 75-100	97 91 65 78	10 97 93 88	(161) (159) (163) (162)

PPV (%), positive predictive value; NPV (%), negative predictive value

I.2.3.1.1. Blood cultures

Clinical sepsis alone (infant with clinical signs, but negative cultures) is much more common, especially in EOS cases (164,165). However, the current gold standard method for confirmation of sepsis in newborns with risk factors, clinical suspicion and abnormal test results remains the identification of the pathogenic organism from a normally sterile site (blood or cerebrospinal fluid) (164,166). When neonatal EOS is suspected, blood cultures are usually obtained on the first day of life, but less than 1% come back positive (167). In other words, the overwhelming majority of blood cultures sampled from newborns evaluated with risk factors or clinical signs of EOS are negative (113,168). The administration of intrapartum antibiotic prophylaxis in mothers with either group B streptococcus colonization or suspected

amnionitis originating from any cause can reduce the ability to detect bacteremia in newborns (156,169). The volume of the sample might also play a part, as ideally 1–3 mL of blood should be obtained, and this is most often extremely difficult, if not impossible in ELBW infants. Organism density is another factor that may influence the chance of pathogen detection in the bloodstream. In infants with low very levels of bacteremia (< 4 colony forming units (cfu)/mL), 1 mL samples are required to ensure a high sensitivity, whereas as little as 0.5 mL may be enough to detect moderate and high grade bacteremia (more than 10 cfu/mL) (170). Brown and colleagues found that only 0.25 mL of placental blood seeded with > 10 cfu/mL E. coli or group B streptococcus was sufficient yield a positive culture (170,171).

Molecular assays (conventional and real-time polymerase chain reaction (PCR)) have the advantage of producing much rapid results, and have proven useful as "add-on" tests, but cannot replace blood cultures as the standard of diagnosis of neonatal sepsis (172,173).

I.2.3.1.2. Cerebrospinal Fluid

Approximately 40% of neonatal EOS cases caused by invasive group B streptococcal infection are associated with meningitis, with E. coli as the second most common pathogen (118). Confirmation of meningitis requires sampling of a cerebrospinal fluid (CSF) specimen by lumbar puncture (LP) for culture, Gram stain, white blood cells count (WBC), glucose and protein levels (169). However, in asymptomatic infants who are being evaluated for EOS based on maternal risk factors, it is appropriate to defer an LP. Nevertheless, all infants with positive culture proven EOS should undergo an LP (118).

The diagnosis of neonatal meningitis in the context of EOS is challenging even when an LP is performed. The difficulties in interpretation of the results may decrease the benefit of the procedure relative to the risk of potentially severe associated complications (spinal hemorrhage and/or hematoma (174), osteomyelitis (175), brain herniation (176). Antepartum or empirical antibiotic therapy for suspected EOS prior to LP may result in false negative CSF culture even when neonatal meningitis is present.

Even though in approximately 20% of newborns with proven meningitis, no bacteria are visualized on the Gram stain, the assay may still be useful for the diagnosis. In bacterial meningitis the WBC concentration is usually elevated with a neutrophilic pleocytosis, but in L. monocytogenes meningitis a mononuclear cellular response is characteristic (169).

Due to the challenges of interpreting CSF parameters to diagnose neonatal meningitis, to increase the reliability of the CSF culture, the LP should be performed prior to administration of empirical antibiotics. If antimicrobial therapy has already been initiated, the clinician should maintain a high suspicion of the possibility of meningitis even in a neonate with negative CSF culture (118).

I.2.3.1.3. Hematological indices

Classically, the limits regarding WBCs for the diagnosis of sepsis are below 5000/mm3 or over 30.000/mm3 (119). Sharma and colleagues claimed that values under 5000/mm3 for WBCs have a high specificity (91%) regarding sepsis diagnosis, but the main weaknesses are the low sensitivity (29%) and the need for correlation with the GA (177). Two articles highlighted that leucopenia (WBCs < 5000/mm3 at more than 4 hours, likelihood ratio of 81) is more suggestive for sepsis than leukocytosis (WBCs > 20000/mm3 at more than 4 hours, likelihood ratio of 0.16) (122,178). Another disadvantage of WBCs resides in the fact that the number of WBCs increases late after the onset of sepsis, hence multiple studies recommend obtaining a sample after 4 to 6 hours from stimulation (119,122,179). WBCs require dynamic follow up and they are more useful in ruling out an infection than in diagnosing it.

PLT and mean platelet volume (MPV) have a low sensitivity and specificity in the diagnosis of EOS (119). Values of MPV greater than 8.6 FL, with a high sensitivity and specificity (97.14% and 100%, respectively) are considered efficient in diagnosis of EOS (156). Increased MPV values are found in respiratory distress syndrome, which makes the interpretation of PLT and MPV difficult in the context of added EOS. Thus, these parameters play only a suggestive role in the diagnosis of NS (157).

Gestational and postnatal age, delivery method, altitude, maternal fever and hypertension, fetal asphyxia, meconium aspiration, periventricular hemorrhage, reticulocytosis, hemolytic disease and pneumothorax affect Absolute Neutrophil Count (ANC's) values, limiting its use in EOS (119,157,178). It is recommended to obtain a sample for ANC after 6 to 12 hours of life in order to reveal a systemic inflammatory response in term newborns (119,156), which importantly delays therapeutic decisions. Neutropenia (ANC < 1000/mm3 at more than 4 hours, likelihood ratio 15) is more frequently associated with EOS, having a higher specificity than neutrophilia (ANC > 10000/mm3 at more than 4 hours, likelihood ratio of 0.31), being less helpful in diagnosing EOS (119,156,177,178). Different values for neutropenia were proposed: ANC < 1800/mm3 at birth, < 7800/mm3 at 12-14 hours after birth and at 72 hours ANC < 1800/mm3 for term and late preterm infants (119), ANC < 1000/mm3 at 4 hours after birth (178). Furthermore, there are some specific situations such as active labor and female gender that lead to neutrophilia in the absence of infection, affecting the immature to total neutrophil ratio and leading to a high false positive predictive value (169,178). Nevertheless, there are some factors such as maternal hypertension, gestational age, delivery method (cesarean delivery without labor) that can decrease the ANC levels, leading to a false negative predictive value (119,157).

Out of all hematological markers, immature to total ratio (I:T ratio) is the most sensitive indicator of NS, but this parameter also varies with GA and postnatal age (119,156). Classically, I:T ratio > 0.2 is criteria for suspected EOS, but significant I:T ratio values for NS are > 0.27 in term newborns and > 0.22 in preterm neonates (156). Increased values of this marker may also be identified in perinatal asphyxia, maternal hypertension and prolonged labor with oxytocin administration (177). An association of low WBCs, low ANC and a high of I:T ratio will lead to a greater odds ratio, suggesting NS (122,177). On the other hand, two normal I:T ratio correlated with a sterile blood culture have maximum NPV (100%) (177).

Hematologic Screening Score (HSS) includes WBCs with differential, PLT, nucleated red blood cell count, assessment of degenerative and toxic changes in PMN. It is mentioned in two studies which both state that the higher the score, the higher the sensitivity (122,156). A HSS >3 in suggestive for NS, but it has the disadvantage of a low PPV (< 31%) (156). Even this score needs association with other biomarkers, in order to validate the EOS suspicion (122,156).

I.2.3.1.4. Acute phase reactants

C–Reactive Protein (CRP)

Inflammatory stimuli of any kind, including infection, trauma or ischemia, generate marginalization, extravasation and activation of the granulocytes and monocytes, resulting in release of pro-inflammatory cytokines like interleukin–1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor (TNF-α), which stimulates the production of acute phase reactants. In the adult patients the reaction times for each of these proteins has been well characterized and it seems that they present similar patterns in neonates. CRP, a cyclic homopentameric protein is an acute-phase reactant 14, that binds phosphorylcholine, a component of teichoic acids in gram-positive organisms, and lipopolysaccharides in gram-negative organisms, but also lysophosphatidylcholine, ribonucleoproteins, chromatin, and histones from apoptotic cells (180,181). CRP functions as an opsonin for neutrophils and macrophages and activates the

classical complement pathway and induces phagocytosis (182). The serum levels of CRP may increase from 100 to 1000 times in response to bacterial infections or other inflammatory conditions and concentrations correlate with severity of illness (183). Protein secretion begins primarily in the liver, at 4–6 hours after stimulation and reaches the maximum level at 36–48 hours (120,184,185). Once the inflammation trigger is eliminated, CRP concentration decreases rapidly, with a half-life of about 19 hours (181). However, due to the delayed response, the sensitivity of CRP increase at the time of evaluation for a clinical suspicion of EOS is low. For a cut-off value of 10 mg/L, the sensitivity for CRP varies between 9%–83%, but the majority of studies reported values of 49%–68% (159). For the same cut-off value, the specificity was consistently above 90% (186). However, in the case of neonatal population there are multiple other pathological situations, aside from infections (bacterial or viral), associated with an increase of CRP, such as rupture of membranes (which induces an increase in CRP levels by 0.4% per hour), active labor (14.5% per hour), maternal administration of steroids (40%) or intrapartum antibiotics (28%) or chorioamnionitis without invasive fetal or neonatal disease. Moreover, trauma, ischemic tissue injury, hemolysis or meconium aspiration syndrome can result in increased CRP concentrations in the first 24–48 hours of life (120,183). In this context, the value of CRP as a diagnostic marker for neonatal EOS is quite low. Even though the accuracy of CRP as a diagnostic marker improves with 3 serial measurements, its positive predictive value for proven EOS is unacceptably low, of 5% for a cut-off value of 10 mg/L and above 10% only for cut-off values exceeding 50 mg/L (159,187,188). However, the reported negative predictive value for EOS was 99.7% which suggests that CRP is more useful for ruling out infection when normal serial values are obtained (120).

Procalcitonin (PCT)

PCT is a 116-amino acid precursor peptide of calcitonin without hormonal activity. It is normally produced only by the C cells of the thyroid gland and circulating concentrations are < 0.05 ng/mL in the serum of healthy subjects. Its levels are not affected by calcitonin levels (177). In healthy neonates, a physiological increase in the plasma PCT concentration occurs shortly after birth. The peak values are attained at 24 hours of age (mean 1.5-2.5 ng/mL, range 0.1–20 ng/mL), followed by a decrease to less than 0.5 ng/mL by 48-72 hours of life (189,190). In the context of sepsis, the PCT is massively produced in the liver and plasma concentrations can increase up to 1000-fold (120). Levels of >0.5 ng/mL suggest systemic infection and possible sepsis and correlate with disease severity (191). PCT synthesis is stimulated by cytokines like IL-6, IL-1β, and TNF-α, or directly by lipopolysaccharides and it is down regulated by interferon-γ which is commonly produced in response to viral infections (120,192,193). This might explain why PCT levels are low during viral infections compared with bacterial and fungal infections (191). PCT concentrations are maximum at 18–24 hours after stimulation and remain elevated for 24-30 hours (122,194). Concentrations decrease rapidly once the inflammation is resolved (191). However, PCT, like CRP, was shown to be increased by several perinatal factors like prolonged rupture of membranes ≥ 18 hours, active labor, maternal administration of steroids or intrapartum antibiotics and by non-infectious perinatal conditions including intracranial hemorrhage and hypoxic ischemic encephalopathy (120,122,189,195). Mode of delivery appears not to influence PCT concentrations (120). PCT levels are not affected by sex, but are influenced by birth weight and gestational age (196). In septic neonates PCT concentrations reported were increased by 5–20-fold compared to the measurements obtained in healthy newborns (120). In an analysis performed by Bell and colleagues, the studies that focused on EOS reported a sensitivity of 0.75 (95% CI, 0.64–0.84) and a specificity of 0.83 (95% CI, 0.71–0.91) for a cut-off value for PCT of 2.5 ng/mL (160). Establishing the optimal cut-off value of PCT for

the diagnosis of EOS is critical, considering the physiologic increase after birth of this marker, which is influenced by both weight and gestational age. Usually, the 95th percentile of normal is typically used as a cut-off point. Eschborn and Weitkamp analyzed three studies that determined the 95th percentile of normal for PCT at different time points during the first 96 hours of life (120). The data showed that at 0 hours of life (HOL) the cut-off value for both term and preterm was 1 ng/mL and at 24 HOL, the values were 10–20 ng/mL for term and 50–60 ng/mL for preterm infants (196–198). All the presently available data emphasizes that the reliability of both CRP and PCT for the diagnosis of EOS requires precise limit values for each assessment time point in the first 48 hours of life (199,200).

Serum Amyloid A (SAA)

SAA, an apo-lipoprotein synthesized by the liver, is an acute phase reactant extensively studied in various acute pathologies in adults (cardiac, renal, degenerative disorders) (201–206). Its levels rise early during the inflammatory response up to 1000 times higher than the baseline serum values but are significantly influenced by the patient's hepatic function and nutritional status. Thus, the value of this molecule is limited in the diagnosis of LOS (157). However, studies that focused on EOS showed that SAA had a higher sensitivity, PPV and NPV compared to CRP (96%, 85%, 99% vs. 30%, 78% and 83%, respectively) but a slightly lower specificity (95% vs. 98%), with an overall better diagnostic accuracy (177,207,208).

I.2.3.1.5. Chemokines and cytokines

Cytokines are divided into pro-inflammatory interferon-gamma (IFN-γ), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-12 (IL-12) and interleukin-17 (IL-17), anti-inflammatory interleukin-4 (IL-4), interleukin-10 (IL-10), tumor necrosis factor soluble receptor (TNF-α), IL-1 receptor alpha and transforming growth factor beta 2 (TGF-β) and multiple functional inflammatory IL-1β, IL-3, monocyte chemoattractant protein (MCP-1) and growth factors (IL-3, G-CSF) (209).

Out of all cytokines, IL-6 is the most studied marker. Its levels rise at 2-4 hours since the onset of infection, right before the clinical signs, symptoms and other diagnostic tests (156). This interleukin has a good sensitivity of 72–100%, a wide specificity of 47–87.5%, a high NPV between 93 – 100% and PPV of 38–100% (157,161,178). IL-6 has its own limitations such as a short half-life and a low sensitivity in case of antibiotic therapy (122,157,161). An advantage is its low value, almost undetectable in healthy newborns when compared with those with sepsis (163,210). Unlike other markers, if there are antenatal risk factors for sepsis (such as chorioamnionitis), IL-6 should be determined in the umbilical cord blood, as its concentration rises significantly in case of infection (157,161,211). However, the umbilical cord level depends on different factors like prematurity, maternal usage of steroids and antibiotics given to the mother. The main weakness is that there is no optimal cut-off value to predict EOS (7–250 pg/mL). The reported values are from either umbilical cord samples or vein samples at different moments in the first 0–36 hours after birth (211). It is obvious that further studies with standardized methodology are needed to precisely determine IL-6 significant cut-off values for EOS. Another disadvantage is the fact that IL-6 levels rise not only in sepsis but also in hypoxia, fetal distress, preterm birth, usage of antenatal steroids and meconium aspiration syndrome (161). It was not clear in the study of Chiesa and colleagues, if the levels of IL-6 were influenced by gestational age and the presence of respiratory distress syndrome (199). In addition, high levels at 24 hours can be associated with the stress of birth, vaginal delivery, active labor, with or without the presence of chorioamnionitis, perinatal asphyxia, fetal acidosis, respiratory distress, low Apgar scores and brain damage (211). The level of IL-6 can be used in evaluating the prognosis of sepsis, as the

higher the value, the severe is the sepsis (211). Conversely, Chiesa and colleagues reported that high levels of IL-6 are not associated with sepsis severity (199). In order to improve the sensitivity and NPV, IL-6 has to be associated with other biomarkers, such as CRP and PCT (119,122,177).

IL-8 presents a rapid increase (in 1 to 3 hours from stimulation), being an early phase marker in the detection of EOS, but has the disadvantage of a short half-life of only 4 hours (122,156,162,163,177). This cytokine has a moderate accuracy, with a sensitivity of 80 – 91% and a specificity of 75 – 100% (156,157,162,163,177). IL-8 does not only correlate with the severity of infection, but is also appears to be more efficient in diagnosing EOS prior to other markers (IL-6, IL-10) (157,159,161,163,177). However, Sharma and colleagues concluded that IL-8 alone is not useful in the diagnosis and prognosis of sepsis, probably because its concentration rises also in necrotizing enterocolitis (NEC), surgery, trauma and meconium aspiration syndrome (114,162). If associated with CRP, the sensitivity and specificity of IL-8 increase (Gilfillan and Bhandari, 2017; Sharma *et al.*, 2020).

Even if IL–10 is not frequently studied as it is less expressed in neonates, an increased value is very suggestive for a severe infection, usually associated with multi organ damage (159,162,163). It can predict the prognosis and survival of a neonate affected by sepsis (162). While in other studies IL-8 is known to be the most useful marker in the diagnosis of EOS, Memar and colleagues stated that IL-10 is the best with a sensitivity and a specificity of 92% respectively 84% for a cutoff value of ≥ 173 pg/mL (163). The value of IL-10 can also increase in the same situations as IL–6 and IL–8 (114). High values of IL-10 (cut-off > 208 ng/L) in association with high values of IL–6 (cut-off > 168 ng/L) are suggestive for disseminated intravascular coagulation (DIC) in neonates with sepsis. This combination of markers leads to a sensitivity of 100%, specificity of 97%, PPV of 85% and NPV of 100%. It is important to note that cut-off values differ in measurements units that impose more studies to precisely decide the accurate value (122,159).

IL–35 is a newly described cytokine from the family of IL-12. It contributes to the regulation of host immunity by suppressing T-helper (Th) 1, Th 2 and Th 17 cell responses. Its levels are increased in systemic sclerosis, allergic rhinitis, and septic shock in adults. In neonates with EOS, IL-35 has not only the advantage of increasing rapidly (6 hours after infection, with a peak at 12 hours) but also of remaining stable for up to 3 days (163,212). In addition, it can be useful for the prognosis of EOS. For a cut-off value of 317 ng/mL, this interleukin showed a sensitivity of 78.48% and a specificity of 66.67% (163).

TNF-α concentration increases fast in 2 to 4 hours in both infection and inflammation, having a sensitivity of 75%, specificity of 88%, PPV of 67% and NPV of 51% for 130 ng/mL as the cut-off value (159). Hence, on its own, it is not a useful marker for the diagnosis of EOS, but in combination with IL-6, its sensitivity rises to 60% and its specificity increases to 100% (177). On one hand, the sensitivity is higher at birth and decreases with the postnatal age (lower at 24 hours), on the other hand, the NPV is more accurate at 24 hours than at birth (73–86%) (209). The main strength of this marker is that its level is not influenced by the gestational or postnatal age (177).

I.2.3.1.6. Presepsin (sCD14-ST)

Presepsin, a cleaved truncated form of soluble CD14 (sCD14), is a surface glycoprotein with a high affinity for lipopolysaccharides, and according to recent studies, may be a better marker that CRP and PCT for the diagnosis of EOS (213). sCD14 level not only increases in the first 24 hours after the onset of infection, just before CRP and PCT but also has a higher AUC (0.97–0.99), being considered an efficient marker in diagnosing EOS (162,212,214,215). In a newborn without signs of infection, the mean value of presepsin differs in term (649 ng/L) compared to premature infants (720 ng/L) (216). In contrast, in case

of infection, its value does not vary with GA, postnatal age or with other perinatal factors (213). The current data also suggests that the value decreases progressively with the administration of antibiotics, thus having the advantage of monitoring the response to therapy (212,215). In order to establish a suggestive cut-off value for EOS, further studies are needed. Cut-off values, sensitivity and specificity differ within EOS from LOS. In EOS, the cut-off varies between 305 and 672 ng/L and has a sensitivity of 81% and a specificity of 86%. Ruan and colleagues suggested higher values of sensitivity and specificity at a cut-off value of 722 ng/L, but the authors do not specify whether they occur in the case of EOS or LOS (212). A higher value, of 788 ng/L, has a sensitivity of 93% and a specificity of 100% (216). Also, a value of 539 ng/L demonstrated a sensitivity of 80%, a lower specificity 75%, a PPV of 91% and NPV of 59%. Elevated levels of presepsin are significantly associated with mortality at 30 days (215). sCD14-ST is efficient in diagnosing bacterial sepsis, especially if gram-negative bacteria are present (159,163). The main bias is that the type of measurements differs between various studies, leading to large range in significance of cut-off values. Parri and colleagues included in a study a large number of neonates and concluded that presepsin has a high accuracy in diagnosing EOS with a sensitivity and specificity around 90% (217).

I.2.3.1.7. Molecular techniques

Molecular diagnostics has the potential of providing results in less than 12 hours with better sensitivity than blood cultures (115,178). These techniques evaluate gene expression in disease and would be most useful for neonates with EOS born to mothers who have received intrapartum treatment with antibiotics. The 16S rRNA (ribonucleic acid) and 18S rRNA genes are preserved in all bacteria and in all candida species, respectively. Using microarray hybridization technique polymerase chain reaction (PCR) can detect the presence of bacteremia and also identify the infecting organism (218). According to a meta-analysis that include 23 studies on PCR-based molecular methods, mean sensitivity and specificity of PCR for bacterial 16S rRNA gene for the diagnosis of EOS were 0.90 (95% CI, 0.78 to 0.95) and 0.96 (95% CI, 0.94 to 0.97) respectively (119,172). The sensitivity of the assay depends on the accuracy of the extraction process and the presence of inhibitors and can be improved by pre-incubation of samples before PCR processing (178). Compared to blood culture, PCR has the advantages of higher accuracy, a significantly shorter time to result (4-6 hours) and a much less amount of required blood for sample (0.2–0.3 mL). However, the main disadvantage is its high cost and reduced availability (218)[129]. Molecular diagnostic techniques represent a promising perspective, but more studies are needed to assess their clinical utility, as there is still uncertainty about whether the detected bacteria actually represent the cause for the sepsis-like symptoms in a specific patient (178). Taking into account the current data available, molecular assays are not sensitive enough to completely replace microbial cultures in the diagnosis of EOS, but are useful as adjunctive tests (119). Blood cultures remain the gold standard for the detection of bacteremia or fungemia, despite their low sensitivity and prolonged time required for results (48 to 72 hours).

I.2.3.1.8. Novel biomarkers currently under investigation

CD64 is a high affinity FC receptor for immunoglobulin G, expressed by inflammatory cells in response to bacterial infection (156,162,163,209). Its value increases 5 to 10 times in the presence of sepsis, at an interval of 1- 6 hours of onset and remains stable over a period of 24 hours (156,162). Its advantages include rapid detection by flow-cytometry, the need for a small amount of blood and the results available in a maximum of 4 hours (119,122,163,219). In addition, the value of CD64 is not influenced by transient tachypnea of the newborn (TTN), respiratory distress syndrome or other non-infectious factors commonly occurring during the first 72 hours of life (219). Its value returns to normal

in a few days after the immune system removes the infection, but a study suggests that the peak of this marker would be at 48 hours (159,162). Repeated dosing is required to guide antibiotic therapy (122). For a cut-off between 2.19 – 3.62, CD64 has a sensitivity of 75–78%, specificity of 59–77%, PPV 29–54% and NPV 81–96% (162). CD64 is able to detect systemic infection 1.5 days before the onset of symptoms due to high sensitivity (89%), specificity (98%) and PPV (99%) (179). Given that on its own it has a moderate accuracy in diagnosing EOS, over the years various combinations with other biomarkers have been tried to increase its diagnostic value. In combination with elevated CRP and interleukin values or CD11b, the sensitivity and NPV of CD64, reaches maximum value (122,157,162,163). Weaknesses of this biomarker include high cost, lack of growth in viral infections, the presence of a moderately high value in premature infants that become similar to normal values in term newborns only after one month of life, high values not only in neonatal sepsis but also in NEC or other digestive pathology (119,122,159). In adults, the value of CD64 is higher in infections with gram-negative bacteria than in those due to gram-positive organisms, which has not been demonstrated in the newborn [109]. In conclusion, CD64 has limited utility on its own, therefore most authors recommend associating it with other markers, clinical signs or even with hematological scoring systems (122,159,219).

