

Selected Lectures of the 13th International Workshop on Neonatology

THE POWER OF EPIGENETICS TWINS: IDENTICAL BUT DIFFERENT

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LECT 1

ASPHYXIA IN THE THIRD MILLENNIUM: FROM VIRGINIA APGAR TO METABOLOMICS

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Virginia Apgar was the first woman to become a full professor at Columbia University College of Physicians and Surgeons; she designed the first standardized method for evaluating the newborn's transition to life outside the womb – the Apgar Score. This was the first standardized method for evaluating the newborn's transition from intra- to extrauterine life [1]. "Five points – heart rate, respiratory effort, muscle tone, reflex response, and color – are observed and given 0, 1, or 2 points". Apgar first planned the score to be taken one minute after birth, as a guide to the need for resuscitation. Eventually, the one- and five-minute Apgar Score became standard. Together with Holaday and James, Virginia Apgar was able to demonstrate that babies with hypoxemia and acidosis had low Apgar Scores.

APGAR SCORE AND OUTCOME

The National Collaborative Project, including 17,221 babies born between 1959 and 1966, established that the Apgar Score, especially the five-minute score, can predict neonatal survival and neurological development. Infants with a normal birth weight and five-minute Apgar scores of 0-3 had a relative risk of infant death of 16 and infants with scores of 4-6 a relative risk of death of 5 compared to infants with normal scores. Relative risks for cerebral palsy were 24 and 5 for children with five-minute Apgar score of 0-3 and 4-6 [2].

In a population based cohort study from Norway by Moster et al. including 233,165 children born between 1983 and 1987 with a birth weight of at least 2,500 grams and no registered birth defects, the association between death or cerebral palsy was related to Apgar scores [3]. At five minutes of age Apgar scores of 0-3 were recorded for 0.1%

and scores of 4-6 for 0.6% of children. Compared with children who had five-minute Apgar scores of 7-10, children who had scores of 0-3 had a 386-fold increased risk for neonatal death (95% CI 270-552), infant death 76-fold (56-103), and death between 1 and 8 years 18-fold (8.5-39). For five-minute Apgar scores between 4-6 the numbers were 45 (30-68), 8.9 (6.4-12) and 2.2 (0.9-5.4), respectively. Regarding cerebral palsy, a five-minute Apgar score of 0-3 increased the risk 81-fold (48-138) and Apgar score 4-6 31-fold (22-44). If Apgar scores at both 1 and 5 minutes were 0-3, the risks of neonatal death and cerebral palsy were increased 642-fold (442-934) and 145-fold (85-248), respectively [3]. Five-minute Apgar scores of 0-3 were 7 times higher and scores of 4-6 were 4 times higher in the Norwegian cohort than in the National Collaborative Study. These results indicate that Apgar score has some predictive value, however the variation from one population to the other is large. There has therefore been a search for other biomarkers of birth asphyxia, which can supplement acid-base status.

BIOCHEMICAL "APGAR SCORES"

In 1975 I published the first study showing that hypoxanthine is a biochemical indicator of birth asphyxia. Hypoxanthine is a breakdown metabolite from ATP and its plasma values therefore reflects the intracellular energy status [4]. Hypoxanthine is a key metabolite in perinatal hypoxia. It reflects the cellular energy status and in addition it is a potential oxygen free radical generator when it is further oxidized to xanthine and uric acid in the presence of xanthine oxidase [5]. This was the theoretical basis for our studies more than 25 years ago in hypoxic newborn piglets testing out the effect of resuscitation with air instead of 100% oxygen. These were followed by clinical studies. In summary it has been shown that resuscitation with air instead of 100% oxygen reduces inflammation in the lung, heart and brain, it reduces kidney and myocardial injury, reduces pulmonary vasoconstriction, neuronal apoptosis and death and reduces mortality [6-10]. A newborn piglet study by Fanos et al. compared resuscitation with 18%, 21%, 40% and 100% oxygen. The urine metabolic profiles indicated that resuscitation with air is the most appropriate concentration to be used for resuscitation [11].

METABOLOMICS

A metabolomic study applying the same piglet model showed that reoxygenation with air or 100% oxygen reduced lactate levels to the same extent. In contrast, metabolites of the Krebs cycle including alpha-ketoglutarate, succinate and fumarate showed

a delay in recovery in the 100% reoxygenation group [12]. Since these metabolites reflect mitochondrial function it seems that resuscitation with 100% oxygen delays cellular recovery. In subsequent studies in newborn mice we showed that 100% oxygen downregulates genes related to oxidative phosphorylation, which presumably leads to a reduced ATP production when resuscitation is carried out with pure oxygen instead of air [13]. This might explain at least partly the increased mortality in these patients. Applying the piglet model in a metabolomics study we identified 46 differentiating variables out of a total of 452 detected features. We identified these variables of importance as biomarkers of neonatal hypoxia: choline, 6,8 hydroxypurine, and hypoxanthine [14]. We therefore constructed a plasma metabolite score consisting of these three metabolites and compared to lactate [15]. For plasma samples withdrawn immediately after the hypoxic insult, the metabolic score performed similar to lactate. However, at 2 hours after resuscitation the score provided and enhanced predictive capability. Using the same model we identified CDP-choline as a marker of retinal hypoxia [16].

CONCLUSIONS

In spite of weaknesses the Apgar score is still widely used and useful more than 60 years after its introduction. However, new biochemical tools have been established which probably will be able to add important information regarding long-term outcome of infants experiencing intrapartum related events such as birth asphyxia. Based on metabolomics studies new biomarkers will be routinely available within the next years.

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LECT 2

INTRAPARTUM FETAL MONITORING: WHERE ARE WE NOW?

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Intrapartum hypoxia still accounts for a considerable number of perinatal deaths and long-term neurologic sequelae. Continuous cardiotocography (CTG) was developed in the 1960s, and in high-resource countries it rapidly gained a central role in intrapartum fetal monitoring and decision-making aimed at avoiding hypoxia. However, the technique

is troubled by poor interobserver agreement in tracing analysis, limited specificity in identification of fetal hypoxia, and an unclear demonstration of benefit. A large number of randomised controlled trials (RCTs) were carried out in the 