Neutrophil CD11b can be detected rapidly by flow cytometry, being considered an early marker of NS (155,220). Its value increases within 5 minutes of bacterial exposure, making it a more accurate marker in the diagnosis of EOS (92% sensitivity, 99% specificity) (115,158,177,220). In addition, due to the high surface density of neutrophils and monocytes, neutrophil CD11b may be a useful marker in diagnosing EOS even in VLBW (158). Although it has very good qualities for EOS detection, the unavailability methods for detection in clinical settings and the cost-effectiveness ratio make this marker a research one and not a clinical use. In a study by Stalhammar et al upregulation of neutrophil CD11b after stimulation with formyl-methionyl-leucyl-phenylalanine (fMLP), generated by organisms like Escherichia coli and Staphylococcus aureus, revealed alterations in receptor expression that were of the same strength in neutrophils from neonates as from adults (220). Moreover, the results of the research presented similar expression of receptors that mediate adhesion, migration, granule activation and phagocytosis determined by fMLP in neutrophils. CD15s, a selectin ligand involved in the inflammation process, appears to be a useful marker in differentiating viral from bacterial infection. A study by Markic et al proposed a model for identifying serious bacterial infection in paediatric patients under 6 months and found that the correlation between percentage of neutrophils expressing CD15s (%CD15S+), CRP and PCT presented a sensitivity of 87% and a specificity of 83% (221). E–selectin (CD62) and L–selectin (CD62L) are selectins activated by acute inflammation (158). Stoll *et al.* showed that for CD62 at 161.7 mg/L, there was sensitivity of 50%, specificity of 93.9% for the diagnosis of EOS (121). In addition, no correlation was observed between the levels of CD62L and infants with bacterial infection (158). Elevated levels of sCD13 (macrophage cell surface glycoprotein receptor), are significantly associated with neonatal infection before the antibiotic use. For a cut-off value of > 896.78 ng/ml the reported sensitivity was 100% and the specificity was 88% (177).

Pancreatic Stone Protein (PSP), a 16 kDa C-type lectin protein, is secreted by the pancreas in response to systemic stress and organ damage associated with sepsis. Observations that PSP level rise in mice and rats in response to septic insults, have led to studies based on adults that demonstrated its role as a potential biomarker in sepsis, sepsis associated with multiple-organ failure in patients with ventilator-acquired pneumonia or post-traumatic sepsis (222). ELMeneza *et al.* published a case control study on 90 newborn infants demonstrating that PSP was significantly higher in EOS compared to normal newborns, with 100% sensitivity and sensibility, PPV and NPV at a cut-off point > 133.8 pg/mL, and a cut-

off value of 125.6 pg/mL for preterm infants, also suggesting a useful value in EOS prognosis, as a statistically significant increase of PSP was observed among non-survival cases (223). Similar data were reported by Rass *et al.* who conducted a hospital-based prospective study on 104 newborn infants, and found a cut-off level of 12.96 ng/mL, with good sensibility (96.2%), specificity (88.5%), PPV (95.8%) and NPV (89.3%) (224). Also, Schlapbach *et al*. reported that PSP had a superior accuracy for EOS diagnosis compared to other markers like CRP and PCT, and provided fast results with a very small amount of blood required for sampling (225). The increase of PSP in septic newborns was explained by promoting proliferative responses in pancreatic cells and activation of polymorph nuclear cells, PSP/reg binds, activating neutrophils and behaving as acute phase reacting protein to early phase injury of infection. The statistically higher levels of PSP in non-surviving infants with EOS support a role for this biomarker in prediction of illness severity and unfavorable outcome (223).

Recently, nanofluidic technology was employed to develop a rapid PSP test for EOS requiring only a few drops of blood and results available within minutes, with a very good precision at about 90% (226).

Soluble intercellular adhesion molecule 1 (sICAM-1) is a protein factor used in the transfer of neutrophils to the site of inflammation in vivo (227). During infection, after activation of endothelial cells by cytokines, a rapid rise (within 1-6 hours) in the serum sICAM-1 levels is noticed. Neonatal sepsis is associated with increased serum sICAM-1 concentrations, which are correlated with severity of disease. The higher the serum value of sICAM-1, the more severe the infection is (228). Zhang et al. reported mean sensitivity and specificity of 76.9% and 82% respectively, but infants with EOS and LOS were evaluated together, without differentiating the two entities (227). Currently there is controversy regarding the usefulness of this marker in diagnosing EOS, as some authors proposed sICAM-1 as a valuable marker only in the first 4 days of life and others have noticed similar or even higher levels in healthy newborns in the first 5 days (229,230). Moreover, the proposed cut-off values vary significantly between studies and the accuracy as a diagnostic marker is questionable. For EOS, a cut-off of 228 ng/mL had a reported sensitivity of 33.3%, and specificity of 95%, with PPV of 50.3% and NPV of 90.35% (230), meanwhile a cut-off value of 400 ng/mL had better sensitivity (64%) and similar NPV (90%), but lower specificity (68%) and PPV (30%) (157). The diagnostic value of sICAM-1 can be significantly improved if used in association with PCT, presenting an AUC of 0.81, as shown by Zhang et al. (227). Considering the controversial data reported for this biomarker, further studies are required to assess its potential utility in EOS diagnosis.

Serum leptin, an immune regulatory hormone that enhances immune response with macrophage effector function, was found to have a higher level in neonates with positive blood cultures compared to those with negative blood cultures, but there was no difference between survivors and non survivors. For a cut-off value of 2.75 ng/mL, the sensitivity and specificity were 75% and 70%, respectively (231).

Progranulin, a 593-amino-acid autocrine growth factor that regulates TNF/TNFR signaling system, was recently studied also in neonates and may significantly predict EOS in neonates >34 weeks of gestation, with a cut-off value of 37.89 ng/mL at which the sensitivity and negative predictive value of 94.34% and 91.7% respectively. When combined to PCT the diagnostic performance was improved to a specificity of 89.06% and positive predictive value of 81.1% (232).

Neopterin is a biochemical marker for immune activity. Increased serum concentrations can be detected in situations when there is cell-mediated immune response. Data from small study groups suggests a better correlation with severity and mortality from sepsis compared to CRP. For a cut-off value of 70.56 nmol/L this marker has a specificity of

88.6% and a sensitivity of 94.7% to detect sepsis. However, the reported results are not specific to EOS (233).

Resistin, also known as adipocyte-specific secretory factor or FIZZ3, is a protein rich in cysteine with a controversial physiological role in obesity and insulin resistance. Some studies on adult and neonatal patients reported elevated serum levels during inflammation and infection. The few studies conducted on newborns suggested that this marker could be an indicator of EOS, but its diagnostic value proved to be less than that of CRP and cut-off value could not be established with accuracy due to several factors such as control group and number of days since the first sign of sepsis (234,235). Some biomarkers like sTREM-1 (human triggering receptor expressed on myeloid cells-1), pentraxin-3 and pro-adrenomedullin, that were found to have high values in infected adults and children, failed to prove their role in neonatal EOS (236).

The vascular endothelium is a component of the innate defense system with an important role in early recognition and limitation of bacterial invasion and a dynamic participant in cellular and organic processes. It controls vascular tone and permeability by expression of surface proteins and secretion of soluble mediators, regulates coagulation and thrombosis and coordinates recruitment and direction of leucocytes towards inflammation sites, with the involvement of surface molecules like E- and P- selectins, intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, whose expression is regulated by pro-inflammatory cytokines like TNF-α and IFN-γ (237,238). However, excessive endothelial activation may lead to systemic overproduction of cytokines and vasoactive substances associated with circulation disturbances and organ dysfunction in severe sepsis and septic shock (239).

Endocan (formerly known as endothelial cell specific molecule-1 or ESM-1) is one of the specific endothelial mediators with a structure of chondroitin/dermatan sulfate glycosaminoglycan and a molecular weight of approximately 50 kDa (240). Normally, endocan is localized mainly within the vascular endothelium, the distal tubules of the kidneys and in the lungs, at the level of small veins, arterioles, alveolar capillaries, bronchial epithelial cells and submucosal glands (241). In healthy subjects the serum concentration of endocan is low, but the levels are significantly increased in patients with sepsis and correlated with disease severity (238,242–245). Moreover, in newborns without infection, during the first 72 hours of life, endocan serum level appears was not to be significantly influenced by sex, delivery method, the presence of meconium in the amniotic fluid, fetal bradycardia/tachycardia or presence of minor birth trauma (ecchymosis, cephalohematoma, clavicle fracture), which have been associated with elevation of CRP and PCT (246). In septic neonates, serum concentrations of endocan are increased, but the insufficient sensitivity and specificity reported suggest that currently the clinical utility of endocan as a single marker for the diagnosis of neonatal EOS is limited. However, serum endocan could prove useful in combination with inflammatory markers as a part of a diagnostic tool for EOS, or if used at a low threshold, for ruling out sepsis, but more studies are necessary to establish the clinical utility of this molecule as a marker for diagnosis of EOS.

I.2.3.2. Endocan – normal values in uninfected neonates

In neonates, the clinical signs of EOS are mostly nonspecific and biomarkers that are routinely used to evaluate for sepsis, such as C-reactive protein (CRP) and procalcitonin, have low sensitivity, specificity and positive predictive value (247). Blood culture remains the gold standard method for sepsis confirmation, but results are usually available 48-72 hours after sampling, which can lead to delay in adequate treatment and negative outcome of newborns with EOS. Endocan is a specific endothelial mediator involved in the inflammatory response

and its role in the diagnosis of sepsis has been studied in adult patients and late-onset neonatal sepsis.

Published papers:

- Zonda GI, Zonda R, Cernomaz AT, **Paduraru L**, Grigoriu BD. Endocan serum concentration in uninfected newborn infants. *J Infect Dev Ctries*. 2019 Sep 30;13(9):817-822

Objective

The aim of this study was to establish normal values range for endocan serum concentration in term and preterm newborns without risk factors for EOS.

Material and methods

We conducted a prospective study on 65 newborns admitted to the Neonatology Department of our tertiary medical center during a 10 months period (from June 2015 to March 2016). The study included both term and preterm infants with GA ranging from 33 to 41 weeks, recruited on the first day of life, based on the following criteria: term or preterm newborn (with GA ≥ 33 weeks) with successful transition to extrauterine life, on the first day of life, without risk factors for/or clinical suspicion of sepsis. The exclusion criteria were: presence of risk factors for infection (rupture of membranes > 18 hours, chorioamnionitis, maternal fever, positive cultures from the amniotic fluid, vaginal or urinary tract infections in the mother during pregnancy, foul smelling amniotic fluid) and/or clinical signs of sepsis (temperature instability, apnoea, need for supplemental oxygen, need for non-invasive or invasive respiratory support, tachycardia/bradycardia, feeding intolerance) (154,248–250). Infants with congenital anomalies were also excluded. The study protocol was approved by the Ethical Committee of the University of Medicine and Pharmacy, and written informed consent was obtained from the parents of the newborns before inclusion in the study.

One milliliter of blood was collected from a peripheral vein of each infant during the first 6 hours of life. A second blood sample was drawn on day 3 of life. The serum was immediately isolated and frozen at -80°C until analysis. Endocan concentration was determined by a sandwich-type enzyme-linked immunosorbent assay using anti-Endocan monoclonal antibodies (Do It Yourself ELISA Kit H1®, Lunginnov, Lille, France). Values were expressed in ng/mL (242).

Results

The study group consisted of 38 term and 27 preterm newborns (with GA ≥ 33 weeks), as presented in Table 24.

Table 24. Clinical and demographic characteristics of the newborns in the study group

Category	Term	Preterm
Number (%)	38 (58.5%)	27 (41.5%)
GA (weeks), m ± SD	38.3 ± 1.0	34.5 ± 1.1
Sex (female/male)	17/21	18/9
BW (g), m ± SD	3,232 ± 376	2,136 ± 387
Delivery (vaginal/C-section)	21/17	19/8
Apgar 1 min – median (IQR)	9 (1)	7 (2)
Apgar 5 min – median (IQR)	9 (0)	8 (2)
Apgar 10 min – median (IQR)	9 (0)	8 (1)

GA = gestational age; BW = birth weight

Following processing of the samples, valid endocan results were obtained for 57 samples on day 1 and 47 samples for day 3. For the rest, the remaining amount of serum was insufficient for the analysis. Out of 38 term infants, there were 36 samples for day 1 and 24 for day 3 to be analyzed; meanwhile, out of 27 preterm infants only 21 samples for day 1 and 23 for day 3 were available for assay. The statistical comparison was possible for 39 sample pairs. In order to establish a trend and to compare the serum levels of endocan on days 1 and 3 for patients included in the study group, we performed the statistical comparison only on these samples.

There were no statistically significant differences of endocan level between the first and the third day of life in either term (p = 0.09) or preterm (p = 0.81) infants, as detailed in Table 25. Also, there were no statistically significant differences of endocan levels between term and preterm infants for either day 1 (p = 0.11) or day 3 (p = 0.79) measurements.

Table 25. Endocan serum concentration in term and preterm newborns on days 1 and 3 of life

Serum endocan (ng/mL) mean ± SD (range; 95%CI)	**n = 22**	**Term newborns**	**Std. error**	**n = 17**	**Preterm newborns (GA ≥ 33 weeks)**	**Std. error**	**p**
Day 1		1.74 ± 0.65 (0.48-3.22; CI: 1.49-2.03)	0.13		2.02 ± 0.49 (1.14-3.20; CI: 1.77-2.27)	0.11	†p = 0.11
Day 3		2.02 ± 0.48 (1.16-2.95; CI: 1.81-2.24) *p = 0.09	0.10		1.97 ± 0.74 (0.77-3.40; CI: 1.59-2.35) §p = 0.81	0.18	‡p = 0.79

GA = gestational age; *significance coefficient for serum endocan between day 1 and day 3 in term newborns; §significance coefficient for serum endocan between day 1 and day 3 in preterm newborns; †significance coefficient for serum endocan on day 1 between term and preterm newborns; ‡significance coefficient for serum endocan on day 3 between term and preterm newborns.

By stratifying the study group using gestational age – week based, we identified a potential downward trend after 39 weeks (Fig. 19) for day 1 endocan concentration – significantly higher among neonates born up to 38 weeks compared to those born at 39 weeks and after, 1.94 vs. 1.46 ng/mL (p = 0.007). There was no difference in endocan levels on day 3 between groups stratified by the 39 weeks threshold 2.10 ± 0.7 vs. 2.10 ± 0.82 ng/mL.

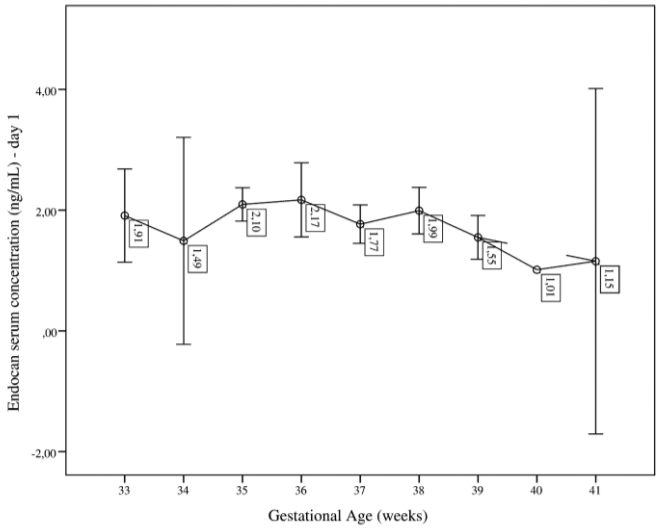

Fig. 19. Endocan serum concentration (ng/mL) variation with gestational age on day 1

Infants delivered by C-section did not have significantly different concentrations of endocan compared to those born vaginally. Evidence of fetal distress or presence of minor birth trauma did not influence significantly endocan serum levels, as shown in Table 26.

Table 26. Endocan serum concentration (ng/mL) variation with delivery method, fetal distress and minor birth trauma

		Median (IQR)
Category	**n**	**Day 1**
C-section	31	1.74 (0.64)
Vaginal delivery	26	1.83 (0.77)
Fetal distress	8	1.53 (1.24)
No distress	49	1.85 (0.65)
Minor birth trauma	8	1.98 (0.59)
No birth trauma	49	1.79 (0.74)

Discussions

The diagnosis of EOS is challenging due to the lack of more specific biomarkers, as many infants present some risk factors or have an intrauterine infection and subsequent inflammation (251). Because the clinical signs that may suggest EOS are nonspecific, the diagnosis is mostly based on laboratory findings. Potential biomarkers, such as CRP and procalcitonin (PCT), are extensively used in clinical settings (105,249), but during the first 24 hours of life their specificity and sensitivity are low (199).

For example, CRP may be elevated in multiple other pathological situations besides bacterial infections (viral infections, trauma, ischemic tissue injury, hemolysis, meconium aspiration syndrome or chorioamnionitis without invasive fetal or neonatal disease). Thus, its sensitivity as a biomarker for EOS varies from 29 to 90%. PCT serum levels rise faster than CRP, but the initial reports of sensitivity close to 100% have not been confirmed by subsequent evaluations (248,252,253).

The complex pathophysiological mechanisms of sepsis involve inflammation and endothelial activation as critical determinants of the host response, leading to the hypothesis that biomarkers of endothelium dysfunction might be used as clinical tools in the diagnosis and follow-up of sepsis (254).

There is compelling evidence for including endocan among the tests that may be used to diagnose and predict mortality in adults with sepsis. The first relevant study was conducted by Scherpereel *et al.*, who found that endocan level was significantly elevated in adult patients with sepsis and was correlated with sepsis severity and mortality (242). Evidence available from several studies indicates a potential role for endocan as a biomarker for the diagnosis of sepsis in adults with a better discriminative power to distinguish septic patients from non-septic in comparison to CRP and PCT (238,243,255).

The utility of endocan as a marker for early onset sepsis is beginning to be investigated. The starting point should be the characterisation of the physiological values and dinamics for this molecule during the first days of life. Studies previously published on neonatal patients compare serum endocan concentrations in newborns with sepsis and systemic inflammation. Due to the complete absence of data concerning values of endocan in healthy newborns, we compared our measurements of endocan concentrations to those reported in healthy adult patients. The serum levels in newborns were significantly higher compared to adults.

Endocan serum levels for both term (1.74 ± 0.65 ng/mL, mean ± SD) and preterm infants (2.02 ± 0.49 ng/mL) are higher than those reported in the literature for healthy adult volunteers (reported mean value 0.77 ng/mL [0.51-0.95]) (242). However, our data show lower median concentrations of endocan (ng/mL) in neonates on the first day of life than

those reported by Hentschke *et* al. for venous cord blood samples in term newborns (2.91, IQR = [2.20-3.66]) (256). The higher cord blood endocan concentration may mirror the higher endocan levels in maternal plasma observed during the third trimester of pregnancy.

Our data does not show any significant difference in endocan levels between term and preterm infants either on day 1 or day 3. However, our study did not include preterm infants with GA ≤ 32 weeks because neonates in this category that were admitted in our unit during the recruitment stage of the study had perinatal risk factors for infection or nonspecific clinical signs that may suggest EOS and did not meet the inclusion criteria.

Similarly, we found no statistically significant differences in endocan serum concentration due to differences in sex and delivery method. Similar results were reported by Aksoy *et al.* who did not find significant differences between newborns delivered vaginally compared to those delivered by C-section with spinal anesthesia, which was also the type of anesthesia used for C-section in our study group (257). Delivery mode and anesthesia were included as potential factors that may influence endocan levels by altering oxygenation and inflammatory status of the newborn (258).

Our data show that endocan serum levels are not significantly influenced by the presence of minor birth trauma or fetal distress. Thus, in newborns without infection, in the first three days of life, endocan serum level appears not to be significantly influenced by obstetrical and fetal factors that are associated with elevation of usual inflammatory markers (CRP, PCT).

Corroborating with data supporting the endocan elevation in neonatal LOS, we might infer that endocan level could be valuable as a biomarker for diagnosis of EOS and might reduce the false positive results. However, these data should be interpreted with caution, given the small size of the study group and individual variability of physiological adaptation to extrauterine life.

While there is no difference in endocan concentration between term and preterm neonates in either day 1 or day 3 of life, some influence of the GA on serum endocan cannot be completely ruled out as far as day 1 of life is concerned. The post-hoc analysis revealed that the 39 weeks gestational age threshold might imply a certain degree of maturity of lung development. This hypothesis is validated by the data supporting the optimal timing for elective C-section at 39 weeks (rather than earlier) which shows a lower risk of neonatal respiratory morbidity: transitory tachypnea of the newborn, respiratory distress syndrome, and persistent pulmonary hypertension of the newborn (259–261). The lack of significant difference on the third day of life is to be expected, considering that the transition to extrauterine life is completed by that time for healthy neonates.

A significant limitation of our study is the small number of samples, which proved difficult to overcome in the clinical setting, where the amount of blood collected is limited and sometimes needed also for other diagnostic tests. In order to minimize possible pain and discomfort to the enrolled newborns, we only took two blood samples for study purposes, one as close as possible to the moment of birth, and the other at 72 hours of life, which marks the threshold of diagnosis for EOS. Only two values might be insufficient to accurately characterize the kinetics of endocan in newborns during the first three days of life.

Conclusions

Our results represent a first attempt to provide a normal range of serum endocan levels in the newborn. This information is anticipated to be useful if endocan proves to have value as a potential marker in neonatal sepsis. Endocan serum levels in neonates during the first 3 days of life are higher in our study group compared to those found in healthy adults, so there is a need for different reference values if this parameter is to be used as a diagnostic tool in EOS. Our study shows no statistically significant variation in endocan level between the first and

third day of life in either term or preterm infants; moreover, endocan level does not appear to be significantly influenced by sex, delivery method, or factors associated with elevation of inflammatory markers such as minor birth trauma or fetal distress. This suggests that endocan could be a useful biomarker for diagnosis of EOS. However, more studies on a larger number of newborns are warranted in order to establish more accurate kinetics of endocan during the first 3 days of life and the potential role of this molecule in the diagnosis of EOS. Endocan levels seem to drop significantly after 39 weeks of gestation, and this finding may provide a physiological explanation for the lower respiratory morbidity in newborns delivered by elective C-section at more than 39 completed weeks of gestation.

I.2.3.3. Endocan – a potential new biomarker for EOS

Current clinical practice employs a mixture of clinical elements and non-specific laboratory tests, which could be improved with the introduction of a new biomarker like endocan. Its role in the diagnosis of sepsis has already been established in adult patients and studied for the diagnosis of late onset neonatal sepsis. Neonatal early onset sepsis assessment is based on the history of pregnancy and delivery and nonspecific clinical signs. None of the biomarkers currently in use for clinical practice has adequate prognostic value, so it is not possible to clearly distinguish neonates with culture-proven sepsis from those with only risk factors or clinical suspicion. Endocan is an endothelial mediator involved in the inflammatory response that is present in low concentrations in the serum of healthy subjects, and in much higher concentrations in patients with SIRS and septic shock.

Published papers:

- Zonda GI, Zonda R, Cernomaz AT, **Paduraru L**, Avasiloaiei AL, Grigoriu BD. Endocan - a potential diagnostic marker for early onset sepsis in neonates. *J Infect Dev Ctries*. 2019 Apr 30;13(4):311-317

Objective

The purpose of this study was to investigate a possible role for endocan in the diagnosis of neonatal EOS, thus facilitating timely initiation of treatment and improving the outcome of these critically ill newborns.

Material and methods

We conducted a prospective study of newborns admitted in the Neonatology Intensive Care Unit of our tertiary care center. Information on the gestational age (GA), weight, gender, mode of delivery, need for resuscitation, Apgar score, risk factors for infection and clinical signs of sepsis were noted. The study included term and preterm infants with GA ranging from 26 to 41 weeks, with postnatal age < 24 hours at admission. Study group inclusion was based on the presence of risk factors and clinical signs (Table 27). Infants with congenital anomalies were excluded.

Within the study group, the newborns were divided between septic and non-septic groups. The septic group included newborns with confirmed infection (positive blood culture) and probable infection (negative blood cultures but with clinical and laboratory evidence of sepsis). The suspicion of infection was assessed on admission in all newborns with clinical signs compatible with infection. Patients who were initially admitted with suspected sepsis but in whom the diagnosis of sepsis was not supported by clinical or laboratory findings were ultimately assigned to the non-septic group (Table 28).

Table 27. Clinical elements for study inclusion

Risk factors	**Clinical signs**	**Clinical and/or biological deterioration during the first 72 hours of life (considered to be due to sepsis)**
Rupture of membranes > 18 hours	Temperature instability	Hypotension requiring volume expanders or inotropic support
Chorioamnionitis	Apnea	Anemia requiring packed red cells transfusion
Maternal fever	Need for supplemental oxygen	Acidosis
Positive cultures from the amniotic fluid	Need for non-invasive/ invasive respiratory support	Necrotizing enterocolitis
Vaginal/urinary tract infections during pregnancy	Tachycardia/bradycardia	Intraventricular hemorrhage
Foul smelling amniotic fluid	Feeding intolerance	
*The presence of at least one risk factor and three or more clinical signs was required.		

Table 28. Criteria employed for defining neonatal sepsis

Confirmed sepsis	**≥ 3 sepsis-related clinical signs (see Table 27)**
	CRP ≥ 6 PCT > 0.5 ng/mL ≥ 2 altered serum parameters* Blood culture: **positive**
Probable sepsis	**≥ 3 sepsis-related clinical signs (see Table 27)**
	CRP ≥ 6 PCT > 0.5 ng/mL ≥ 2 altered serum parameters* Blood culture: **negative**
Possible sepsis	**< 3 sepsis-related clinical signs (see Table 27)**
	CRP < 6 PCT ≤ 0.5 ng/mL < 2 altered serum parameters* Blood culture: **negative**
No sepsis	**No sepsis-related clinical signs***
	CRP < 6 PCT < 0.5 ng/mL No altered serum parameters Blood culture: **negative**
*Serum parameters other than CRP or PCT: white blood cells count; absolute neutrophil count, platelet count.	

Within the septic group, 22 infants had severe sepsis according to internationally accepted definitions (105,106). Antibiotic treatment was initiated on admission for all patients in both the septic and non-septic groups according to existing internal protocols (262,263). In the non-septic group antibiotics were discontinued based on clinical and laboratory findings after 72 hours, while in patients with clinically diagnosed sepsis or with positive cultures,

antibiotic therapy continued for at least 7 days. The study protocol was approved by the Ethical Committee of the University of Medicine and Pharmacy, and written informed consent was obtained from the parents of the newborns before inclusion in the study. Data were anonymised prior to analysis.

From each newborn included in the study, 1 mL of blood was collected from a peripheral vein on the first day of life, at admission in the neonatal intensive care unit (NICU) at the time of initial laboratory workup before any treatment, and then on days 3 and 7. Serum was immediately isolated and frozen at – 80°C until analysis. The concentration of endocan was determined by a sandwich-type enzyme-linked immunosorbent assay using anti-Endocan monoclonal antibodies (Do It Yourself ELISA Kit H1, Lunginnov, Lille, France). Values were expressed in ng/mL (242).

Results

The study group consisted of 24 term and 35 preterm newborns evaluated on the first day of life for suspicion of sepsis, for which the parents granted their informed consent. According to the criteria already mentioned, 32 newborns were assigned to the septic group (10 with sepsis and 22 with severe sepsis) and 27 were included in the non-septic group (Table 29).

Table 29. Stratification of the newborns included in the study group based on the severity of sepsis

	Sex	Gestational age		Total
		Preterm	**Term**	
Non-septic	Female	3	4	7
	Male	5	15	20
	Total	**8**	**19**	**27**
Sepsis	Female	5	1	6
	Male	2	2	4
	Total	**7**	**3**	**10**
Severe sepsis	Female	5	0	5
	Male	15	2	17
	Total	**20**	**2**	**22**
Total		**35**	**24**	**59**

Septic patients had lower gestational age ($p < 0.01$) and lower birth weight ($p < 0.01$) (Table 30).

Table 30. Clinical and demographic characteristics of the newborns in the study group

Group	Non-septic	Septic
No	27	32
Gestational Age	36.5 ± 3.0	31.5 ± 4.3
Preterm/Term	8/19	27/5
Sex (female/male)	7/20	11/21
Birth Weight (g)	2812.9 ±780.2	1795.3 ± 955.8
Delivery (vaginal/c-section)	10/17	13/19
Apgar 1 min (median) IQR	8 (2)	5 (4)
Apgar 5 min (median) IQR	8 (2)	7 (3)
Apgar 10 min (median) IQR	8 (2)	7 (3)

Sepsis was confirmed by positive blood culture in 3 newborns. Two infants died from severe sepsis: one at 28 hours since birth and the other on the 7th day of life. Neither had a positive blood culture.

Table 31. Mean serum endocan (ng/ml) in septic versus non-septic neonates measured on days 1, 3 and 7

Serum endocan (ng/mL) mean +/-std. dev.	**Group 1 (non-septic)**	**Group 2 (septic)**
Day 1	1.77 +/- 0.57	2.43 +/- 0.95
p (95%CI of mean difference)		***0.004 (**0.22-1.1**)***
Day 3	2.32 +/- 0.3	2.92 +/- 1.2
p (95%CI of mean difference)		*0.3(0.6-1.8)*
Day 7	1.97 +/- 0.57	2.04 +/- 0.75
p (95%CI of mean difference)		*0.8(0.6-0.8)*

In septic compared to non-septic neonates the mean serum concentration (ng/mL) of endocan was significantly higher at admission to the NICU on day 1 (Table 31). In both non-septic and septic newborns the mean endocan concentration continued to rise on day 3, but even though the mean serum level was still higher in septic patients, the difference between the two groups was no longer statistically significant.

The mean endocan concentration measured on day 7 was lower compared to day 3 in both groups, but the decrease was statistically significant only in septic newborns (2.04 vs. 2.92, p = 0.01) (Fig. 20).

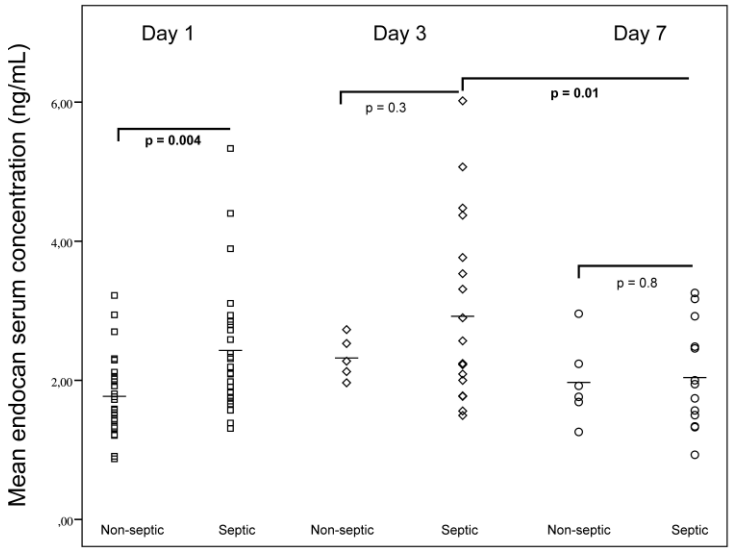

Fig. 20. Comparison between endocan serum concentration (ng/mL) on day 1, day 3 and day 7 in non-septic and septic newborns.

Mean endocan serum levels measured on the first day of life were significantly higher in neonates with sepsis (2.39 ± 0.82) and severe sepsis (2.45 ± 1.02) compared to those included in the non-septic group (1.77 ± 0.57) (Fig. 21A). Mean endocan serum levels measured on the third day of life remained higher in neonates with sepsis (2.53 ± 0.94) and severe sepsis (3.11 ± 1.42) compared to non-septic newborns, but the difference was not statistically significant (Fig. 21B).

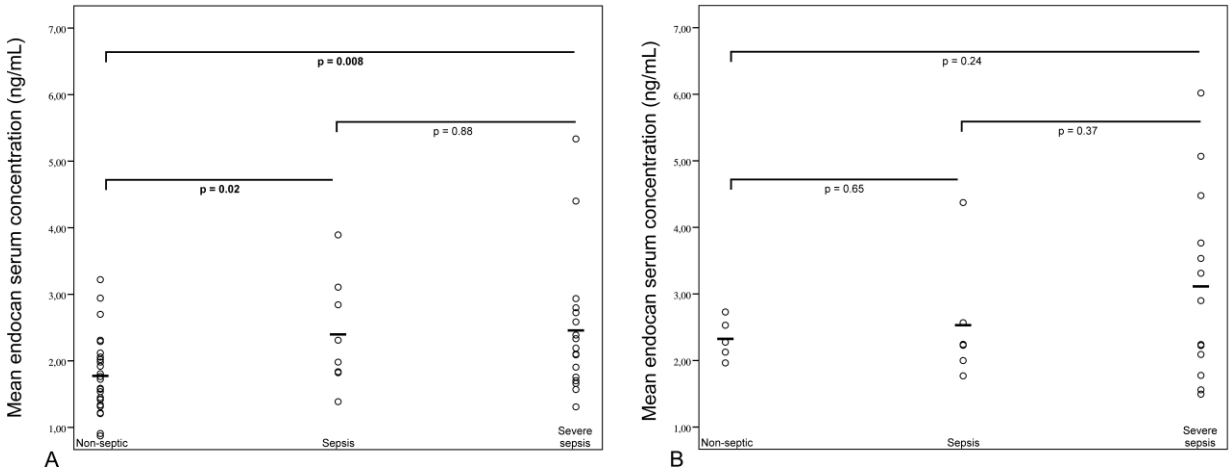

Fig. 21. Comparison of mean serum endocan concentrations measured on day 1 (A) and day 3 (B) in newborns with sepsis and severe sepsis versus non-septic neonates.

ROC curve analysis for the utility of endocan in differentiating between septic and non-septic newborns returned an area under the curve (AUC) of 0.73 (p = 0.004, 95% CI = 0.597- 0.871) (Fig. 22). Based on maximum Youden index, an optimum threshold value of 1.62 ng/mL corresponds to a sensitivity of 88% and a specificity of 50%.

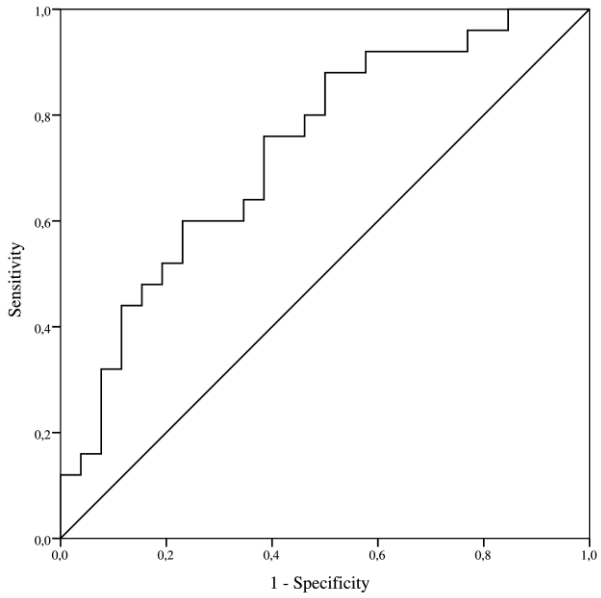

Fig. 22. ROC curve analysis for the differentiation between septic and non-septic newborns based on the endocan serum concentration measured on day 1.

The serum concentration of endocan on the first day of life was statistically correlated with the number of days of mechanical ventilation in non-septic neonates (Pearson correlation coefficient = 0.958, p = 0.01); however, it must be noted that only 5 of 27 patients in this group required mechanical ventilation. We did not find a similar correlation between the mean endocan level and the number of days of mechanical ventilation for newborns with sepsis or severe sepsis. The mean number of days of mechanical ventilation in septic newborns was 9.9 ± 15.5, with a non-normal distribution and a range of 70.

Hypotension was present in 12 infants, 1 with sepsis and 11 with severe sepsis. Inotropic support was necessary for 9 newborns, all preterm infants with severe sepsis, but there was no statistical correlation between the mean serum endocan level and the cardio-vascular status.

Discussions

Currently, there is no test or panel of markers usable for neonatal EOS diagnosis with an acceptable sensitivity and specificity range. It is not possible to clearly distinguish neonates with culture-proven sepsis from those with only risk factors or non-specific clinical signs, and none of the biomarkers currently in use for clinical practice has adequate prognostic value. In critically ill newborns, the decision of initiating antimicrobial therapy is usually formulated on clinical considerations, regardless of the laboratory results.

The vascular endothelium is a component of the innate defense system involved in early recognition and limitation of bacterial invasion. It controls vascular tone and permeability by expression of surface proteins and secretion of soluble mediators, regulates coagulation and thrombosis, and coordinates recruitment and direction of leucocytes towards inflammation sites (237). The activation of endothelium in the presence of microbial components generates production of cytokines, chemokines and adhesion molecules which attract circulating leukocytes. Activated leukocytes, platelets and endothelial cells lead to the release of vasoactive substances. Excessive endothelial activation may generate vasodilation and organ dysfunction in severe sepsis and septic shock (106).

Endocan is one of the specific endothelial mediators involved in the inflammatory response (241). It is present in low concentrations in the serum of healthy subjects, but the levels are much higher in patients with SIRS and septic shock and are correlated with illness severity and prognosis (238,242,243).

Our study shows that septic subjects have significantly higher mean endocan serum concentration than non-septic patients on day 1, which points to endocan as a potential marker for timely diagnosis of neonatal EOS. This is consistent with previously reported data on septic adults (242) and neonates with LOS (254).

When looking at the mean serum levels of endocan reported for adult patients with sepsis (1.95 ng/mL) our data show higher mean concentrations in septic newborns (2.43 ng/mL on day 1 and 2.92 ng/mL on day 3). We determined endocan concentrations on different days and compared both day 1 and day 3 values of endocan in newborns to reported data for adults, taking into account the difficulty in appreciating the exact moment for onset of sepsis in adult patients. The blood was sampled at the time of admission for adults, but this moment most likely does not coincide with day 1 of early-onset neonatal sepsis. Similarly, our data show higher mean endocan concentrations in non-septic newborns (1.77 ng/mL for day 1 and 2.32 ng/mL for day 3) compared to adults with systemic inflammatory response syndrome (0.72 ng/mL).

The significant decrease of serum endocan level on day 7 compared to the concentration measured on day 3 is compatible with the resolution of the systemic inflammatory response following specific treatment, as there are data linking the synthesis and release of endocan to pro-inflammatory cytokines (237,240,241).

The significantly higher level of endocan in septic vs. non-septic newborns on day 1 suggests a role of endocan in detection of potential cases of EOS. However, the cut-off value of 1.62 ng/mL has a sensitivity of 88% and a specificity of only 50%, which limits the practical utility of endocan as a single marker for the diagnosis of neonatal EOS. Using a higher threshold value (> 2.15 ng/mL) improves specificity to 81%, but decreases the sensitivity to 52%. For diagnostic purposes, a marker should have a very high sensitivity (approaching 100%) and good specificity (> 85%), or if it is unable to satisfy both criteria, then the optimal cut-off should be chosen so that both the sensitivity and the specificity approach 80% (264,265). In this context, serum endocan could probably be integrated with other inflammatory markers and clinical elements in order to develop a composite diagnostic tool for EOS, or it might prove to be useful at a low threshold for ruling out sepsis.

We also found a correlation between the value of endocan on the first day of life and the number of days of mechanical ventilation in non-septic neonates suggesting that endocan is related to the severity of respiratory disease, but the value of this analysis is limited by the small number of patients in this group that were mechanically ventilated. Among septic patients, we had a few outliers with 21, 37 and 70 days of mechanical ventilation, but in these cases the need for invasive respiratory support was determined also by other factors besides the severity of infection.

Our data does not show a correlation between the mean serum endocan level and the cardiovascular status as reflected by hypotension requiring volume expanders or vasopressor agents, suggesting that endocan may be more related to sepsis itself. Such a correlation would have been expected, as the data published on adult patients suggests a correlation between endocan serum concentration and septic shock. However, in neonates, especially preterm infants, hypotension requiring inotropic medication may reflect the contribution of other factors besides the inflammatory process, such as the decreased contractility due to the immature myocardium, deficient transition from intrauterine to extrauterine circulation, patent ductus arteriosus, perinatal hypoxia, or decreased systemic blood flow due to increased intrathoracic pressure in infants requiring positive pressure ventilation (266).

One limitation of our study is the low number positive blood cultures, so newborns with probable sepsis were assumed to be similar to those with culture-proven sepsis. Exposure of neonates to maternal antibiotic treatment during labor and delivery and the small blood volumes collected when obtaining blood cultures (0.5-1.0 mL) may have led to decreased ability to diagnose sepsis by blood culture. On the other hand, neonatal sepsis cannot be ruled out solely based on a negative blood culture result (267,268).

Another limitation of our study is the small number of samples which had to be synchronized with the usual blood tests performed according to the internal protocol. In septic newborns the serum concentration of endocan seems to show an accelerated rise from the first day of life, followed by a less marked increase up to the third day of life and then a significant decrease by day 7, probably due to the resolution of the systemic inflammation as a result of antibiotic treatment. However, only 3 values from each infant might be insufficient to accurately characterize the kinetics of endocan in newborns with EOS.

Conclusions

Serum endocan levels are significantly increased in septic compared to non-septic neonates in the early stages of sepsis; the levels continue to rise up to 72 hours from the onset of sepsis and then decrease by the seventh day of treatment. This suggests a potential role for endocan serum level as a marker for early diagnosis and follow-up in neonatal EOS. Further studies on a larger number of cases are needed in order to establish the diagnostic role of this molecule in practice.

CHAPTER I.3 NEONATAL HYPOXIA AND ITS LONG-TERM CONSEQUENCES

I.3.1. State of the art

Hypoxia is the major life-threatening event both in prenatal and postnatal life. During pregnancy and immediately after birth, main efforts are directed to prevent, detect, and treat hypoxia, as it has major consequences for the entire body, especially for vital organs like heart and brain (269,270). This condition represents an emergency, whether it is of antenatal, intrapartum, or postnatal origin, as when it occurs, hypoxia will eventually generate the same alteration in function and will lead to severe organ failure and respiratory and cardiac arrest.

Specific situations like some congenital anomalies and/or genetic syndromes may complicate with conditions like obstructive sleep apnea (OSA) that will enhance hypoxic

status and exacerbate neurological damage (271). Recent studies demonstrated and explained the link between obstructive sleep apnea and development of dementia, earlier in life. Both intermittent hypoxia and sleep fragmentation during OSA interfere with brain structure and function, thus enhancing vulnerability to neurodegenerative diseases (272). A biphasic pattern of neuroimaging findings could be in play in OSA (273), with acute transitory or compensatory responses (grey matter hypertrophy, restricted white matter diffusivities) followed by evidence of cellular damage (grey matter atrophy, lower white matter fractional anisotropy, higher white matter hyperintensity burden, higher water diffusivities). Moreover, OSA has been recently associated with increased amyloid and tau burden (274,275), two proteins involved in Alzheimer`s disease (AD) pathophysiology. Several mechanisms underlie these neuroimaging or pathological findings and include inflammation, oxidative stress, metabolic disturbances, cerebral edema, metabolic disturbances and endothelial dysfunction (276) and all these mechanisms are common in neonatal asphyxia. Indeed, inflammation is involved in neurodegenerative processes, notably by triggering a positive feedback loop that increases amyloid beta production and oxidative stress, facilitating amyloid and tau pathology (277,278).

Every effort must be directed to preservation of oxygen supply at every level, in order to avoid lactic acidosis and progressive anaerobic metabolism.

At birth, hypoxia may occur in different grades: mild, moderate, or severe. Mild hypoxia results in a transient delay in transition to extrauterine life, without major consequences on further brain function. Most of the cases will spontaneously recover only with small amount of supplementary oxygen. Moderate hypoxia impairs the function of different organs and systems, in various degrees, generating transient lesions and organ failure status, potentially affecting all organs (269,279). Severe hypoxia may lead to asphyxia that can start even from intrauterine life, may occur during delivery, or may take place even after birth in certain situations.

Neonatal asphyxia has been defined by the guidelines of the American Academy of Pediatrics (AAP) and the American College of Obstetrics and Gynecology (ACOG) as a neonatal condition following any episode of fetal or neonatal hypoxia that leads to all of the next criteria (280,281):

1. profound metabolic or mixed acidemia (pH <7.00) in umbilical artery blood sample
2. persistence of an Apgar score of 0–3 for longer than 5 min
3. neonatal neurologic sequelae (e.g., seizures, coma, hypotonia), and
4. multiple organ involvement (e.g., kidney, lungs, liver, heart, intestines).

Perinatal asphyxia occurs when an antenatal, intranatal or postpartum neurologic insult or any combination of the three leads to:

- hypoxemia (decreased oxygen flow to the fetus/newborn)
- hypercapnia (altered O_2/CO_2 exchange)
- ischemia (inadequate perfusion of tissues and organs)(282).

The main factors that may be involved in generating neonatal hypoxic-ischemic events are summarized in table 32.

Systemic hypoxia and cerebral ischemia lead to a decrease in oxygen and glucose. The consequence is anaerobic glycolysis, followed by the decrease of ATP and acidosis, all of these contributing to impairment of brain (283). Various intertwined mechanisms (excessive excitatory amino-acids receptors synthesis, intracellular calcium accumulation, free radical generation) influence the outcome of hypoxic-ischemic injury. DNA is damaged by oxidative destruction but also by structural alterations, such as chain breakage, deletions of bases and chromosomal anomalies (284). Moreover, reactive oxygen species modulate the transduction of cellular proliferation pathways, and an excessive accumulation can lead to cellular death

through necrosis and apoptosis (285). Reactive oxygen species can promote the expression of adhesion molecules, leading to activated granulocyte accumulation, which amplifies cellular destruction (286).

Table 32. Main factors that may generate neonatal hypoxic-ischemic events

Maternal conditions	Uterine and placental factors	Fetal factors	Labor & delivery factors
Diabetes Hypertension Heart/lung diseases Infections Anemia Epilepsy Drugs/medication during pregnancy	Placenta praevia Placental abruption Placental infarction/fibrosis Umbilical cord compression Umbilical cord prolapse Uterine malformations	Genetic/congenital anomalies Prematurity IUGR Post-term newborn Multiple pregnancy Hemolytic anemia Fetal infections Polyhydramnios	Abnormal presentations Prolonged/precipitated labor Forceps delivery Emergency cesarean section Maternal sedation during labor Meconium stained amniotic fluid

Post-asphyxia syndrome may include several impairments for different organs:

1. BRAIN

Hypoxic ischemic brain injury is the most important consequence of perinatal asphyxia when prolonged hypoxia overwhelms compensatory mechanisms. The following lesions may be seen after moderate or severe asphyxia:

- focal or multifocal cortical necrosis
- watershed infarctions
- selective neuronal necrosis
- necrosis of thalamic nuclei basal ganglia

Hypoxic-ischemic encephalopathy (HIE) has a spectrum of clinical manifestations from mild to severe, including:

- hypotonia/hypertonia
- abnormalities of the primary reflexes (Moro, suckling)
- periodic breathing
- tonic or multifocal clonic seizures which occur 6-24 hours after the hypoxic insult
- progressive deterioration in CNS function, with prolonged apnea and coma, in severely affected infants

2. CARDIOVASCULAR SYSTEM

Infants with perinatal asphyxia may have *transient myocardial ischemia.* They develop respiratory distress and cyanosis shortly after birth. They will have signs of congestive heart failure, such as:

- tachypnea
- tachycardia
- enlarged liver
- gallop rhythm

In its severe form, cardiac dilatation and tricuspid valve incompetence may accompany congestive heart failure.

3. LUNGS

- increased pulmonary vascular resistance
- pulmonary hemorrhage
- pulmonary edema secondary to cardiac failure
- inhibition of surfactant synthesis due to persistent acidemia with secondary respiratory distress syndrome
- meconium aspiration syndrome
- persistent pulmonary hypertension

4. KIDNEY

Whenever a neonate develops severe asphyxia, kidney damage may result. Proteinuria is common and in association with myoglobinuria, lead to acute tubular necrosis and subsequent renal failure.

5. LIVER

The liver also may be damaged by the asphyxic insult with necrosis, clotting factor deficiency and deficiency of enzymatic processes.

6. BLOOD

- polycythemia
- anemia
- disseminated intravascular coagulation (DIC)

7. GASTROINTESTINAL EFFECTS

The asphyxiated infants are at the risk for intestinal ischemia and necrotizing enterocolitis (NEC).

8. THERMOREGULATION

Hypoxia inhibits thermoregulation, causing a very difficult transition to the extrauterine life and inducing hypothermia.

I.3.2. Main impact of neonatal hypoxia on the brain

The most severe outcome of asphyxia is hypoxic-ischemic encephalopathy (HIE), a syndrome characterized mainly by abnormal muscle tone and reflexes, an altered level of consciousness and commonly by convulsions (287).

The American Academy of Pediatrics (AAP) and American College of Obstetrics and Gynecology (ACOG) recommend using HIE because this term accurately describes the clinical condition, encephalopathy from asphyxia, without implying the time of brain injury. The AAP and ACOG also advise not using the terms perinatal asphyxia or birth asphyxia because it is difficult to identify the time of brain injury and nearly impossible to ascertain that the brain had been "normal" before such injury (288).

Published paper:

- Mogos M, Serban A, Stan CI, Tarniceriu CC, **Paduraru L.** The impact of hypoxia over fetal and neonate brain. *Revista Română de Anatomie funcţională şi Clinică, Macro şi Microscopică şi de Antropologie*, 2015, Vol XIV, nr 4, 660-665

HIE remains a common cause of death in newborns and those who survive are exposed to develop serious neurological disorders such as cerebral palsy (289). As the clinical assessment of the neurological state of newborns in the early postnatal period is the gold standard in the diagnosis, the widely used staging system, known as Sarnat and Sarnat scoring system following a revision is considered to be an important method in the initial assessment of newborns with asphyxia and in the prediction of the prognosis (290).

According to this staging system, HIE may have three stages based on the level of certain criteria: consciousness, muscle tone, deep tendon reflexes, presence of seizures, appearance of the pupils, duration of symptoms, posture and conventional EEG (Table 33).

Another scoring system (Thompson score) was developed as a clinical tool comprising of a set of clinical signs associated with CNS dysfunction, used to assess status and prognosis of a child with HIE following birth asphyxia (Table 34). In the scoring system, a score of 0 is normal and the maximum is 22, which signifies the worst possible status of HIE (291).

Table 33 Clinical stages of asphyxia by Sarnat and Sarnat

Stage I	Stage II	Stage III
Hyperalert	Lethargic	Stuporous, comatose
Normal muscle tone	Mild hypotonia	Flaccid, intermittent decerebration
Weak suck Weak Moro	Weak/absent suck Weak Moro	Absent suck No Moro
Mydriasis	Miosis	Poor pupillary light response
No seizures	Focal/ multifocal seizures	Seizures are uncommon

Severe HIE has a very bad prognosis, with a survival rate 14 times smaller than infant with asphyxia at birth which does not develops HIE. Thus, efforts must be made in order to early detect the occurrence of brain damage, as clinical signs may take up to 72 hours to install completely and active treatment should be applied to preserve and protect the neurons as much as possible.

Table 34. Thompson score

	0	1	2	3
Tone	Normal	Hypertonia	Hypotonia	Flaccid
Level of consciousness	Normal	Hyperalert	Stare, lethargic	Comatose
Fits	None	< 3 per day	> 2 per day	
Posture	Normal	Fisting, cycling	Strong distal flexion	Decerebration
Moro	Normal	Partial	Absent	
Grasp	Normal	Poor	Absent	
Suck	Normal	Poor	Absent ± bites	
Respirations	Normal	Hyperventilation	Brief apnea	Apnea on MV
Fontanel	Normal	Full, not tense	Tense	

Nowadays, researches work on numerous biochemical markers to use them in the diagnoses of the perinatal asphyxia and HIE in order to initiate treatment as soon as possible (292–296). A retrospective study done between 2007-2012 in the Department of Obstetrics & Gynecology of The Institute for Mother and Child Care „Prof. Dr. Alfred Russescu" in Bucharest, aimed to evaluate the frequency of hypoxia at birth, followed by perinatal hypoxic ischemic encephalopathy (HIE), to identify most frequent causes of asphyxia at birth and the severity degree of HIE. The secondary objective was to establish the value of predictive biochemical tests usually used as markers of HIE for selected cases.

Eligibility criteria for the study included the association of at least two of the following: markers of fetal stress, markers of neonatal asphyxia, HIE criteria. Exclusion criteria were infants with HIE associated with other conditions, major congenital anomalies, congenital infections, sepsis, obstetrical instrumental delivery trauma (cranial birth trauma), extremely severe condition (brain damage may have occurred antepartum), congenital metabolic disease. Thus, from the total of 21933 newborns born in the specific time period, only 261 (1.12%) were included. For the current secondary analysis were selected 89 term infants (41 girls and 48 boys) presenting complete clinic and paraclinical data. HIE was classified after international criteria (Sarnat and Sarnat), recognized worldwide as mild, moderate and severe. Infants with HIE were found by recording the hospital health databases.

Data related to maternal and obstetrical history, parturition, pregnancy, fetal heart rate, type of delivery, gestational age, neonatal clinical status, birth weight, Apgar score at 1 and 5 minutes, need and time of resuscitation, neonatal outcome were included. Paraclinical data were also evaluated: blood tests including complete blood count, biochemical values, blood gas analyses, ventilation variables. Outcome data were determined from the records of neonatal follow-up. The statistic evaluation was performed with EXCEL and SPSS version

16.0. Following analysis of collected data, HIE was diagnosed on 89 infants from 21933 newborns admitted in the Maternity Unit during 2007-2012 (0.4%). From those, 74.15% presented moderate and severe signs of disease (stage 2 and 3 Sarnat) and 25.84% had mild HIE (stage 1 Sarnat) with very good outcome. Infants with moderate/severe HIE had a higher mortality frequency, registering 31 deaths (34.8%).

Evaluation of acid-base balance and blood gas analyses as prognostic markers for the evolution of newborn with HIE, showed that the most utilized parameter, the pH, does not estimate accurately the exposure of the infant to hypoxia. pH is a logarithmic value and does not reflect the linear progression of acidosis. Also, the value of lactate was not found to be a reliable predictive marker. Concerning the enzymatic panel, there was a certain correlation between LDH, ASAT, ALAT and the evolution of HIE, high elevated values in first 12 hours after birth being an indicator of this severe condition.

Fetal heart rate (FHR) was correlated with Apgar score, low pH, low oxygen saturation (SaO_2), time until first spontaneous breath. Regarding the pattern of the CTG during labor, the subjects were divided in the normal pattern group and modified pattern group. From 89 infants, 72 (~80%) had abnormal pattern, most frequently with late deceleration, with significant difference to the normal pattern group. Bradycardia was nearby the limit of statistical significance and will no further be regarded.

The median of the Apgar score in the first minute for infants who required resuscitation was 3 (IQR [1 – 7], CI = 95%) and after five minutes elevated to 7 (IQR [4 – 8], CI = 95%). There were 65,9% infants with Apgar score less than 4 in the first minute and after five minutes decreased at 31.8%. Apgar score less than 7 correlated well with abnormal FHR (severe/variable deceleration or transitory tachycardia). For this, FHR and Apgar score can be co-used as prognostic factors for the selection of infants which will necessitate interventional therapy.

The study also identified as antenatal risk factors: extreme maternal age, socio-economic status, obstetrical causes. The intrapartum risk factors were: meconial amniotic fluid, abnormal FHR, induced labor, uterine rupture. Abnormal CTG pattern is associated with unfavorable neonatal outcome, and it is correlated with Apgar scores less than 7. LDH gives important information for the diagnostic and evolution of HIE. Research in the field of biomarkers seems to show promise for early diagnose and appropriate treatment.

I.3.3. Gut consequences of neonatal hypoxia

Necrotizing enterocolitis (NEC) is an acquired, multifactorial and potentially severe gastrointestinal disease characterized by ischemia, necrosis and inflammation of the bowel wall. Being one of the most common neonatal gastrointestinal emergencies requiring surgery, this disease presents high mortality rate (10–30%), particularly among preterm infants (297,298). Some studies report that the incidence of NEC remained stable in recent years (~10%) (299,300), but due to the enhanced survival of extremely low birth weight (ELBW) newborns, the incidence seems to be increased (11-15%) (301–303).

Published paper:

- Hincu MA, Zonda GI, Diaconescu S, **Paduraru L**. Dilemmas Around Necrotizing Enterocolitis in Late Preterm Neonates. *Med. Surg. J. – Rev. Med. Chir. Soc. Med. Nat., Iaşi* 2020; 124(4):630-637

The risk of developing NEC depends on the gestational age (GA), the lower the GA, the higher the risk (304). For the survivors affected by NEC, there is high associated morbidity with neurodevelopmental sequels and retinopathy of prematurity (298). Being one

of the main causes of mortality and morbidity in neonatal intensive care unit (NICU), a better understanding of the pathogenesis of NEC is critical for an early diagnosis.

Very low birth weight (VLBW) preterm infants represent the vast majority of patients affected by NEC, however about 10% of the cases are diagnosed in late preterm and term infants with risk factors for mesenteric ischemia like intrauterine growth restriction (IUGR), polycythemia, birth asphyxia, chorioamnionitis, sepsis, or congenital gastro-intestinal or cardiac defects (305–308). In table 35 the risk factors for different etiopathogenetic mechanisms of NEC are summarized, as well as the most common laboratory and radiological findings related to this complication.

Table 35. Risk factors for different etiopathogenetic mechanisms of NEC and common laboratory and radiological findings

	ISCHEMIC NEC	INFECTIOUS NEC
Medical history	Full-term/late preterm infant with IUGR Caesarean section 5 min Apgar score < 7 Severe metabolic acidosis Hypoglycemia Severe anemia/ polycythemia 36ºC at 1 h of life Mother with preeclampsia or diabetes Placental abruption Gastroschisis/omphalocele Congenital heart disease (patent ductus arteriosus, hypoplastic left heart syndrome etc.) (309) Umbilical arterial catheterization (310)	Preterm infants (< 32 weeks GA) VLBW infants (< 1500 g) Chorioamnionitis Premature rupture of membranes
Drugs	Indomethacin ± dexamethasone Ibuprofen Red blood cell transfusions (311)	H2 blocker (ranitidine/famotidine/nizatidine) (312,313) Empirical antimicrobials
Time of onset	Short term after birth (5.3 days) (307)	Later onset (15.3 days) (307)
Laboratory studies	• Blood culture negative • Negative gastric aspirate • Normal WBC • Low serum bicarbonate (due to poor tissue perfusion – isolated, without metabolic acidosis) • Inflammatory syndrome is absent - normal serial CRP values would favor aborted antibiotic therapy and early resumption of feedings (314).	• Blood culture may be positive in sepsis • Gastric aspirate may be positive (a negative gastric aspirate does not exclude a bacterial infection) • Inflammatory syndrome present - persistently elevated CRP after initiation of appropriate medical management suggests associated complications (314). Moderate to profound neutropenia – sepsis • Low hematocrit or hemoglobin may suggest infection or are low due to blood loses caused by consumptive coagulopathy • Low serum bicarbonate (associated with severe metabolic acidosis in sepsis) • Thrombocytopenia - reaction to gram-negative organisms and endotoxins or as part of consumptive coagulopathy (in this case associated with high levels of PDF) • Fecal volatile organic compounds (VOCs) (315)
Radiography	• Abnormal gas pattern • Dilated bowl loops • Thickened bowel walls	Radiological signs from ischemic NEC associated with: • Pneumatosis intestinalis • Portal venous gas • Abnormal free air • Intraperitoneal free fluid

A clinical example of challenging diagnostic and treatment is presented in the case report bellow:

Prenatal and birth history

We present a preterm female infant, GA 34 weeks, birth weight 1800g, first twin of a dichorionic diamniotic pregnancy, born vaginally in a level II maternity to a 20 years old mother with inadequate prenatal care. The mother had an urinary tract infection with *Escherichia coli* in the last month of pregnancy treated with antibiotic (Cefixime). APGAR scores were of 8/9/9 at 1, 5 and 10 minutes, requiring early CPAP in the delivery room for 15 minutes.

The second twin was also female, with a BW = 2150 g, and Apgar scores of 9 at 1 minute and 10 at 5 minutes. Twin 2 had an uncomplicated course in the neonatal intensive care unit.

Hospitalization course prior to transport

The infant was admitted in the level II NICU at 20 minutes of life and the initial physical exam revealed intercostal and subcostal retractions, oxygen saturation (SpO_2) of 88%, a heart rate (HR) of 135 beats per minute (bpm), respiratory rate of 38 beats/min and blood pressure (BP) 63/24/35 mmHg. The neonate required free-flow oxygen in order to maintain SpO_2 within normal range, parenteral nutrition and prophylactic antibiotic (Ampicillin). Enteral feeding was initiated with formula on day of life (DOL) 2.

Initial laboratory studies revealed hemoglobin (Hb), hematocrit (Ht) white blood cell count (WBC) within normal range, negative C-reactive protein (CRP) and initial peripheral cultures (skin, external auditory canal, gastric liquid and nasopharyngeal swabs).

On the fifth day of life the patient became unstable and clinical examination revealed recurrent desaturations (lowest value of SpO_2 = 50%) and coffee ground followed up by bilious emesis. Blood gas analysis showed mild respiratory acidosis (pH 7.26 and PcO_2 52.7 mmHg). Chest and abdomen X – Ray was normal. Blood and stool were sampled for bacterial cultures (and were negative after 72 hours). Feeds were stopped and ampicillin was replaced by intravenous gentamicin and colistin. At this point then transfer to level III center was requested. During transfer the neonate was stable with free flow oxygen.

Neonatal course at level III facility

The clinical examination at admission in the level III NICU revealed pale–greenish skin, hypotonia, shallow breathing, systolic murmur grade IV/VI, tachycardia (175–181 bpm) and bilious drainage with streaks of old blood. Immediately after admission, she presented severe recurring apneas requiring positive pressure ventilation (PPV) followed by intubation and mechanical ventilation (SIMV). Blood gas analysis showed respiratory acidosis with normal oxygenation (pH = 7.26, pCO_2 = 52.8 mmHg, PO_2 = 58 mmHg). Laboratory workup on admission revealed anemia, leucopenia (I/T = 0.47), elevated CRP and PCT (Table 36). The X–Ray on admission revealed distended bowels with wall edema. Peripheral and blood cultures, as well as cerebrospinal fluid were collected.

Table 36. Laboratory workup on admission and during hospitalization in the level III NICU up to DOL 17

DOL	Hb (g/dl)	Ht (%)	WBC x10^3/mm^3	PLT x10^3/mm^3	CRP	PCT	Direct bilirubin	Urea
6	10.6	33.2	2.8	187	44.1	20.7	0	69.8
8	13	41.1	6.7	104	50.6	6.86	0.2	110.7
9	10.1*	31*						
10	12	37.9	9.8	120	125.2	1.06	0.4	66.3
14	10.6	33.1	17.9	249	87.5	1.11	1.4	79.5
17	14.3	43.7	16.3	174	37.6	0.79	0.9	71

*values from the arterial blood gas assay

A nasogastric tube was placed for decompression of the dilated bowels and total parenteral nutrition (TPN) and intravenous antibiotics with broad-spectrum coverage (colistin, meropenem and gentamicin) were started. The cranial ultrasound revealed supernumerary ventricular cavity. Echocardiography showed Tetralogy of Fallot (TOF) with mild pulmonary stenosis, patent *foramen ovalae* and *ductus arteriosus*. Blood cultures and lumbar puncture were negative, and the peripheral cultures were positive for *Escherichia coli*. The newborn required mechanical ventilation for 21 days, three packed RBC's transfusions (15 ml/kg) for anemia and completed a 15 days course of antibiotic therapy for presumptive sepsis despite negative blood culture. Following treatment clinical status improved slowly and the laboratory tests normalized by DOL 17. Enteral feeds were restarted on DOL 15 with full feeding established on DOL 29.

Onward the clinical course of the infant was uneventful, however on DOL 55 the neonate presented marked abdominal distension and the X-ray revealed dilated intestinal loops. Following surgical consult, the newborn was transferred to the pediatric surgery department for further evaluation.

Discussion

The etiology of NEC is not entirely understood as multiple factors appear to be involved, including hypoxia, acidosis and hypotension, leading to ischemic damage of the mucosal barrier of the small intestine. Gram negative bacterial invasion of the mucosa may be another factor involved in the pathogenesis of NEC. Repeated radiographs are recommended, pneumatosis intestinalis and portal venous gas being pathognomonic signs, caused by anaerobic bacteria, particularly by Clostridia.

Newborns with very low birth weight (<1,500 g) and very preterm infants (<32 weeks) are the most affected by this pathology (299,316), as they have decreased ability to suppress the exaggerated inflammatory response or are unable to develop an inflammatory response to pathogenic bacteria (317). This case report presents a late preterm infant with a low birth weight and congenital heart disease. Hence, intestine mucosal injury and ischemia might have played a part in the development of NEC.

The signs and symptoms of necrotizing enterocolitis are highly variable, nonspecific and subtle (318). Physical examination findings may include abdominal distention, abdominal tenderness, visible intestinal loops, decreased bowel sounds, palpable abdominal mass and erythema of the abdominal wall. Systemic findings may include respiratory failure, circulatory collapse, and decreased peripheral perfusion. The clinical course of this patient was marked by multiple days of parenteral nutrition and mechanical ventilation.

The role of inflammation in the pathogenesis of NEC is increasingly recognized. Antenatal inflammatory processes like chorioamnionitis may increase the susceptibility of preterm infants to develop NEC. Decreased intestinal mucosal thickness and increased mucosal permeability, as a result of inflammatory mediators, may explain this association (317). This twin derived from a mother with urinary tract infection in the third trimester, which seems to have complicated the course of the disease.

A recent meta-analysis investigating the outcomes of preterm infants affected by IUGR compared with those without growth restriction found that the incidence of NEC was at least 2.5 times higher in preterm infants with growth restriction (318). It is suggested that in such fetuses, blood flow to the brain is preserved at the expense of blood flow to other organs, including the gastrointestinal tract. Although the evidence suggests that newborns with IUGR and abnormal antenatal Doppler are at increased risk of developing NEC, early initiation of breast milk feeds does not appear to increase the incidence of NEC in this population (319).

Generally, this condition occurs after the first week post-partum when the intestine has been colonized (320). In this case, rapid initiation of enteral feeding with formula was a risk factor for NEC, due to changes in enteric blood flow and oxygen requirements during feeding.

An exclusive human milk diet might be protective due to growth factors, antibodies, cellular immune factors, probiotic bacteria, oligosaccharides, and stem cells. Fresh maternal breast milk is superior to frozen or donor breast milk (321). A prospective multicenter study concluded that the incidence of NEC was 6–10 times higher in exclusively formula-fed infants when compared with exclusively breast milk-fed newborns, and three times higher when compared with infants who received breast milk and formula (322). Early initiation of enteral feeds in preterm infants with very small non-nutritive volumes (known as trophic feeding or minimal enteral nutrition) is thought to have several advantages over complete fasting as a result of "priming" the gastrointestinal tract and aiding in its maturation processes (323–325). Initially, one of the differential diagnosis considered was feeding intolerance caused by lactose intolerance. Neonatal lactose intolerance syndrome is based on a series of digestive system symptoms caused by the lack of lactase, leading to the inability of digesting the lactose in breast milk or cow milk. Symptoms of lactose intolerance include loose stools, abdominal bloating and pain, flatulence and vomiting (326). In this particular case, the neonate presented gastric residuals, abdominal tenderness and recurrent apnea/bradycardia, which together with the systemic signs and abnormal blood work raised the suspicion of sepsis.

The reported frequency of NEC in infants with critical congenital heart disease (CCHD) ranges from 3% to 9% (327–330) with no relationship to feeding practices. However, in many centers infants with CCHD are managed with TPN and enteral feedings are deferred in the preoperative period (331,332) as hypoxia, poor mesenteric perfusion, acidosis and hemodynamic instability are high risk factors for NEC. In our case the diagnosis of congenital heart defect was made on DOL 7, after the onset of symptoms and the deterioration of the infants' clinical status. TOF with mild pulmonary stenosis is unlikely to be a risk factor for NEC, but in this particular case it might have played a part in the intrauterine growth restriction, considering the fact that the other twin without cardiac pathology had an adequate birth weight and an uneventful clinical course. The growth restricted twin was at risk for both NEC and sepsis, making the differential diagnosis extremely challenging, as clinical signs are frequently non-specific and overlapped and laboratory test results may show similar abnormalities. However, the medical treatment in both cases follows the same general principles. The suggested antibiotic regimen includes ampicillin, gentamicin, and either clindamycin or metronidazole. While the patient is NPO, total parenteral nutrition must be provided. Antimicrobial therapy should always be adapted to the pathogen causing the disease. It is recommended to be justified by clinical, paraclinical and imagistic arguments in order to prevent bacterial antibiotic resistance.

If this conservative therapy is effective, infants may resume enteral feedings once signs of infection or abdominal pathology have disappeared. In some cases, this may take several days to a week. The presence of normal bowel movements suggests the return of bowel function. In infants with confirmed NEC who have worsening condition or bowel perforation or who do not respond to medical therapy, surgical intervention is indicated. Laparotomy is the standard approach, surgery being as conservative as possible, with removal only of portions of unquestionably necrotic or perforated intestine in an attempt to preserve as much intestine as possible (333).

For the neonatologist, the decision to refer the case to the pediatric surgeon and the timing of the referral are real challenges when faced with a neonatal patient with clinical deterioration despite maximal medical therapy, in the absence of free air in the abdominal cavity, as in the present case.

The peculiarity of this case consists in the association of multiple risk factors, as well as several etiological contexts – ischemic and infectious – which make inclusion into classical etiopathogenetic mechanistic patterns extremely difficult, if not impossible.

I.3.4. Potential treatment options in neonatal asphyxia

The treatment tools in neonatal asphyxia are few, and none can restore the neuronal destruction caused by the main aggressive events: hypoxia and ischemia. The following interventions showed therapeutic benefits:

- appropriate resuscitation at birth
- respiratory therapy, including different modes of mechanical ventilation including high frequency ventilation or nitric oxide ventilation in case of severe pulmonary persistent hypertension that occurs as a severe complication
- hemodynamic support therapy
- correction of metabolic and acid-base imbalances
- protection with large-spectrum-antibiotics (ampicillin +/- gentamycin)
- parenteral nutrition in order to prevent NEC, with an important challenge for assuring an appropriate perfusion for the gut, but to proceed to a restrictive fluid regimen to protect against cerebral edema. Renal perfusion should be assured but not overloaded, as severe hypoxia may affect kidney function
- prevention of HIE complications or reduction of their severity by cerebral/systemic hypothermia is currently the standard for treatment (Fig. 23). It mainly consists in reducing the core temperature to 34°C in order to reduce basal metabolism and neuronal death and was proved to have beneficial effects, both on the short-term and on the long-term outcome (334).
- specific treatment of seizures

The first line of treatment for neonatal seizures is phenobarbital. However, in some cases the response is inadequate, and the infant requires association of multiple anticonvulsant drugs, with long term severe potential adverse effects (ex. respiratory depression due to benzodiazepines, especially diazepam, which in neonates has a therapeutic dose very close to the toxic dose).

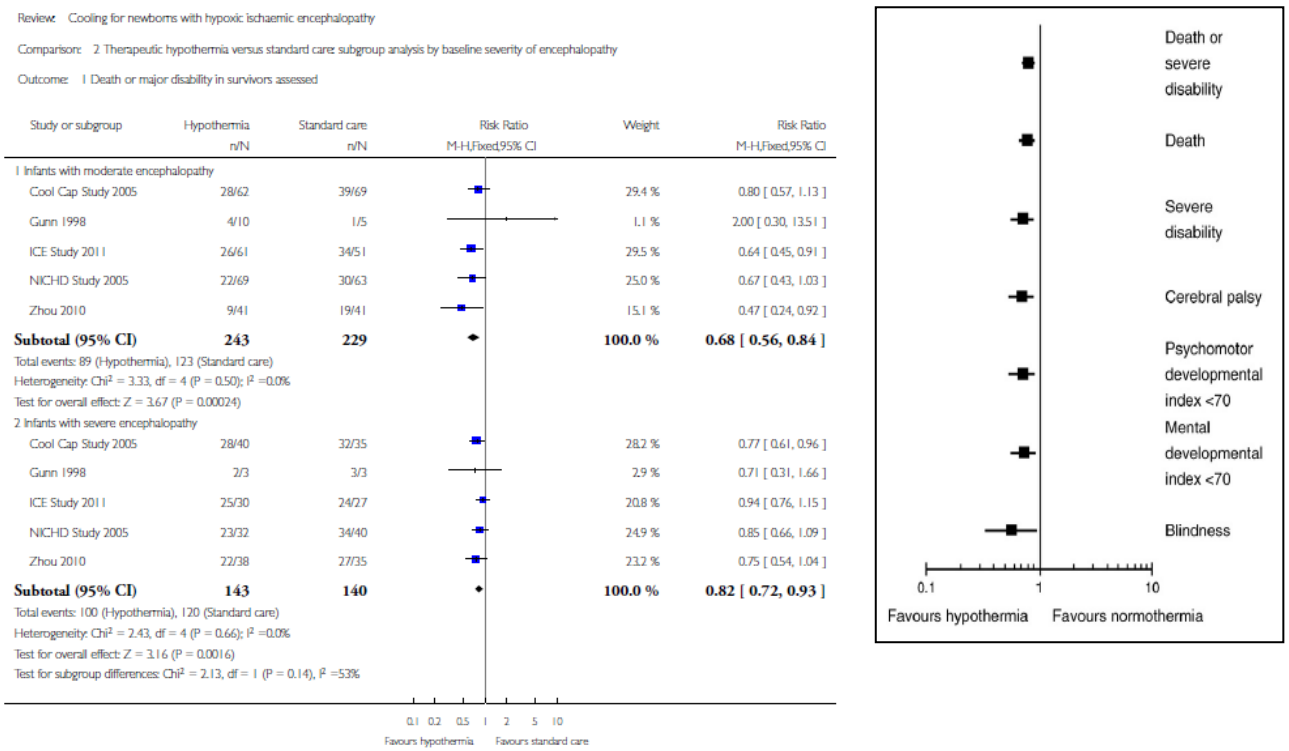

Fig. 23. Benefits of early selective controlled hypothermia in asphyxiated neonates (334)

Thus, researchers focused on alternative drugs or combination that would have a potential preventive role against permanent neuronal damage.

Therapeutic cooling of the whole body or the head reduces the long-term adverse effects of hypoxic ischemic encephalopathy, but many infants still die or suffer neurologic impairment despite cooling (334–337). Therefore, newer and more effective neuroprotective therapies are badly needed. Phenobarbital and erythropoietin are two additional therapies that might be used in addition to or instead of therapeutic hypothermia. Phenobarbital acts through suppressing the oxidative cerebral metabolism and diminishing the neuronal response to glutamate.

High-dose intravenous Phenobarbital, administered 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 (338). Erythropoietin has been shown to have potential for ameliorating the neurological sequels of hypoxic-ischemic encephalopathy (339,340).

Published paper:

- Avasiloaiei A, Dimitriu C, Moscalu M, **Paduraru L**, Stamatin M. High-dose phenobarbital or erythropoietin for the treatment of perinatal asphyxia in term newborns. *Pediatr Int*. 2013;55(5):589-93

Objective

The purpose of this study was to examine the potential beneficial effects of phenobarbital and erythropoietin for infants with perinatal asphyxia who had signs of hypoxic-ischemic encephalopathy.

Material and methods

We conducted a prospective randomized study of 67 term neonates with perinatal asphyxia, admitted from January 1st, 2010 to September 30th, 2011 in the "Cuza-Vodă" Clinical Hospital of Obstetrics and Gynecology Neonatal Intensive Care Unit (NICU) in Iasi, Romania. Perinatal asphyxia was diagnosed using the criteria of the American Academy of Pediatrics and the American College of Obstetricians and Gynecologists (280). The study was approved by the hospital's Ethical Committee, and informed consent was obtained from the infants' mothers. Preterm infants (with gestational age less than 37 weeks), infants with major congenital malformations, and infants with hemolytic disease due to Rh incompatibility were excluded from study.

The following data were collected and analyzed: birth weight, gestational age, Apgar scores at 1, 5 and 10 minutes, cord blood pH, RBC activity of antioxidant enzymes (superoxide dismutase – SOD, glutathione peroxidase – GPx), total antioxidant status in serum (TAS) and malon-dialdehide (MDA).

TAS was analyzed using the ABTS® technique and RANDOX reactants. SOD was measured by the degree of formazan inhibition using RANSOD kits with control serum. GPx was measured using RANSEL reactants through the Paglia and Valentine method. Malon-dialdehide was analyzed using the thiobarbituric acid reaction.

The samples were collected at 4, 24, 48, and 72 hours and at 7 days of life, and the values were determined using a Beckmann spectrophotometer. A neurologic clinical examination was performed as soon as possible after birth and periodically thereafter, noting the presence or absence of neurologic abnormalities and their duration. In addition, the presence or absence of seizures throughout the admission was documented. Treatment was started as soon as the diagnosis was confirmed.

All subjects were included in the "Cuza-Vodă" follow-up program, examined at discharge using the Amiel-Tison assessment and at 3, 6, 9, 12, 18 months, using Bayley Infant Scales of Development, edition II.

The infants were randomly assigned to supportive treatment (oxygen, volume expanders, inotropes, diuretics, antibiotics), a single dose of intravenous phenobarbital, 40 mg/kg, during the first 4 hours after birth plus supportive treatment, or subcutaneous erythropoietin, 1000 UI/kg/day, for the first three days plus supportive treatment. Infants were allocated to treatment groups by unblinded, random-draw, numerical assignment. No infant was treated with whole-body or head cooling, as these therapies were not available in our center during the period of study.

The data was analyzed using SPSS V.19.0. (SPSS, Chicago, IL). The descriptive statistics was used to express characteristics and tendencies of studied parameters. The independent variables – treatment-derived differences among parameters – were analyzed using the ANOVA test, used 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 at P values of less than 0.05.

Results

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 (Fig. 24). This rate is similar to the incidence reported worldwide (0.1-0.8%) (341,342). These infants comprised 3.8% of all infants admitted to the "Cuza-Vodă" NICU. All 67 infants were enrolled in the study: 23 were randomized to the control group (supportive care), 22 to phenobarbital, and 22 to erythropoietin.

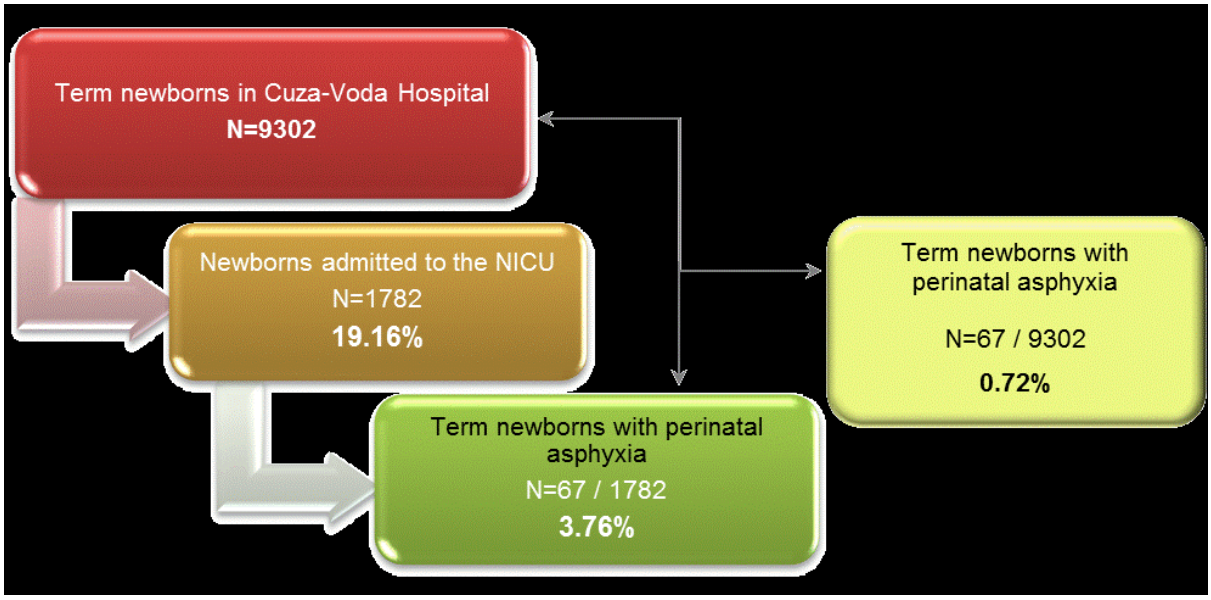

Fig. 24. Flowchart for study group

The newborns in our study group had a mean gestational age of 40.4 weeks and a mean birth weight of 3278 grams (Table 37). Apgar scores at 1 minute were between 3.04 and 4.12 and had a mean of 3.58. As resuscitation is continued, Apgar scores at 5 minutes rise slightly to a mean of 5.33, and at 10 minutes – 6.61. Low values of cord blood pH confirm the diagnosis of perinatal asphyxia (7.00-7.09).

Antioxidant enzymes (SOD and GPx) values were lower for infants treated with phenobarbital or erythropoietin compared to control infants (Fig. 25). When compared to reference values for healthy term newborns (SOD=216-310U/L, GPx=4171-9881 U/L), the

high values in our control infants suggests the existence of oxidative stress following perinatal asphyxia.

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Table 37. Statistic indicators of parameters evaluated at birth

	Mean	Mean -95%	Mean +95%	SD	SEM	Min	Max	Q25	Median	Q75
Gestational age (weeks)	40.4	39.6	41.1	1.9	0.4	36	43	40	41	42
Birth weight (g)	3278	3121	3435	643	79	1360	5000	2900	3240	3650
Apgar 1 min	3.58	3.04	4.12	2.22	0.27	0	7	1	3	6
Apgar 5 min	5.33	4.85	5.80	1.95	0.24	1	9	4	6	7
Apgar 10 min	6.61	6.18	7.04	1.76	0.21	2	9	5	7	8
Cord blood pH	7.04	7.00	7.09	0.15	0.02	6.53	7.39	6.96	7.04	7.14

The values of TAS are higher, although insignificantly so ($p < 0.5$), phenobarbital or erythropoietin groups compared to control group (Fig. 25). This represents the effort of antioxidant systems to counterbalance free radical injury, generated by hypoxia-ischemia. The mean reference values for TAS in healthy term newborns are 1.20-1.30 mmol/l.

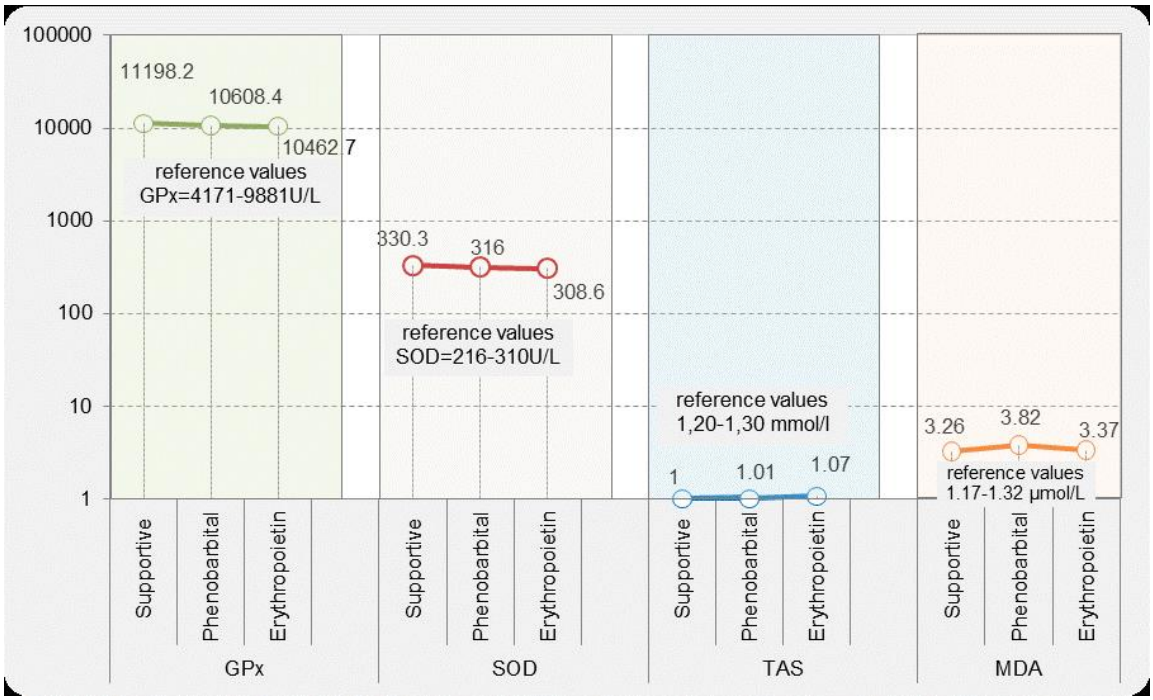

Fig. 25. Antioxidant enzymes values in phenobarbital and erythropoietin groups compared to control group

Lipid peroxidation measured by malondialdehyde in plasma 7 days after the initial hypoxic insult was high in all our patient groups compared to normal ranges for healthy term newborns (1.17-1.32 µmol/L) (Table 38).

There was a descending trend in abnormal findings on neurologic examination. At birth all infants had clinical neurologic abnormalities, and this aspect persisted at 6 and 12 hours, after 72 hours neurologic disorders can be found in only 53.7% of the newborns (Table

39). This finding suggests the recovery of cerebral metabolism following the early transient failure of cerebral blood flow. There was a higher incidence of seizures during the first 12 hours after perinatal asphyxia (Table 39).

Table 38. Statistical indicators of biochemical determinations

Treatment		Mean	Mean		SD	SEM	Min	Max	Q25	Median	Q75
			-95%	*+95%*							
GPx*	Control	11198.2	10375.3	12021.1	2432.1	405.4	4607.7	15871.0	10191.9	11609.5	12900.8
	PHE	10608.4	9335.4	11881.3	2204.7	589.2	4585.7	13268.0	9772.8	10778.0	12153.0
	EPO	10462.7	9129.8	11795.6	2406.9	621.5	4984.3	13473.3	9829.3	11382.5	12050.8
		10901.4	10314.9	11488.0	2367.0	293.6	4585.7	15871.0	10156.5	11382.5	12413.0
SOD†	Control	330.3	317.5	343.2	37.9	6.3	250.3	420.5	305.0	330.3	357.2
	PHE	316.0	296.5	335.4	33.6	9.0	217.7	360.0	307.5	319.2	335.3
	EPO	308.6	287.3	330.0	38.5	10.0	224.0	380.3	281.5	300.7	338.5
		322.2	312.9	331.6	37.8	4.7	217.7	420.5	300.7	323.0	349.0
TAS‡	Control	1.00	0.95	1.05	0.14	0.02	0.77	1.40	0.90	0.96	1.06
	PHE	1.01	0.91	1.10	0.15	0.04	0.79	1.27	0.93	0.95	1.11
	EPO	1.07	0.98	1.16	0.17	0.04	0.83	1.33	0.90	1.03	1.23
		1.02	0.98	1.05	0.15	0.02	0.77	1.40	0.90	1.00	1.16
MDA§	Control	3.26	2.97	3.55	0.82	0.14	2.10	5.40	2.70	3.00	3.80
	PHE	3.82	3.26	4.39	0.97	0.26	2.60	5.60	2.90	3.67	4.60
	EPO	3.37	2.84	3.90	0.88	0.24	2.30	5.30	2.80	3.10	3.80
		3.41	3.19	3.64	0.88	0.11	2.10	5.60	2.75	3.10	3.90

* - Glutathione peroxidase
† - Superoxide dismutase
‡ - Total serum antioxidant status
§ - Malon-dialdehide
PHE - Phenobarbital
EPO - Erythropoietin

Four deaths occurred in the control group (17%); 1 (5%) in the phenobarbital group; and 1 (5%) in the erythropoietin group.

Table 39. *Incidence of neurologic abnormalities and seizures*

Age at examination	Number of infants with abnormal examination	%	Number of infants with seizures	%
At birth	67	100%	1	1.5
6 h	67	100%	10	14.9
12 h	67	100%	11	16.4
24 h	52	78%	6	9.0
48 h	52	78%	6	9.0
72 h	43	64%	4	6.0
>72 h	36	54%	1	1.0

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 the receptive language (RL), expressive language (EL), and cognitive development (CD) (Fig. 26).

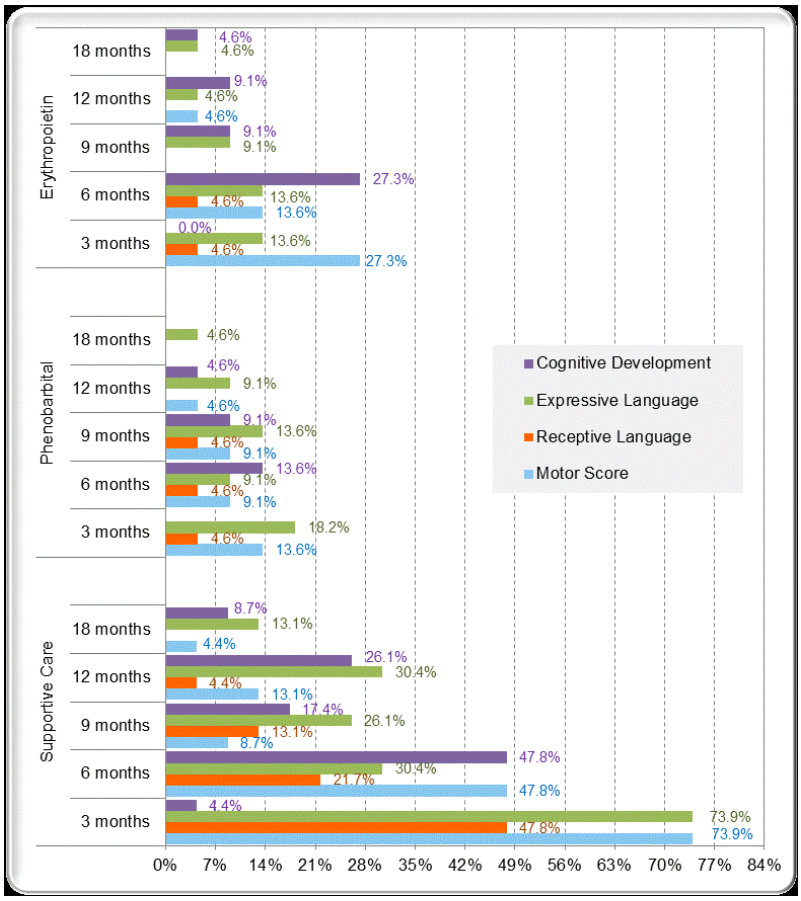

Fig. 26. Long term neurologic outcome

Discussions

The decrease of the 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 (343,344). Phenobarbital decreases cellular metabolic rates and oxidative stress, favoring the decrease of antioxidant enzyme activity (338) TAS values are higher in the Erythropoietin Group than in the other groups, but TAS may be greatly influenced by other medications administered during the first 72 hours after perinatal asphyxia.

Lipid peroxidation is a slow process, and it is a good reflection of oxidative stress generated by both perinatal asphyxia and oxygen therapy. As 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 (345), as 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.

MDA is lower in the Erythropoietin Group compared to the other groups, although the difference is statistically insignificant ($p>0.05$). This could be explained by the various models of neonatal hypoxia which showed that in doses ranging from 1000 UI/kg to 30,000 UI/kg, erythropoietin has anti-apoptotic and anti-inflammatory effects in the acute post-injury period, with neurogenic and vasculogenic effects in the recovery period (346–350).

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. The mortality rate was lower in the Phenobarbital and Erythropoietin Groups (both 4.6%) than in the

Control Group (17.4%) ($\chi 2$=7.26, p=0.0087, 95%CI). This finding may be explained by the facilitation of antioxidant defense mechanisms through the administration of phenobarbital or erythropoietin.

Long-term neurologic follow-up showed a high incidence of sequelae in the Control Group compared to the Phenobarbital and Erythropoietin Groups. In the Control Group, delay in the achievement of motor and expressive skills prevailed (each 73.9%) at 3 months but became less frequent over time, as described by Ment and others (351). At 18 months, delayed cognitive development (8.7%) and expressive language (13.0%) were more frequent. Receptive language delays became less frequent when the same infants were tested repeatedly over time, and by 18 months, receptive language was normal in all subjects.

In the Phenobarbital Group, expressive language delays were found in only 18.2% of infants at 3 months, a significantly lower rate than in the Control Group. Receptive language delays were constant until 9 months of age (9.1%) but absent by 12 months.

In the Erythropoietin Group, motor development was significantly affected at 3 months (27%), although at the final evaluation only cognitive and expressive language disorders existed (each in 4.6% of infants). Receptive language disorders were very rare and were absent by 9 months of age.

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 with expressive language. However, at 18 months the differences between these two groups were insignificant. Our results regarding the efficacy of high-dose phenobarbital during the immediate recovery period were consistent with the work of Gathwala et al, which showed decreased oxidative stress after phenobarbital administration (338). Our study demonstrates that high-dose phenobarbital improves long-time neurologic outcome, as shown by Hall and coworkers (352).

Conclusions

To our knowledge, this is the first study to compare the efficacy of high-dose phenobarbital and erythropoietin for the neuroprotection of infants with post-asphyxic encephalopathy. 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 its low cost and simplicity. Unlike therapeutic cooling, phenobarbital can easily be given in any NICU, even in low or modest-resource countries. Our study was limited by its small size and the lack of blinding the caregivers and examiners. Larger studies are needed to confirm or refute our findings.

I.3.5. An eye open to the role of neonatal hypoxia-ischemia in brain development, cognitive functions, and neurodegeneration

Alzheimer's disease (AD) is a progressive and irreversible neurodegenerative condition associated with gradual memory loss, different degrees of cognitive deficits and intellectual disabilities that interfere with quality of life (353,354). Currently, worldwide, over 50 million people have been affected by dementia, AD accounted for 60-80% of these cases. Over the next three decades, this number is estimated to more than triple to 150 million (355).

In 2018, globally, the total cost of care for patients with AD was estimated at $ 1 trillion, which corresponds to a gross domestic product of 1.2%, and this figure will rise to $ 2 trillion annually by 2030; costs that can weaken economic and social development and could overwhelm social and health services (353,356). Furthermore, this chronic condition is no longer a problem only for the developed countries. More than two-thirds of all AD patients live in Brazil, India, the United States of America and China, and this percentage will increase by 2050 (353).

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- Stanciu GD, Ababei DC, Bild V, Bild W, **Paduraru L***, Gutu MM, Tamba BI. Renal Contributions in the Pathophysiology and Neuropathological Substrates Shared by Chronic Kidney Disease and Alzheimer's Disease. *Brain Sciences*. 2020; 10(8):563
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Based on the ''*amyloid hypothesis*'', the formation and deposition of soluble amyloid-β protein (Aβ) in the brain is one of the crucial events driving cognitive decline and later dementia (357,358). The key risk factor for AD onset and progression is represented by increased age (359–361). In addition to the family history, obesity, degeneration or vascular dysfunction, low levels of education, diabetes, kidney failure, hypotension or hypertension, hyperlipidemia, physical inactivity and the existence of epsilon 4 allele of the apolipoprotein E gene also contribute to the onset and development of AD pathology (201,203,362–365). Growing evidence indicates that AD is a chronic neurological condition throughout the lifespan with critical influences beginning with conception and early life (366–368). A large-scale of epidemiological, clinical and preclinical studies have suggested a central role of gestational factors in promoting cognitive impairments that accelerate the onset and evolution of AD-like pathology later in life (369,370).

The "*fetal origins of adult disease*" hypothesis has shown that the adverse prenatal environment can alter the developmental trajectory of organs/tissues in early life and may intensify the risk of disorders later in life, including neurobehavioral conditions, heart and metabolic disorders (371–376). Prenatal hypoxia is a common form of fetal stress, which leads to the fetal growth restriction (reduced birth weight) and in the most important periods of brain formation results in essential changes in the development of cognitive functions in different stages of postnatal life, which correlates with morphological changes in the cerebral structures involved in learning and memory (377,378). Moreover, hypoxia can also leads to a reduction in plasticity and brain adaptive potential due to interruption in the process of development of novel contacts between cells and propagation of neural stimuli, mainly in the hippocampus and cortex (379). Investigating the impact of prenatal hypoxia and fetal stress on the onset of AD-associated pathology in 5xFAD mice offspring, Shen and colleagues (380) showed that fetal hypoxia significantly decreased brain and body weight in the fetal and the early postnatal period, which recovered in young adult mice. Using a comprehensive behavioral test battery to evaluate different aspects of cognitive impairment (working memory, object discrimination, spatial memory, learning and episodic-like memory), the authors found that antenatal hypoxia intensified cognitive decline in offspring of 5xFAD compared with control groups. Interestingly, fetal hypoxia did not alter intraneuronal soluble Aβ oligomer accretion in the hippocampus and cortex in offspring mice, demonstrating that antenatal hypoxia amplified the brain's vulnerability to synaptotoxic Aβ in the onset of

disease later in life. Consistent with the early onset of cognitive impairment, they also found loss of synapse but not neuronal death at the cerebral cortex level and an exacerbated growth of astrogliosis and microgliosis in the first stages of AD. Taken together, these results revealed a causal link between fetal stress and the accelerated onset of AD-related pathology, providing mechanistic information about the origin of the development of neurodegenerative disorders related to aging.

Despite exhaustive studies and the collection of significant amounts of research data, the mechanisms underlying developmental alterations caused by prenatal pathologies are still not well understood. More and more lately, the concept of epigenetic programming of neurological disorders seeks to describe how prenatal stressful experience through epigenetic modifications in the developmental program in the fetal brain exerts long-term consequences on the future mental health (381). This concept suggests subsequent regulatory mechanisms related to the early life induced by the environmental agent's in utero or early in postnatal period which could underlie the onset of neurodegenerative disorders, including Alzheimer's disease (382).

Neurological deficiencies greatly reduce the quality of life of children affected by neonatal hypoxic-ischemic brain injury (HIE) and increase the socio-economic burden on families, caregivers and society. Hypothermia is currently the only standard treatment for full-term infants with moderate to severe level of this disease. With this therapy, infants with the most severe type of HIE may not be saved, and this approach implicates the risk of severe disability or death, as almost 50% of the cases treated still die (336) and up to 1/3 up to 1/2 of patients have a demonstrate low Intelligence Quotient at the age of 6 to 7 years or constant neurological deficits (383,384). Because, hypothermia is expensive and its therapeutic window is thin, there is an urgent need to develop more effective alternatives and therapeutic strategies. Thus, it is generally believed that a series of natural agents extracted from plants (fig. 27, 28, 29, 30) have neuroprotective effects against HIE. These natural compounds with the anti-oxidative, anti-inflammatory, neurofunctional regulatory properties and anti-apoptotic display preventive or therapeutic outcomes against neonatal HIE obtained experimentally.

Grape seed extract (GSE) includes numerous phenolic compounds, mostly proanthocyanodins monomers, polymers, procyanidins and their gallate ester and resveratrol, which is the main compound that is extracted from the skin and seeds of grape. Investigating the neuroprotective effect of GSE therapy (50 mg/kg) against neonatal hypoxia-ischemia brain injury, Feng et al., found that brain weight loss was diminished from 20.0% in vehicle rats to 3.1% in treated ones, as well as improvement in the histopathologic brain score in hippocampus, thalamus, and cortex after GSE pretreatment. In addition, 8-isoprostaglandin F2alpha and thiobarbituric acid reacting substances considerably augmented due to hypoxic ischemia (385).

In a study by Yu et al., evaluating the anti-amyloidogenic activity of gallic acid through gavage against cerebral Aβ/β-amyloid pathology at a daily dose of 30 mg/kg for 30 days in APP/PS1 double transgenic mice, the authors revealed that a the brain sections of mice showed a significant reduction in Aβ1-42 plaques size, not in their numbers (386). An exploration of whether rosmarinic acid may also influence the changes of white matter fibers and cognitive decline caused by hypoxia-ischemia injury in Sprague-Dawley rats was performed by Li at al., (387). The data showed that rosmarinic acid ameliorates cognitive dysfunctions (anxiety behavior, locomotor activity, spatial learning and memory deficits) induced by perinatal hypoxia-ischemia injury. This protection seems to be achieved by improving the differentiation and maturation of oligodendrocyte progenitor cells and restoring myelin sheaths in the corpus callosum. The clinical feasibility and the most important benefits of rosmarinic acid therapy of cognitive damages after perinatal hypoxia-ischemia injury make us hope this compound will be used soon to benefit more patients.

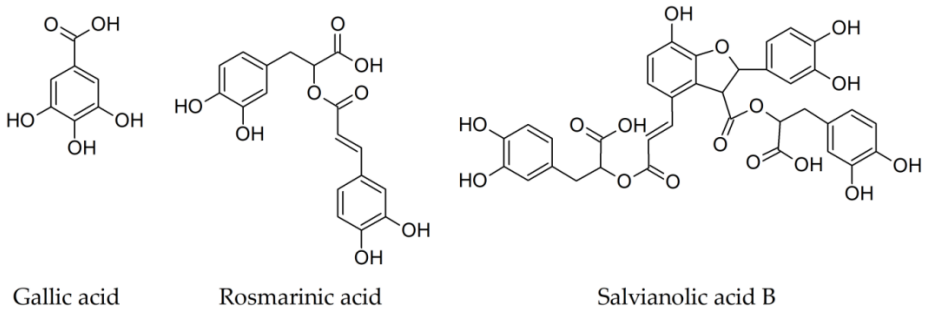

Fig. 27. Chemical structures of phenolic acids from plants whose inhibitory activity towards beta-amyloid fibrillogenesis (205)

In order to explore the neuroprotective effect of quercetin, Qu et al., conducted a study on postnatal 3-day-old rats and revealed that oral gavage of 20 or 40 mg/kg of this compound from 2 hours after hypoxia-ischemia to the day rats were euthanatized significantly improved hypoxia-induced myelin damage through strengthening survival of oligodendrocytes (388). The use of a 7-day-old neonatal rat model of hypoxia-ischemia and oral administration of quercetin daily for 7 days (40 mg/kg/day) resulted in an increase in anti-apoptotic (Bcl-2) and a reduction of pro-apoptotic (Bax) proteins in cortical cells, as well as a significant attenuation of the DNA-strand breakage induced by hypoxia-ischemia, which confirmed the antiapoptotic effect of quercetin.

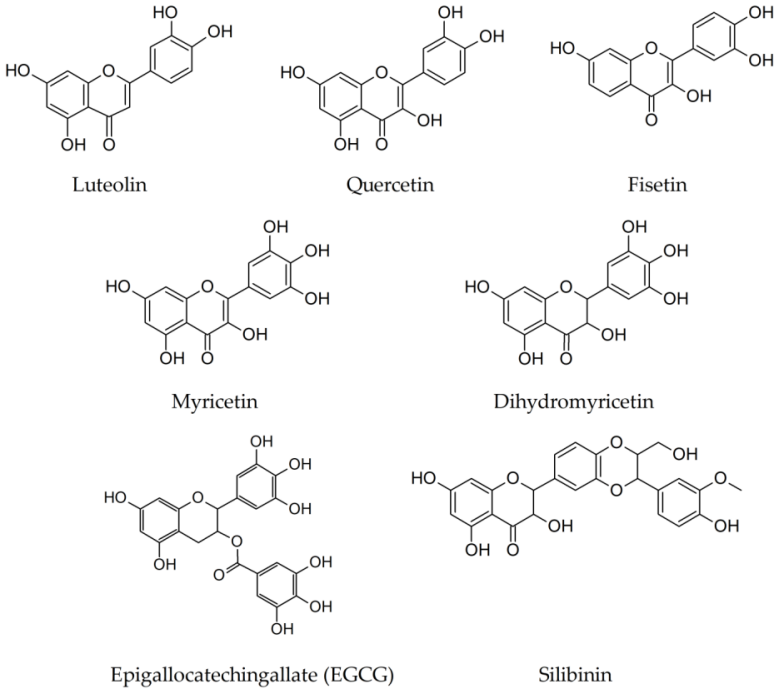

Fig. 28. Chemical structures of flavonoids and flavanol-lignan silibinin from plants whose inhibitory activity towards beta-amyloid fibrillogenesis (205)

The results also showed that quercetin therapy reduced microgliosis and astrogliosis; and down-regulated the inflammatory factors' expression in rat cortex, highlighting the anti-inflammatory properties of this compound and its potential ability to protect brain cortex tissue from further damage (389).

In a preclinical model of hypoxic-ischemic brain injury using a 1-week-old Sprague Dawley rats, curcumin therapy (150 mg/kg by gavage for 3 days) efficiently decreased the

brain injury score, augmented myelin basic protein expression and increased the quantity of neuronal cells in neonatal rats. At the same time, curcumin treatment significantly attenuated the alterations in superoxide dismutase activities and malondialdehyde levels, suppressing nitric oxide synthase protein expression induced in neonatal rats by hypoxia-ischemia damage. Taken together, the results of this study designated that curcumin attenuates hypoxic-ischemic brain injury in neonatal rats through the induction of nuclear factor erythroid-2-related factor 2 and heme oxygenase-1 (390). In a study that tested the neuroprotective properties of oleuropein, Lee et al., found that combining hypothermia with oleuropein therapy in 2- to 4-day-old piglets neonatal hypoxic-ischemic model improved post-hypoxia-ischemia subcortical white matter protection by preserving myelin density, myelinating oligodendrocytes and oligodendrocyte markers (391).

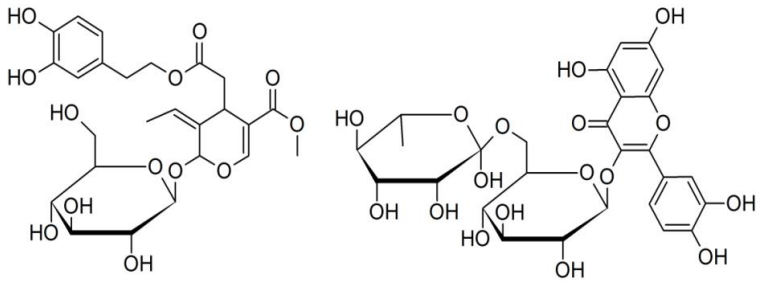

Fig. 29. Chemical structures of oleuropein and rutin (205)

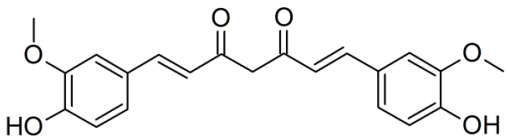

Fig. 30. Chemical structure of curcumin (205)

Given the restricted approaches currently available to treat newborns suffering from neonatal hypoxia-ischemia and considering the limiting pathology that affects a young population, often related with mental and physical disabilities on throughout life, the plant extracts and plant-derived natural compounds could provide the ideal platform for therapeutic strategies that can be safely and prophylactically administered to prevent and reduce the brain damage and neurological damages.

Despite the recognized association between cognitive conditions and renal failure or diabetes, direct evidence connecting kidney/pancreas to brain injury is still missing. In this context, various hypotheses have been projected as supplementary mechanisms in the kidney–brain and/or pancreas-brain communication, including vascular injury, insulin resistance, mitochondrial dysfunction, impaired carbohydrate metabolism, oxidative stress, inflammatory response, renin–angiotensin system and amyloidosis (392). It is worth highlighting that the crosstalk between brain and kidney/pancreas seems to be bidirectional since central nervous system conditions. In this context, a growing number of preclinical and now clinical studies have provided evidence of drugs authorized to treat diabetes or renal failure as having protective on the brain (393–396). The neuroprotective properties of diabetes compounds were first documented by positive neurological effects in diabetes patients under treatment and now in various studies for the treatment of different neurological conditions, such as Alzheimer' disease (Table 39).

Table 39. Comparative efficiency and acceptability of antidiabetic compounds for Alzheimer's disease (203)

Antidiabetic medication	Experimental model	Findings	References
Biguanides			
Metformin	mouse neuroblastoma cell lines under sustained hyperinsulinemic conditions treated with different concentrations of metformin (0.4–3.2 mM)	resensitization of insulin signaling; prevention of the molecular and pathological alterations detected in AD neurons	(393)
	transgenic APPswe/PSd1E9 mouse model of AD; intraperitoneal delivery of 200 mg/kg metformin for 14 days	amelioration of spatial memory deficits, neural cellular proliferation, in the cortex and hippocampus reduction of local inflammation, decrease of Aβ plaque deposition	(394)
	PDAPP (J9) mouse model of AD; 350 mg/kg/day metformin delivered in drinking water for several months	attenuation of memory impairment in female subjects and intensified it in males	(397)
	longitudinal aging study in adults with diabetes	long-term metformin therapy (over 6 years) could diminish the risk of developing AD	(398)
	case-control study, older adults with an incident diagnosis of AD; 1–9, 10–29, 30–59, or ≥60 metformin prescriptions	long-term treatment (60 or more prescriptions) has been correlated with a slight augmented risk of developing AD	(399)
Sulphonylureas			
Glibenclamide	Aβ25-35-induced rat AD model; 6 mg/kg/day of glibenclamide for 20 days by gavage	reduction of Aβ25-35-treated behavioral anomalies	(396)
Thiazolidinediones			
Pioglitazone	meta-analysis of randomized clinical trials; 15 to 30 mg of pioglitazone, as adjunct therapy for AD	doses of 15 to 30 mg pioglitazone but not 45 mg improve cognitive capacity	(400)
	transgenic APPswe/PSEN1dE9 AD mouse model; combined therapy with 0.03 mg/kg/day of leptin intranasal delivery + intraperitoneal administration of 10 mg/kg/day pioglitazone for 2 weeks	decrease of spatial memory impairments and brain Aβ levels	(401)
	APPV717I transgenic mice, a model for AD; acute 7 days gavage therapy with 40 mg/kg/day of pioglitazone	reduction of soluble Aβ1–42 peptide levels by 27% and glial inflammation	(402)
	controlled trial in cases with mild Alzheimer and an accompanying diagnosis of diabetes; daily doses of 15-30 mg pioglitazone for 6 months	cognitive and functional improvements and stabilization of the disease in diabetics with AD	(403)
	controlled pilot trial in individuals with AD without diabetes; daily 45 mg of pioglitazone	18 months of pioglitazone therapy were well tolerated by patients, but no important efficacy data were detected	(404)
Rosiglitazone	meta-analysis of randomized clinical trials; 2 to 8 mg of rosiglitazone, as adjunct therapy for mild to moderate AD patients	pro-cognitive effects	(400)
	pilot study which randomized individuals with AD or amnestic	better delayed recall and selective attention	(405)

	mild cognitive damage		
	large study in population with mild to moderate AD; 2, 4, or 8 mg of rosiglitazone for 6 months	in week 24 was registered an improvement (−2.9 points) of cognition in apolipoprotein Eε4-negative people treated with 8 mg of rosiglitazone	(406)
	phase III trials of rosiglitazone in AD; 2 mg or 8 mg rosiglitazone for 48 weeks, as adjunctive agent to ongoing acetylcholine esterase inhibitors	rosiglitazone does not lead to an improvement in cognition or overall function	(407)
Glucagon-like peptide-1 receptor agonists			
Lixisenatide	transgenic APPswe/PSd1E9 mouse model of AD; intraperitoneal injection with 1 or 10 nmol/kg of compound for 10 weeks	several biomarkers have been improved such as learning, inflammation or plate loading	(408)
	cell culture, 100μM of lixisenatide were applied 24 hours before Aβ25–35 application rat model of AD; 5 nmol/μl of lixisenatide before intrahippocampal application of Aβ25–35 (5 nmol/μl)	reversal Aβ25-35-triggered cytotoxicity, normalization of intracellular calcium levels prevention of memory loss caused by amyloid intracerebroventricular injection	(409)
	transgenic APP/PS1/tau mouse model of AD; daily intraperitoneal injection of 10 nmol/kg lixisenatide for 60 days	reduction of amyloid plaques, neuroinflammation and neurofibrillary tangles	(410)
Dulaglutide	intracerebral injection of streptozotocin induced mouse AD-like condition; 0.6 mg/kg/week of dulaglutide with intraperitoneal delivery for 4 weeks	amelioration of learning and memory deficits	(411)
Liraglutide	transgenic APPswe/PSd1E9 mouse model of AD; intraperitoneal injection with 2.5 or 25 nmol/kg of drug for 10 weeks	improvement of learning, reduction of amyloid plaque deposits by 40–50% and decrease inflammatory response	(408)
	methylglyoxal-induced mouse Alzheimer-like condition; daily subcutaneous administration of 25 nmol/kg liraglutide for 2 months	attenuation of hippocampal damage and cognitive deficits in C57BL/6J mice	(412)
	cell culture; liraglutide (300 nm) was added to cultures 40 minute before Aβ oligomers Aβ oligomers-induced AD mouse model; daily intraperitoneal injections of liraglutide (25 nmol/kg) for 7 days Aβ oligomers-induced non-human primate model of AD; subcutaneous delivery of liraglutide (0.006 mg/kg/day for the first week and 0.012mg/kg thereafter) for 24 days	reduction of Aβ oligomers - induced synaptotoxicity, protective effects on synapses; prevention and reversal of cognitive abnormalities and insulin receptors loss produced by intracerebroventricular injection of Aβ oligomers; the agent was less effective, but still provided partial protection against insulin resistance loss, synapses and phosphorylation of tau	(413)
	Aβ protein-induced rat model of AD; 2 μL liraglutide trough intrahippocampal administration	liraglutide pre-therapy remarkably protected against Aβ-induced damage of spatial memory and long-term potentiation	(414)
	transgenic 3xTg-AD female mice; 0.2mg/kg/day of liraglutide,	reduction of cortical Aβ1–42 levels, partially attenuation of	(415)

	intraperitoneal injections	cerebral estradiol, inflammation and oxidative/ nitrosative stress	
	a pilot clinical trial in AD patients lasting 26 weeks. In the first week, the drug was daily delivered subcutaneously at a dose of 0.6 mg; hereafter 1.2 mg daily for another week before finally increasing to 1.8 mg daily	prevention of brain glucose metabolism decline; there were no important cognitive changes compared with placebo group	(416)
Dipeptidyl peptidase-4 inhibitors			
Saxagliptin	intracerebral injection of streptozotocin-induced rat model of AD; 0.25, 0.5 and 1 mg/kg of saxagliptin administered orally for 60 days	reduction of amyloid plaques formation, a marked decrease of Aβ42 level and phosphorylation of tau protein; total reversal of cognitive impairments	(417)
Vildagliptin	intracerebral injection of streptozotocin-induced rat model of AD; daily orally doses of 2.5, 5 and 10 mg/kg vildagliptin for 30 days	attenuation of Aβ, phosphorylation of tau protein and inflammatory markers	(418)
	Aβ protein-induced rat model of AD; daily gavage of 5 or 10 mg/kg vildagliptin for 4 weeks	anti-apoptotic effect, attenuation of memory abnormalities, reduction of tau phosphorylation and increase of neurotrophic proteins expression	(419)
	streptozotocin-induced rat diabetes model associated cognitive decline; daily gavage of 5 mg/kg vildagliptin for 4 weeks	prevention of memory impairment and diminution of apoptosis in hippocampal neurons	(420)
Sitagliptin	APP/PS1 AD mice model; 20 mg/kg/day of sitagliptin for an 8-weeks period	protective effect of cognitive function, reduction of amyloid plaque deposits	(421)
	transgenic APPswe/PSd1E9 mouse model of AD; daily gavage of 5, 10 and 20 mg/kg sitagliptin for 12 weeks	much more obvious effects for the 20 mg/kg sitagliptin dose: reduction of nitrosative stress and inflammation markers; an important diminution in the number and area of APP and Aβ deposition	(422)
Linagliptin	3xTg-AD mouse model of AD; daily oral administration of 5, 10, and 20 mg/kg linagliptin for 8 weeks	improvement of cognitive performance; reduction of Aβ42 levels, but not Aβ40; diminution of tau phosphorylation and neuroinflammation	(423)
	human neuroblastoma SK-N-MC cell culture; exposure to 10 to 100 μM linagliptin for 24 hours	protection of cells against Aβ-induced intracellular reactive oxygen species accumulation and mitochondria dysfunction	(424)
Amylin analog			
Pramlintide	SAMP8 mice, a model of sporadic AD; subcutaneous infusion of 0.24 mg/kg/day pramlintide for 5 weeks	may improve memory, decrease neuroinflammation and reduce oxidative stress	(425)
Sodium-glucose cotransporter 2 (SGLT-2) inhibitors			
Canagliflozin	scopolamine-induced rat model of memory impairment; daily oral gavage of 10 mg/kg for 2 weeks	improvement of memory dysfunction	(426)
Insulin analogues			
	intracerebral injection of streptozotocin rat model of cognitive decline; 0.5 units = 12 nmol of detemir	alleviating cognitive dysfunction with a significant increase in learning ability; change in insulin degrading enzyme, insulin	(427)

		receptor and somatostatin	
	patients with early AD; intranasal administration of 20 or 40 IU insulin	facilitation of verbal memory recall in memory-impaired ε4− patients; no influence on glucose or plasma insulin levels	(428)
	patients with early AD; intranasal administration of 20 or 40 IU insulin for 21 days	improvement of attention, functional status and verbal memory; modulation of Aβ peptide	(429)
	placebo-controlled pilot clinical trial in people with AD; intranasal delivery of 20 or 40 IU insulin for 4 months	improvement of cognition and functional ability compared to control group	(430)
	clinical trial; 20 or 40 IU of insulin detemir for 21 days, intranasal administration in AD	therapy effect for the memory composite outcome for the 40 IU patients, influenced by the APOE status	(431)

Besides its neuroprotective effects, metformin has been shown to support neurogenesis by proliferation and differentiation, improving the neuronal precursor self-renewal. In a hypoxia–ischemia neonatal rat's model, metformin therapy remarkably diminished brain edema, infarct volumes and attenuates cognitive impairments via improving remyelination (432). Furthermore, a murine model of neonatal hypoxia-ischemia lesion revealed better metformin-induced neuroprotection in females after early lesions compared to males. Accordingly, long term metformin therapy leads to cognitive improvements in females, but not males following early hypoxia-ischemia injury. The mechanism may be related to the sex hormones but further studies of the pathway underlying this effect is required (433).

The neuroprotective effect of sulfonylurea compounds is not completely known but glibenclamide appears to be able to block the sulfonylurea receptor 1, a regulatory subunit of the microglial KATP channel. Clinically, glibenclamide is effective in preventing edema and improving the outcome after focal ischemia (434). In a rodent model of hypoxia-ischemia injury, glibenclamide improved some neurological parameters at 3 weeks of therapy, but failed to attenuate brain edema, brain tissue loss or infarct volume. This may be due to a significant reduction in blood glucose induced by the dose of glibenclamide used, which may exacerbate ischemic brain damage (435).

Also called gliptins, dipeptidyl peptidase-4 (DPP-4) inhibitors, although they have shown neuroprotective effects in preclinical studies, their ability to cross the blood-brain barrier is still unclear. However, they might indirectly growth the active glucagon-like peptide-1 levels in the brain that crosses from the blood (436). There are no data in an experimental neonatal hypoxia-ischemia model, but is well-known that DPP-4 enzyme activity increase in the blood serum of term and preterm neonates with cerebral ischemia (437).

Thiazolidinedione, also called peroxisome proliferator activated receptor agonists or glitazone improve insulin sensitivity and decrease serum glucose in patients with diabetes, without notable changes in serum glucose of non-diabetic animals or humans. Troglitazone, rosiglitazone and pioglitazone therapies could relieve oxygen glucose deprivation induced hypoxia injury *in vitro* and exert neuroprotective effect (438). In the context of adult hypoxia-ischemia injury, thiazolidinedione agents have shown therapeutic efficiency in Ob/Ob mice, a model for diabetes and obesity. This preclinical model has a high risk for stroke and augmented risk of brain injury. Darglitazone therapy in this adult diabetic mouse showed significant neuroprotection accompanied by complete restoration of the initial microglial response and decrease of infarcted brain size at 24 hours of recovery (439). To date, no

studies have been performed on neonatal models of hypoxia-ischemia, but the recognized neuroprotective properties and the strong anti-ischemic effects of this class of diabetes drugs it could be a promising option.

Despite extensive research into the molecular pathways that affect brain development and functioning caused by hypoxia during pregnancy and birth, we are still far from a comprehensive assessment of all modifications in molecular and epigenetic levels which shape individual development in postnatal life. Subsequent studies using preclinical models of hypoxia will allow us and others to gain a more detailed perspective on the mechanisms of neuronal functions dysregulation during fetal development and design innovative preventive approaches to restore brain integrity and cognitive functions.

I.3.6. Postpartum hypoxic context and a specific long-term consequence in particular severe cases

General considerations

Certain neonates may have a normal oxygenation and development in the intrauterine life, but due to restrictive ventilation after birth, may experience a hypoxic status. There are several situations, mainly genetic disorders, and malformations prone to develop postnatal hypoxia, acute or chronic, generating life threatening status or accidents. Such a long-term challenging consequence is the obstructive sleep apnea syndrome (OSAS), a disease common in adults same as in children, that has been extensively studied over the few last decades and classified according to its pathogenesis and severity.

It is defined as sleep-disordered breathing (SDB) that is characterized by prolonged partial upper airway obstruction and/or intermittent complete obstruction (obstructive apnea) that disrupts normal ventilation during sleep and disturbs normal sleep patterns (440). Although obstruction of the upper airway during sleep remains its main feature (441), other symptoms are common in clinical practice, such as frequent snoring, sleep enuresis, headaches on awakening, daytime sleepiness, and attention-deficit and learning problems (440). Physical examination may reveal characteristic signs, such as being underweight or overweight, tonsillar hypertrophy, adenoidal facies, micrognathia/retrognathia, or a high-arched palate. Nocturnal hypoventilation may produce nocturnal arousals because of hypoxemia or hypercarbia, awkward sleeping positions, night sweats, morning headaches, irritability, and other symptoms (442,443).

The disease affects 1–10% of children in certain populations (444). The prevalence is differently reported when considering various age groups, gender, and social and genetic factors (445). Genetic disorders are a category of diseases that include certain types of birth defects, chronic diseases, developmental problems, and sensory deficits, and could appear de novo or are inherited from one or both parents.

The presence of congenital craniofacial anomalies can be associated with a higher incidence of obstructive sleep apnea than in the regular pediatric population (446). In these children with congenital malformations or genetic disorders, which are the main cause of OSAS, SDB is not always recognized and reported by the parents (447). Some studies have shown some degree of association between parental concern and severity of the disease (448).

The connection between craniofacial anomalies and sleep apnea could reside in the anatomic obstruction of the upper airways, which these patients sometimes present. Mucopolysaccharidoses, Down syndrome, muscular dystrophies, and other neurologic disorders have been associated with obstructive sleep apnea (449,450). Impaired sleep is a frequent problem in these subjects and it is commonly either missed or underestimated.

The severity of the disease is usually classified according to existing guidelines (451). Unrecognized OSAS in children may lead to complications whose severity is even more important as OSAS evolves over time. Intermittent hypoxia has been demonstrated even in

preterm neonates and severe apnea/bradycardia in infants (452). Long-term sleep disturbances can produce cognitive impairment, attention-deficit/hyperactivity disorders, and social disabilities that could prove challenging to cope with (443,444). It is difficult to decide which factor contributes the most to long-term cognition complications of OSAS.

A multidisciplinary approach for both the diagnosis and therapeutic interventions is required and recommended due to the complexity of comorbidities, patients with genetic syndromes (453). The management of obstructive sleep-disordered breathing can be surgical or conservative. A tonsillectomy is one of the most used procedures for improving airway patency in children (454,455). Continuous positive airway pressure (CPAP) or other forms of non-invasive ventilation (NIV) are appropriate therapeutic approaches when indicated (456,457). CPAP is very helpful in OSA since it improves the quality of sleep and alleviates daytime sleepiness and caregiver concern (452,457). Bilevel positive airway pressure (BiPAP) is preferred when there is associated hypoventilation, which involves a complex set of sleep-disordered breathing mechanisms: hypotonia, craniofacial anomalies, obesity, and patients with genetic disorders (458). For these patients, the benefits from NIV could be different from otherwise healthy children and are focused on the palliation of symptoms and quality of life improvement, both for children and their families (452,459). In neurologically impaired pediatric patients, different therapeutic protocols can be discussed.

Published paper:

- Oros M, Baranga L, Plaiasu V, Cozma SR, Neagos A, **Paduraru L,** Necula V, Martu C, Dima-Cozma LC, Gheorghe DC. Obstructing Sleep Apnea in Children with Genetic Disorders—A Special Need for Early Multidisciplinary Diagnosis and Treatment *J. Clin. Med.* 2021;10(10): 2156

Objective

The aim of this study was to analyze the prevalence and severity of OSAS in a peculiar group of children with complex neurological, musculoskeletal, and genetic disorders to show that sleep-disordered breathing frequently affects this particular population and to argue for the need for a multidisciplinary approach in the diagnosis and therapeutic management of OSAS that is adapted to the special needs of cases with various genetic diseases.

Materials and Methods

The present retrospective study was conducted on children that were diagnosed with neurological, musculoskeletal, and complex genetic syndromes which were referred from August 2017 to March 2020 to the pediatric sleep unit of Regina Maria Clinics and Ponderas Academic Hospital Bucharest for complete sleep studies using polysomnography (PSG). For children diagnosed with OSAS with indications for surgical treatment, it was performed at the M.S. Curie Hospital. The protocols for diagnosis and subsequent management were discussed with all the specialists involved and were approved by the Ethics Committee of Ponderas Academic Hospital (no. 245b/05/10/2020). All subjects gave their informed consent for inclusion before they participated in the study and the study was conducted in accordance with the Declaration of Helsinki.

Inclusion criteria were as follows: age under 18 years and the presence of at least one neurological, neuromuscular, or genetic comorbidity. Patients with an uncertain diagnosis or with traumatic or iatrogenic lesions of the upper airway were excluded.

The overnight video PSG was performed using System Alice 6 LDx, Phillips Respironics, with Sleepware G3 software. Transcutaneous CO2 (TcCO2) values were obtained over the entire PSG recording period using the Radiometer Monitor system.

Respiratory parameters, including apnea–hypopnea index (AHI) and sleep stages, were scored according to the AASM 2012 guidelines (451). AHI is the sum of apneas plus hypopneas per hour of sleep (events/h).

The OSAS was defined as follows: mild for 1 < AHI _ 5, moderate for 5 < AHI _ 10, and severe for AHI > 10, with oxygen desaturation index > 3%.

Hypoventilation was defined at TcCO2 > 50 mmHg for >25% of the total sleep time (TST) according to guidelines (451).

In this study, non-invasive ventilation was defined as any form of ventilatory support whenever CPAP or BiPAP was applied.

CPAP was chosen in the presence of isolated OSAS and BiPAP was selected in the presence of OSAS in neuromuscular patients or with associated hypoventilation. In all patients, NIV was started in the pediatric respiratory ward or the pediatric sleep laboratory.

The final goal was achieving uninterrupted supine rapid eye movement (REM) sleep by manually selecting the NIV pressure. The pressures were titrated according to international guidelines until the complete elimination of respiratory events and the normalization of pulse oximetry and TcCO2 with good sleep efficiency (SE). The patients and their parents were trained on how to use ventilatory masks and how to prevent adverse effects.

In children with an obvious clinical anatomical obstacle (adenoid vegetations, chronic tonsillar hypertrophy), surgical treatment of the obstruction was proposed.

The following parameters were collected for the study: the presence or absence of different symptoms of OSAS, the results of the clinical otorhinolaryngological examinations, polysomnographic studies data, CPAP or BiPAP parameters, and complications associated with the NIV. Endoscopic evaluations were also recorded when available, as well as the performed surgical procedures, which included an adenoidectomy or/and a tonsillectomy. The ENT clinical assessment used the drug-induced sleep endoscopy (DISE) only in particular cases where the level of airway obstruction was difficult to be appreciated on a clinical basis.

Adherence to treatment was evaluated by analyzing the parameters of the machine's data logging. Good adherence was considered to be achieved in cases where the NIV time was higher than 4 h per night. Quantitative absolute and relative data were expressed as a median and interquartile range (IQR). The statistical analyses were performed using the Microsoft Excel software package, version 16.16.14.

Results

The demographic data and the distribution of diagnosed disorders in the study population are reported in Table 40. The study population included 108 children that were identified with neuromuscular diseases or complex genetic conditions, with 65 boys (60.2%) and 43 girls (39.8%).

Neuromuscular disorders were detected in 78 patients (72.2%) and other multiple congenital anomalies were detected in 30 patients (27.8%). Obesity was observed in 23 patients (21.3%) and 43 subjects (39.8%) had scoliosis.

Some subgroups from our study included a small number of cases that were part of rarer genetic disorders. OSAS was detected in 87 patients (80.5%) with different prevalence rates among various groups of genetic disorders in the study population, as shown in Table 41.

Table 40. Demographic and clinical characteristics of the study population

	Patients *n* = 108 (%)[a]	Gender		Age at Time of First PSG (Years) Median [IQR]
		Female *n* = 43 (39.8%)[a]	Male *n* = 65 (60.2%)[a]	
Neuromuscular pathology	**78 (72.2%)**			
Spinal muscular atrophy (SMA)	**45**	**24**	**21**	-
SMA 1	12	4	8	7
SMA 2	25	14	11	8
SMA 3	8	6	2	11
Duchenne muscular dystrophy	**25**	-	**25**	14
Ulrich muscular dystrophy	**1**	**1**	-	11
Merosin deficient muscular dystrophy	**1**	-	1	5
Other myopathies	**6**	**2**	**4**	15
Skeletal disorders	**4 (3.7%)**			
Achondroplasia (ACH)	**3**	**3**	-	4
Marfan syndrome	**1**	**1**	-	17
Complex abnormalities	**26 (24.1%)**			
Craniosynostosis (Crouzon syndrome)	**2**	**1**	**1**	4
Prader–Willi syndrome (PWS)	**19**	**8**	**11**	4
Arnold–Chiari syndrome	**5**	**3**	**2**	11

PSG: polysomnography. IQR: interquartile range. [a] The percentage refers to the total number of patients in the study (N = 108).

The respiratory parameters recorded using PSG are presented in table 42. The median of obstructive apnea–hypopnea index (AHI) was 7.7 events/h with IQR = 5–14.2 events/h; the highest AHI = 117.5 events/h was observed in a patient with achondroplasia. The lowest SatO2 in patients with OSAS was 60% in a Crouzon disease patient. Sixty-two patients (71.3%) had SatO2 < 90% and 13 patients (15%) had hypoventilation. The highest TcCO2 of 78 mmHg was recorded in a neuromuscular patient.

Table 41. Prevalence of OSAS

Genetic Disorder	Prevalence of OSAS
Neuromuscular diseases	69.2%
Prader–Willi syndrome	94.7%
Arnold–Chiari syndrome	80%
Achondroplasia	100%
Crouzon syndrome	100%

As reported in table 43, otorhinolaryngological surgery was performed in 15 patients (17.2%). Thirty-five patients (40.2%) started NIV, where 32 started BiPAP (36.8%) and 3 started CPAP (3.4%).

In the study population, 93.8% of the patients that were treated using BiPAP had neuromuscular disorders, while among the patients who used a CPAP therapeutic approach, two had Prader–Willi syndrome and one had achondroplasia. From the group with neuromuscular disorders, 40 patients (51.3%) had associated scoliosis.

Three patients started NIV during a respiratory upper airway distress episode and hence, without a previous polysomnographic study, all other patients performed a formal sleep study prior to the implementation of the ventilatory support. Five patients were referred

for another management option: neurosurgery for achondroplasia and Arnold–Chiari syndrome and ocular–orbital reconstruction for a patient with Crouzon disease.

Table 42. Respiratory parameters recorded using PSG

	Patients with OSAS *n* = 87 (80.5%)	**Respiratory Characteristics in Polysomnography Median [IQR]**			
		AHI (events/h)	**Index > 3% (ODI/h)**	**SpO_2 < 90% *n* (%)***	**$SatO_2$ Nadir (%)**
Neuromuscular disorders	**59 (67.8%)**			**41 (69.4%)**	
Spinal muscular atrophy (SMA)	29	8.3 [3.1–15.9]	4.8 [2.9–1.7]	22	77
Duchenne muscular dystrophy	23	6.3 [6.3–9.5]	6.2 [2.9–0.5]	13	80
Ulrich muscular dystrophy	1	16	16.4	1	85
Merosin deficient muscular dystrophy	1	8.3	6.9	1	89
Other myopathies	5	13 [5.8–16.3]	15.3 [7.2–2.1]	4	82
Skeletal disorders	**4 (4.6%)**			**4 (100%)**	
Achondroplasia (ACH)	3	6.5 [3–117.5]	9.3 [5.3–100.3]	3	71
Marfan syndrome	1	8.4	6.1	1	84
Complex abnormalities	**24 (27.5%)**			**17 (70.8%)**	
Craniosynostosis (Crouzon syndrome)	2	27.5 [17.3–37.8]	26.7 [26.2–27.2]	2	60
Prader–Willi syndrome (PWS)	18	5.7 [3.7–9.6]	7.6 [5–15]	14	73
Arnold–Chiari syndrome	4	5.1 [1.3–9.1]	16.5 [15.1–17.5]	1	70

*IQR: interquartile range. Obstructive apnea–hypopnea index (AHI) events/h. Oxygen desaturation index > 3% (ODI)/h. Number of patients with SpO_2 < 90% is given as n (%), * percent from the group of disorders.*

The PSG parameters recorded at the post-therapeutic follow-up, which are presented in table 44, confirmed the success of the treatments performed. Minor complications of NIV were recorded in five (14.3%) patients as local skin irritation. All patients (100%) with sustained NIV had clinical improvement, as reported in the clinical files at the end of the sleep monitoring/titration process.

Table 43. The therapeutic approach and post-therapy follow-up in the study groups.

	Patients with OSAS	**OSAS-Treated Patients *n* = 50**			**PSG after Therapy**
		ENT	**CPAP**	**BiPAP**	
	***n* = 87**	***n* = 15 (17.2%)**	***n* = 3 (3.4%)**	***n* = 32 (36.8%)**	***n* = 29**
Neuromuscular disorders	**59 (67.8%)**				
Spinal muscular atrophy (SMA)	29	3	-	14	10
Duchenne muscular dystrophy	23	1	-	11	6
Ulrich muscular dustrophy	1	-	-	1	1
Merosin deficient muscular dystrophy	1	-	-	1	1
Other myopathies	5	-	-	3	2
Skeletal disorders	**4 (4.6%)**				
Achondroplasia (ACH)	3	2	1	-	2
Marfan syndrome	1	1	-	1	1
Complex abnormalities	**24 (27.5%)**				
Craniosynostosis (Crouzon syndrome)	2	-	-	-	-
Prader–Willi syndrome (PWS)	18	8	2	1	6
Arnold–Chiari syndrome	4	-	-	-	-

Although confirmed as having OSAS, 37 patients (42.5%) did not accept any therapeutic proposal and did not return for clinical follow-up. The reasons for minimal compliance are beyond the scope of this paper, but we suggest that multiple social and psychological factors contributed to this result.

For some patients, a specific therapeutic approach was advised: neurosurgery for achondroplasia and Arnold–Chiari syndrome and ocular–orbital reconstruction for a patient with Crouzon disease. Those cases did not come for follow-up either.

Table 44. PSG parameters during the post-treatment follow-up

Treatment	AHI (events/h)	SpO_2 < 90%, *n* (%)	$SatO_2$ Nadir (%)
ENT surgery	1.33 [1–2]	0	92
BiPAP	1.8 [1–4]	0	95
CPAP	3 [2–4]	0	95

Discussions

The results of the present study showed that OSAS is a pathological condition with a significantly high prevalence in a selected population of children with neuromuscular, skeletal, genetic syndromes, and/or craniofacial abnormalities. The high prevalence of OSAS reported in this study (80.5%) is consistent with previously published studies for different populations and highlights the increased risk of OSAS among children with complex genetic disorders (453,457,458,460) compared with a much lower value of the prevalence of sleep-related breathing disorders (9.73%) in a general pediatric population (461). Polysomnography played a key role in identifying OSAS in our pediatric population. The comparative analysis of the results of the present study indicated a prevalence of 69.2% of OSAS in children with neuromuscular pathology in our group, which is a significantly high value that confirmed the trend published by other authors that report prevalence of over 40% (460,462–464). In particular, OSAS was present in our study in 92% of children with Duchenne muscular dystrophy; this is a higher prevalence than that found in most previous studies, which ranged from 31 to 63% (465). Our work identified OSAS in all children diagnosed with achondroplasia (100%); this is a result in disagreement with those published in the literature, which mentions prevalence between 54 and 59% for achondroplasia cases (464,466). This discrepancy may be due to the small number of cases of the pediatric population enrolled in our study.

In cases of children with complex abnormalities, the prevalence of OSAS observed in our study is in line with the trend of values published by other authors, but with a higher incidence for all studied diseases. We identified OSAS in 100% of children with Crouzon syndrome, a higher value than the published ones, which are between 74 and 77% (467,468). In children with Prader–Willi syndrome OSAS prevalence was 94.7%. Results published by other researchers show values between 79.9 and 92.9% (463,464). We recorded an OSAS prevalence of 80% in cases with Arnold–Chiari syndrome, which is also higher than the published values of 60 to 72% reported by other authors (469,470).

A significantly higher prevalence of OSAS, both in our study group and in other previous publications, suggests a possible occurrence of neurocognitive complications on long-term evolutions. Some literature reports have demonstrated an abnormal central nervous system development in children with obstructive sleep apnea, followed by degradation in their quality of life (471–473). This is why investigating sleep-disordered breathing in children with genetic diseases is important for delineating secondary neurocognitive deficits from their main associated pathology (453). As a consequence, the recommendations for the proper

diagnosis and management of OSAS come as a priority for children with complex neurological and genetic conditions (474).

In our group of patients, the association of scoliosis in 51.3% of children with neuromuscular disorders worsened the health condition of these children. Scoliosis was found to be associated with hypopnea or apnea with decreased oxygen saturations and hypoventilation, especially during rapid eye movement (REM) sleep (475). It is also a risk factor for disease progression to daytime respiratory failure (442).

In a population of children with genetic abnormalities, the instabilities in the ventilator control system can generate temporary arrest of the respiratory drive, hence the term central sleep apnea, which is also a consequence of the obstruction of the airway that triggers an elevated chemoreceptor sensitivity (476). Both clinical forms, namely, mixed-dominant apnea and obstructive-dominant sleep apnea, could be met in a group of children with genetic disorders, but the mixed apnea could make the therapeutic approach more challenging (477).

In patients with neuromuscular diseases, NIV is the standard approach for sleep-disordered breathing because of specific characteristics, such as pharyngeal neuropathy or weakness, macroglossia, scoliosis, or small lung volumes. In our study, the use of CPAP and BiPAP has shown a complete control of sleep-disordered breathing, relieving the symptoms of OSAS. These results were achieved via careful monitoring, with manual titration of the positive airway pressure (PAP) that was conducted in 91.4% of the patients. PSG allowed us to produce accurate recordings during the NIV management. It is noteworthy that the non-invasive ventilation can also trigger specific sleep-disordered breathing events, such as air leaks, patient–ventilator asynchrony, and central sleep apnea (459). After the therapy by CPAP, the mean value for AHI in the group in our study was 3 events/h, significantly higher than other studies for a special pediatric population that show values of 1.1 events/h (477), although the symptoms of our patients were totally controlled.

In the therapeutic approach taken for our patients, ENT surgery was used in those cases with obvious enlargement and obstructing pharyngeal lymphoid tissues. As a result, 73% of ENT surgeries were performed in patients with skeletal disorders and complex abnormalities (60% of our Prader–Willi patients), underlining the connection and summation influence of craniofacial anomalies with the obstruction of the upper airways in these patients.

Analyzing the PSG posttreatment parameters, the children from the group who benefited from ENT surgery showed a follow-up mean AHI of 1.33 events/h, which is a result that is consistent with data published by other studies on special pediatric populations that report values between 1.1 to 1.6 events/h in groups of patients with adenotonsillectomy (478).

In the end, multiple factors are associated with producing more severe diseases in children with genetic disorders. Allergic rhinitis should be medically controlled as an adjuvant measure, as rhinitis can also interfere with NIV compliance, with worsening nasal obstruction and mucosal dryness being frequent side effects that are also associated with NIV (457).

The low compliance with treatment recommendations was present in our study group in a large number of children (42.5%) with confirmed OSAS who did not accept the proposed treatment solutions and who did not continue follow-up monitoring of the disease's evolution. We can explain this fact only in terms of the difficult access to health facilities of the medical system or by educational deficiencies of the parents or their lack of understanding of the long-term evolution of their child's disease.

The gold standard for OSAS diagnosis remains PSG examination. However, in areas where PSG is not readily available, overnight pulse oximetry with continuous CO2 monitoring can also be used to record nighttime gas exchanges. A recent study demonstrated that nocturnal PSG contains more information about breathing than respiratory parameters alone, for example, snoring, mouth-breathing, and flow limitation. These symptoms can also

indicate a disturbance of breathing and occur earlier than apnea/hypopnea events associated with a decrease in oxygen saturation by 3% or more (478).

As in all retrospective studies, our research may have limitations, such as the availability of all categories of data for each patient, a limited number of patients with some specific pathology, or the lack of a uniform therapeutic approach, but the results may guide the design of future improved studies.

Conclusions

The results of the present study suggest, in agreement with the literature data, that OSAS is a serious and frequent clinical condition for pediatric patients with complex genetic diseases and plays a pivotal role in their disease symptomatology and worsens the quality of life of these children.

We showed that in genetic diseases, sleep-disordered breathing should not be considered an incidental complication. Because clinical symptoms cannot be used as predictors or markers for OSAS or nocturnal hypoventilation, the screening of overnight gas exchanges, at least to detect nocturnal hypoxemia or hypercapnia, with or without a complete sleep study, if possible, should be a priority in all children with neuromuscular or genetic diseases. Detecting and treating sleep-disordered breathing in these patients may improve their already impaired quality of life (457).

Due to cognitive necessities and the rapid growth processes of children, the early diagnosis and treatment of OSAS is a priority for children with complex genetic conditions and requires a holistic understanding of the diagnosis, technology involved in diagnosis and therapy, prognoses, and long-term care. Our study strengthened the evidence for a multidisciplinary approach toward obstructing sleep apnea syndrome in children with neuromuscular or complex genetic disorders.

I.3.7. Ethical aspects in life threatening hypoxic diseases in newborn

Neonatology is one of the specialties that has recently acquired a recognized importance and has had a rapid development in the last 25 years. Currently, the most difficult cases such as extreme premature infants, at the limit of viability, or severe perinatal asphyxia are successfully recovering, cases that a few years ago would have evolved to death (479). These achievements are not only due to modern equipment, but especially to the high professional training with updated notions and well assimilated knowledge of the medical staff in this specialty. Along with the progress, there have been ethical difficulties in approaching and managing borderline situations. Thus, it came to the identification of particular situations faced by the neonatology practitioner.

Published paper:

- Stamatin M, **Paduraru L**. Specific medical and ethical aspects in the care of life – threatening illnesses in newborn. *Rev Rom Bioet*. 2009;7(1):67-73

The impossibility of direct communication with the patient

The newborn does not verbalize, and the clinical symptoms are often nonspecific, presenting as respiratory distress syndrome which is a sign for a wide range of etiological entities, most often life-threatening (480). To identify the main risk factors, a rigorous monitoring of the pregnancy and close communication between general practitioner - obstetrician - perinatologist - neonatologist focused on the pregnant woman are required. The extreme situations are due either to the pathology of the pregnancy despite a rigorous monitoring, but also to the lack of adequate perinatal care caused by insufficient access to

medical care in regions with low resources. However, there are some situations when the mother does not seek medical care, even when available, due to lack of education. Thus, the problems of the fetus and newborn come as a surprise that must be dealt with quickly and properly, in the child's best interest. The newborn is part of the category of vulnerable people (defined in the International Ethical Guide to Biomedical Research Involving Human Subjects since 2002 as "persons absolutely incapable of protecting their own interests"). Therefore, the decisions are transferred to the parents or to the hospital ethics committees.

Difficulties in contacting and informing parents correctly and quickly about life-threatening pathology and rapid deterioration of the newborn's condition

Given that the mother is going through a difficult period after birth, the information provided may be misperceived. Life-threatening pathology requires a minimum of medical knowledge, making it difficult for parents to understand. Most of the time, the father is not in the immediate entourage, so he cannot be informed. The neonatologist must break the difficult news with care, patience, but at the same time should be brief and fast and prioritize the care of the patient who needs his/her presence and sustained action. On the other hand, it is known that information about the patient's condition must be communicated as soon as possible and preferably in the presence of a third-party, family, or social worker, who can provide emotional support. Parents should be kept informed of both the therapeutic measures and the course of the disease, so that the news of the eventual death would not come as a "lightning strike" that could raise doubts about the quality, promptness, and professionalism of the medical act. The duty of confidentiality is not only part of the Hippocratic Oath, but also an imperative by law and most often requested by the family. Thus, doctors often must refuse to provide information to people who claim to be related to the newborn. These situations must be well evaluated, because most of the time the parents are seriously affected by the bad news, refuse to accept it, and ask for reconfirmations from relatives or friends in the medical field. Unlike other countries, in Romania we have the advantage that there are no communication difficulties due to language differences, the population being mostly of Romanian nationality. Only in special situations like deaf-blindness or psychiatric disorders, major difficulties of communication with the mother arise, but fortunately these situations are rare.

Difficulty in obtaining real informed parental consent regarding therapeutic options

The Patients' Rights Act (Law No. 46 of January 2003, updated in 2019) stipulates the rights of the patient or of the parents to be informed about available therapies, those considered necessary, the state of health, the evolution of the disease, the policy of the hospital, but it also states the right to refuse certain therapeutic procedures. Informed consent of the parent or of the legal guardian is thus mandatory in cases such as initiation or maintaining life support therapy in borderline cases. When the parents do not agree with each other, or when they are not immediately available, it is necessary to consult the hospital's ethics committee. Unfortunately, it is not available at any time either, especially when the progression of the disease is too fast and the need for a swift decision is paramount. Therefore, in neonatology more than anywhere else, the practitioner is forced to face a pressure of ethical-medical decision in an emergency alone, without any legal protection and with important psychological, professional and even social implications (481).

The lack of extensive experience that would allow us to give the parents a more realistic prediction of the chances of survival, decreases the possibility of an accurate information. In these situations neonatologists often resort to international statistical results, communicated and recognized by the main authorities in neonatology (482). However, these cannot be extrapolated to our conditions of care or monitoring, which are not always similar

to those from centers with high-end facilities. Moreover, parents ask for guarantees and certainties, percentages and forecasts in days or hours, which are in fact impossible to offer. The risk of giving personal predictions, from the experience or subjective intuition of each doctor, does nothing but offer false hopes on the basis of fixed ideas that the parent is inclined to cling to. The disappointment will be even greater, and the blame will return with even greater force on the doctor.

Lack of clear law on the declaration of the newborn and the opportunity to initiate resuscitation or intensive care maneuvers at decreasingly lower gestational ages

Regardless of gestational age, the current legislation states that any fetus with minimal signs of life immediately after delivery is considered alive. This leads to the initiation of resuscitation in any newborn with minimal cardiac activity, even if the degree of immaturity will lead to exhaustion of the organic response in a time period varying from a few hours to days or weeks postnatally. For newborns diagnosed antenatally with severe congenital malformations, as well as for those with confirmed gestational age of less than 22 weeks, resuscitation should not be initiated because it is considered un unethical treatment option (483). This recommendation is based on the fact that intensive medical interventions will not improve survival on the long term and will have a negative impact on the quality of life for both the patient and the family. The decision to not resuscitate or stop intensive care when the chances of survival are minimal or when, despite survival, the neurological outcome of the newborn is permanently compromised, is a difficult one to make (484). Children with extremely low birth weights, less than 500 grams and gestational ages under 24 weeks, who were recovered and cared for intensively for weeks or months with therapies that involved enormous costs, developed long-term complications which required repeated surgery and subsequent prolonged hospitalizations. Eventually, they were lost after months or years of sustained medical and family efforts. Another category of newborns in which the ethical decision is required at a certain time, is that of newborns with severe perinatal asphyxia. After cardio-respiratory stabilization, some of them show irrecoverable neurological decline, with persistent comatose state, motor, and cognitive deficits, extremely difficult to manage and discouraging for the care takers. The presence of such a case that requires continuous and adequate care in a family, is not only depressing, but can also be an important destabilizing factor for a couple, having a significant negative impact on the psychological and affective development of the child's siblings, in addition to important cost that the entire family and even society must cover. The survivors' quality of life of should be an important element of decision-making, especially for the newborn. The right to life is inalienable, and the doctor is compelled to exercise his/her full professional capacity to save every life. However, the continuation, at any cost, of resuscitation, despite a severe hypoxia with neuronal destruction demonstrated by EEG, will lead to severe encephalopathy, with vegetative life without the conscious human component. The most common situation is the following scenario: the newborn, either premature or full-term, with severe asphyxia defined according to the current criteria, with prolonged complex resuscitation at birth, will require artificial ventilation and drugs to support cardiac activity, parenteral nutrition and complex monitoring, but will not show of stability or EEG activity. Following discussions with the parents, they request that the hospital staff make every effort to keep the infant alive, regardless of the consequences. After a variable time of intensive care, cardio-respiratory stabilization is obtained, allowing feeding by palliative methods, enterally by gavage, but without clinical improvement or neurological progress. After a long hospitalization, which aims to educate the mother in the necessary care for the child, the infant is discharged and referred to the neurology specialist who monitors neurological progression periodically and the physiotherapist who indicates methods that may be useful in recovery. With passage of time, the enthusiasm of the family

gradually decreases, and disappointment becomes overwhelming. As the situation continues, blame towards the medical staff surfaces and frequently the parents' reply is "if I had known from the beginning that he/she would suffer like this, I would not have insisted on keeping him/her alive at all costs".

Further on, there are some clinical cases with different ethical and social implications, originating from different clinical situations.

Case 1: newborn SE, severe asphyxia with permanent consequences and forensic implications

SE, female term newborn, birth weight (BW) = 3600 g, Apgar scores of 4/7/8 at 1, 5 and 10 minutes, born vaginally from breech presentation at a level II unit and transferred at 24 hours of life for perinatal asphyxia to the level III Regional Center for Neonatal Intensive Care. Complex explorations established the diagnosis of hypoxic-ischemic encephalopathy stage II with seizures from the first days of life, severe respiratory distress, subarachnoid hemorrhage, brachial plexus paralysis after traumatic shoulder hematoma, post-hypoxic hypertrophic cardiomyopathy, post-hypoxic hepatic insufficiency. The infant required 5 days of mechanical ventilation, complex therapies like red packed blood transfusions, parenteral nutrition, antibiotic prophylaxis, anti-seizure medication, with apparently good evolution in the first 12 days. At the age of 18 months, the patient was monitored in a neurological follow-up program and, despite physiotherapy, even if the seizures have stopped, the baby showed psychomotor retardation, flaccid tetraplegia, no verbalization, the only functions being vegetative. Being with a high intellectual and socio-economic level, the family barely accepted the reality, and began legal procedures to inflict responsibility on the medical staff involved in the delivery and treatment of the newborn.

Case 2: newborn CT, prolonged resuscitation with death in the late perinatal period

CT, male newborn, BW = 2500g, delivered by C-section, with Apgar scores of 1 to 1 minute, 3 to 5 minutes, 4 to 10 and 20 minutes. After a complex and prolonged resuscitation (one hour) in the delivery room, chest movements were barely present, accompanied by tonic-clonic seizures. The neonate required mechanical ventilation for 8 days, correction of severe metabolic acidosis with repeated high doses of bicarbonate, inotropic support with dopamine and dobutamine for three days, early and aggressive treatment with two anticonvulsants. Severe perinatal asphyxia was accompanied by renal failure, bronchoplegia, absence of reflexes and generalized spasticity, feeding only by gavage. The aEEG specified peak-wave and micro voltage complexes, and the computed tomography examination showed diffuse cerebral edema with poor differentiation between white and gray matter. After 40 days, the newborn was transferred to pediatric neurology department and subsequently discharged without any progress or neurological acquisition. He died at home at the age of two months.

Case 3: DR, newborn diagnosed with hypoxic-ischemic encephalopathy stage III

DR, term newborn, BW = 4000 g, delivered by C-section at a level II center for decrease of the fetal activity, to a mother with adequate prenatal care. Apgar scores were 8/7/7 at 1, 5 and 10 minutes. Immediately after birth, the infant developed hypotonia, cyanosis, systolic murmur, bradycardia, shallow breathing, and was transferred to a level III facility intubated and mechanically ventilated. Over time, he developed clinical signs of hypoxic encephalopathy with increased biochemical markers and severe respiratory distress of neurological origin which required mechanical ventilation for 10 days. On the 13th day of life, he developed tonic-clonic seizures which did not respond to the usual anticonvulsants. The computed tomography exam showed post-hypoxic cerebral atrophy and the aEEG showed a flat trace. The echocardiography revealed post-hypoxic hypertrophic cardiomyopathy. The muscle biopsy indicated accentuated and persistent hypotonia, specific

for post-hypoxic muscle atrophy. After 52 days of persistent seizures without any neurological improvement, following an episode of continuous seizure activity, he was transferred to the pediatric ward where he died at 3 months of age, despite all intensive care measures.

Case 4: newborn MI, diagnosed with hypoxic ischemic encephalopathy stage III, no apparent explanation, with severe prognosis and socio-familial implications

MI, female term newborn, GA = 40 weeks, BW = 3600g, born vaginally at a level II center. Apgar scores were 1 to 1 minute, 4 to 5 and 10 minutes, 5 to 20 minutes. The infant required prolonged and complex resuscitation for severe perinatal asphyxia and after 30 minutes showed gasping respirations, with spontaneous breaths installed 10 minutes later. The infant was admitted to the NICU and the clinical examination revealed cephalohematoma, as well as tone, reactivity and reflex disorders. At 4 hours of life he developed generalized tonic-clonic seizures which lasted for 48 hours under sustained treatment with two anticonvulsant drugs. The lab work revealed post-hypoxic syndrome with hepatic-renal failure and metabolic acidosis. At 14 days of life the neonate was transferred to a superior care center for further investigations. The aEEG showed a normal amplitude trace alternating with a flat trace on both cerebral hemispheres, and inconsistent bilateral seizure activity. Cranial ultrasound did not objectify the presence of any type of hemorrhage. Cerebral computed tomography revealed hypodensity of the cerebral hemispheres due to hypoxic or metabolic cause. The baby was discharged at 45 days of life with severe neurological impairment, increased spasticity, hyperexcitability, alteration of the primitive reflexes (sucking reflex included). At home, under continuous treatment with anticonvulsants, he did not have patent seizures, but the neurological status did not improve, as he maintained cognitive and motor disorders, remaining in a vegetative status even at the age of 7 months, under physical therapy.

From a social point of view, the newborn belonged to an illegitimate family, with a 21-year-old mother and a 38-year-old father, who did not show understanding of the newborn's condition anyway and were unable to provide the necessary care. As a result, the maternal grandmother took over the responsibility of care. The difficulty also consisted in the impossibility of the family to accept and understand the origin and unfavorable prognosis of the disease of the only child in the family, apparently without justification, considering the seemingly normal pregnancy, without risks finalized with term delivery.

Case 5: newborn BA, severe complication of extreme prematurity, with unfavorable outcome

Female preterm neonate, BW = 530 g, GA = 25 weeks, born vaginally at a level II center and transferred immediately to a level III unit. Conventional mechanical ventilation and surfactant therapy was initiated for severe respiratory distress and considering the suspicion of infection, treatment also included triple antibiotic therapy. After an apparent favorable cardio-pulmonary evolution, on the 15th day of life the neonate developed grade III intraventricular hemorrhage, with subsequent progression towards hydrocephalus and ventricular leukomalacia. Despite the surgical treatment in a specialized neurosurgery center, the ventricular-peritoneal shunt required repeated de-obstruction procedures and the hydrocephalus progressed. Secondary, stage II retinopathy of prematurity was also associated, and could not be surgically treated due to the risk of anesthesia. At 10 months of age, with severe dystrophy, severe anemia, impressive hydrocephalus and encephalopathy, after repeated and prolonged hospitalizations, the girl remained comatose in the pediatric intensive care unit, with an extremely severe prognosis. However, she has constantly benefited from emotional support and care by the devoted mother, of average social condition.

Case 6: newborn MM, prematurity with severe complications and particular social implications

Female preterm newborn, GA = 24 weeks (borderline prematurity), BW = 650 g, delivered by C-section, to a 30-year-old mother, with 2 previous abortions and advanced Hodgkin's disease. The parents decided to maintain the current pregnancy at any cost, although they were informed about the risks of decompensation of the mother's disease during the pregnancy, but also about the high probability of premature birth caused by the maternal condition. They were also informed in detail about the neonatal complications of prematurity. Expectant parents have expressed their firm choice to do everything possible to have a child, regardless of the consequences. Following delivery, the newborn was promptly resuscitated and admitted to NICU. The Apgar score was 4 at 1 minute, 5 at 5 minutes and 6 at 10 minutes. The infant developed respiratory distress due to surfactant deficiency, grade III intraventricular hemorrhage complicated with hydrocephalus, patent ductus arteriosus, necrotizing enterocolitis, requiring 40 days of intensive care. She was transferred to the neurosurgery department, for the ventricular-peritoneal shunt. The infant required repeated surgeries, with subsequent decompensations. Meanwhile, there was a deterioration of the maternal disease and she died within the first week after birth. This had huge familial implications that included the father being forced to move to rural areas, far from the hospital. After 2 and a half years after birth, the girl was still in coma, artificially kept alive.

Conclusions

The cases presented may be subjected to multiple comments and observations. Each of them has its own particularity, and this emphasizes the important moral, ethical, and bioethical challenges that the team of neonatologists had to face, aspects that are sometimes difficult to perceive, unexpected or even unknown. From this type of practice derives the need to pay more attention and consistent psychological and moral support required for both the family of the severely affected newborn, and sometimes for the participants in the medical act, witnesses to such drama that undoubtedly leave their mark in time on the human, mental, moral and professional attitude of any individual. The lack of legal normative to help regulate attitudes, to standardize medical practices, leaves room for interpretations and especially errors. There is a clear need for a more sustained concern in the specific bioethical field of neonatology, and this dissertation aims to enhance discussions and collaboration in the medical world on ethical aspects and the particular aspects in the neonatal period.

SECTION II. FUTURE PROJECTS IN THE PROFESSIONAL, ACADEMIC AND SCIENTIFIC FIELD

II.1. PERSPECTIVES IN THE PROFESSIONAL ACTIVITY

A university clinician needs professional performance for at least two reasons. First, for balancing his/her personality, originally designated toward medical profession. Secondly, the clinical maturity constantly demonstrated is the credible basis of dialog with the student or the resident doctor. Therefore, the next years represent a bolstered effort toward the target of acquisition and enlargement of clinical skills and personal medical performance.

Because of the continuous advances in neonatology field and need for progress in our daily practice, I am convinced that for a better outcome of our little patients there is an increased need for acquiring novel strategies of diagnostic and treatment. The recent pandemic context has withdrawn the access to on site clinical exchange of experience between different national or international neonatal intensive care units and neonatal specialists, which I consider it is one of the most useful learning tools for new achievements and specialization. Although online technology allowed us to access conferences and seminars lead by renowned opinion leaders in different specific and up-to-date area of interest, practical and hand-on exercise is the most powerful and useful in gaining the ability to perform procedures without experiencing on critically ill patients. There is still a high need for improvement of my abilities in areas like:

- ✓ Mechanical ventilation and new techniques for avoiding barotrauma and volutrauma in neonates
- ✓ Implementing modern available strategies for monitoring brain activity at the bedside – near infrared regional spectroscopy (NIRS)
- ✓ New better strategies for lung recruitment from the first minutes of life
- ✓ Neonatal pulmonary ultrasound: acquiring the best possible images of the lung, grading lesions and best interpretation of challenging images for a more accurate differential diagnostic and prompt intervention
- ✓ Interpreting the amplitude integrated EEG in term and preterm neonates for detecting abnormal brain activity

All these goals can be reached by acceding available programs that will restart after the epidemiological context clears-up.

- ✓ Completing PoCUS (point-of-care ultrasound) formation: PoCUS has been described as the "third hand of emergency physicians and surgeons." Significant efforts have been made to improve the speed and accuracy of diagnostic testing, particularly by using point-of-care testing, to minimize the delay between onset of symptoms and initiation of definitive therapy. PoCUS answers specific clinical questions that narrow differentials, guide clinical therapy, and direct consultations and disposition. Applications for PoCUS in the NICU include the evaluation and serial monitoring of common pulmonary diseases, hemodynamic instability, patent ductus arteriosus (PDA), persistent pulmonary hypertension of the newborn (PPHN), necrotizing enterocolitis (NEC), and intraventricular hemorrhage (IVH), among others. Also some procedural applications refer to vascular access, endotracheal intubation, lumbar puncture, and fluid drainage (485–489).

Since deafness continues to be an important pathology for the high-risk neonates from NICU, I am considering maintaining and further developing the collaboration with colleagues from the ENT and Audiology services, as this is extremely important for the successful management of these cases. We have been successfully having a traditional and collaborative relationship with audiology specialists for over 12 years now. Based on this, the authorities

understood the importance of the approach and supported the implementation of universal neonatal hearing screening throughout Romania in 2019, grace of the deep implication and high dedication efforts made by the neonatology-audiology team. Over 50% of children who are diagnosed with hearing loss in preschool and school screening are coming from the category who passed the screening tests after birth. Only acoustic emissions may not detect all types of deafness types. As a future project, I aim to continue the development of the auditory screening program by completing the protocol of universal maternity screening tests with auditory evoked potentials in order to avoid undetected auditory neuropathies, which can pass the acoustic emission test. Another advantage is represented by the immediate information on the probability of a diagnosis of deafness.

Currently, the national registry of the RENSA deafness conducted at the same time as the national screening is not yet operational. One of the future projects is the mobilization of resources and join efforts with ENT and audiology specialists, to activate this very useful registry in terms of managing this pathology on a national scale.

II.2. PERSPECTIVES IN THE ACADEMIC ACTIVITY

Transmitting knowledge to students and residents is one of my personal talents, trained for so many years. It became for me a goal for daily activities. Driven by the passion to understand physiological and pathological processes, and the desire to implement in medical practice the novelty in diagnosis and intervention in pathology from our specialty, I am stating that in my short-term and long-term future projects, these desiderata will always be present.

The publication of revised textbooks for students of the Faculty of Medicine in Romanian, English and French, series of lectures that I am currently teaching, is a project for the near future.

For the not very distant future, I am considering the development of online medical education resources on the university's e-learning platform, with the inclusion of various educational materials, lectures, and virtual clinical cases. I am adamant that learning from clinical cases remains a powerful tool and for this reason I included several clinical examples in the present habilitation thesis. Regular updating of these resources, as well as the materials used is mandatory for maintaining a high-quality level of teaching, respected and sought after by students and residents.

Encouraging students and residents to participate in clinical trials and to present research results or clinical cases in specialized scientific events will continue to be a priority for me. Also, I will assist and support students who address me for the completion of their bachelor theses.

The recent adapted curricula for residency in Neonatology has recommended novel references, translated into Romanian after well renowned international manuals. In my opinion, a corollary of clinical cases, based on our experience from the largest NICU in Romania, would be of real and practical benefit not only for the young doctor, but also for other specialists from our country, as it is well known that “there are no illnesses, but patients” and learning from our peers experience is more beneficial than having to learn from our own errors. My project is to include clinical presentations, sorted by pathology, with photos and graphs of modern ventilatory strategies adapted to different situations and clinical context, integrated with novel therapeutic methods. Also, this approach would be useful in providing a model for training in clinical oral presentation required for passing the specialty and consultant exam. A selection of multiple-choice questions should be added after each chapter. Additionally, rare cases and challenging situations should be presented, to raise awareness that neonatologists may be confronted with high-risk emergent situations, and a continuous training is required in order to be able to face them properly.

As a lead instructor in the S.T.A.B.L.E. Program, I will continue to spread the knowledge toward residents, physicians, nurses, and connected specialities, as resuscitation and neonatal stabilization should be one of the primary goals for all professionals in the field. The program is updated every four years and new protocols are developed and disseminated worldwide.

In addition, I strongly encourage direct involvement (with supervision) of residents in medical care provided to patients as a quick acquiring path of the skills necessary to every specialist. Gradually, depending on the demonstrated capabilities of the resident, he/she should be enabled to make medical decisions and be awarded tutorial competencies over colleagues from smaller training years.

High quality training for residents can be achieved by optimizing the lectures:

- ❖ Presentation of critical national and european guidelines
- ❖ Lectures with previously announced topics, preferably requested by residents, followed by examples of real cases and imaging
- ❖ All lectures should include a final evaluation form, as feedback to the lecturer.
- ❖ Teaching interaction ensures flexibility to the considered priority theme and gives attractiveness and efficiency.

Last but not least, there is the issue of educating residents and PhD students in the field of scientific documentation and medical writing. Lectures and practical examples on practical topics can be implemented, as in the following examples:

- ✓ Type of medical articles: examples, discussion.
- ✓ Online documentation:
  - Access of SpringerLink, BlackwellSynergy, OxfordJournals, Ovid.
  - International databases (ISI Web of Knowledge, PubMed, Scopus, IEEExplore).
- ✓ Notions of scientific publications (ISI Web of Knowledge, impact factor, h index, PageRank, Publish or Yalda, cited half-life).
- ✓ Presentation and discussion about Evidence Based Medicine (EBM) and the Cochrane Library. Utilities, access ways, limits, etc.
- ✓ Student-lecturer interaction using on-line tools, with individual feedback to each student.

Students access the databases and choose 3 significant items according to the topic of interest and would be required to analyze the articles according to peer-reviewing criteria.

The development of international relations has been one of my most beneficial professional achievements in all my work. I am convinced that this could represent an engine of progress for any academic and scientific community, a source of inspiration for modernizing the means and methods of work in health care and in education and in scientific activity. Thus, I plan to continue and develop the interuniversity relations established with colleagues and universities from other countries. These new contacts are necessary for overcoming the existing gaps in our local practice. Experienced specialists from prestigious universities around the world can bring the impulse that we need.

II.3. FUTURE PROJECTS IN THE SCIENTIFIC ACTIVITY

The scientific projects that I have approached and carried out so far have already been presented above. The realization of successful research is an invitation to continue on other levels. On the other hand, pending projects which have the potential for scientific and clinical exploitation can be the focus of future development, along with my colleagues teaching staff working in our services or those who wish to pursue doctoral studies.

In the presentation of the future plans for my research activity, I will follow the main topics that I have presented in the habilitation thesis and that I aim to develop, of course together with other topics that will concern me in the future.

II.3.1. Research on impact of prolonged premature rupture of membranes on neonatal sepsis

Severe infections during the neonatal period are responsible for over 1 million newborn deaths every year, worldwide (111,490,491). Neonatal sepsis (NS) often represents a diagnosis and treatment challenge for the neonatologists, due to its variable and non-specific presentation, rapid progression to multiorgan failure and severe consequences in the survivors, like post infectious encephalopathy, seizures, ventriculomegaly, hydrocephalus, encephalomalacia, brain infarction, neurodevelopmental delay and sensorial deficits.

Despite the abundance of data already published regarding biomarker identification for EOS, there is no consensus yet concerning a diagnostic protocol, as many factors may affect values interpretation of each marker. Detection and currently available validity of an EOS clinical diagnosis is still unsatisfactory and emphasize the need for further improvement of clinical criteria for EOS using modern biomarkers.

In this setting, an important and useful research direction is to assess the value and accessibility of relatively new biochemical diagnostic tools, such as presepsin, endocan, or IL-6. Some neonatal centers from Romania already use presepsin and IL-6 as part as their sepsis workup panel, but there aren't any significant publications on the subject. Also, on the role of endocan determination in neonates with EOS, our intention is to continue on the path opened by the two already published works, by exploring the mother-infant dyad and the utility of endocan along with the above mentioned biomarkers in the diagnosis of sepsis in neonates of mothers with prolonged rupture of membranes.

II.3.2. Research on different factors that affects human milk composition and some drugs transmitted through the milk

Antibiotics are probably among the most prescribed drugs during lactation. Mastitis is one of the most frequent problems that occur during lactation, and the prevalence of mastitis treated with antibiotics is estimated between 16 and 49%. Except for quinolones, chloramphenicol, aminoglycosides, metronidazole, most antibiotics are considered to be safe during breastfeeding. Subtherapeutic transmission of antibiotics via breast milk to the newborn could possibly contribute to antibiotic resistance later on, that is a major concern and challenge in practice. However, no data establishing this relation could be found. Furthermore, recent research shows that the neonatal microbiome is affected by intrapartum antibiotics and maternal use of antibiotics in the lactation period. In turn, the microbiome may affect brain development, behavior and obesity. Further research concerning drug concentration in mother`s milk and their effect in neonates are needed.

II.3.3. Research on congenital malformation secondary to isotretionin mother treatment for acne

The teratogenic potential is an important characteristic of isotretinoin. The first cases of congenital anomalies after isotretinoin use during pregnancy were documented as early as 1983. It is known to cause birth defects with a characteristic pattern of craniofacial, cardiac, thymic and central nervous system malformations among babies of women exposed to isotretinoin in early pregnancy. We are currently under collaboration with a team of dermatologists from Slupsk, Poland in a clinical research study. The objective of our study is to evaluate the effects of isotretinoin taken by women of reproductive age on subsequent births and health of born children. The results are in the final stage of statistical analysis and will be published in the future.

II.3.4. Research on intranasal use of human milk in preventing severe intraventricular hemorrhage in very preterm newborns

Neurotrophins are molecules that have the capability of promoting growth and survival of neural cells. They include brain-derived neurotrophic factor, glial derived neurotrophic factor, nerve growth factor, insulin-like growth factor-1, and hepatic growth factor. It turns out that not only are these found in high concentrations in breast milk, but that a woman who produces breast milk at early gestational ages has higher amounts of these compounds in her milk. Stem cells are pluripotent cells, meaning that they can develop into pretty much any cell type that they need to in the body. These are also present in mother's milk and in fact can represent as much as 30% of the population of cells in breast milk. Recently Keller (2019) opinionated in a small clinical study that early intranasal application of breast milk could have a beneficial effect on neurodevelopment in preterm infants (492).

II.3.5. Research on congenital deafness

Congenital hearing loss is the most common disease present at birth (1-3/1000 newborns). This problem is usually hidden at birth or in early childhood, being an invisible disability. Consequently, the timely diagnosis cannot be made by actively detecting hearing impairment. The universal newborn hearing screening is the only method we can identify infants with possible hearing problems. Due to the neonatal hearing screening, the access to early diagnosis of deafness can be ensured. Hearing loss may be favored by the presence of risk factors. Thus, it is known that deafness can occur both in children with and without risk factors present, neonatal hearing testing of children with risk factors for deafness is insufficient, and can exclude from diagnosis a significant number of children, representing, according to some statistics even over 50% of the newborn population (493).

In the direction of diagnosis strategies in the pathology of the auditory system, I will continue to research the risk factors for deafness present in Romanian maternity hospitals, sensitizing the medical staff in neonatology. The final goal is to reduce the prevalence of neonatal and perinatal deafness by implementing strategies of early detection.

In summary, my future research activity will focus mainly on the continuation of the study directions in which I have gained experience over the past years, as well as on the initiation of new study and research directions in the field of Neonatology. The ideas expressed above will give rise to research programs funded according to the priorities and opportunities that I must identify.

"The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires." (William Arthur Ward)

Section III. REFERENCES

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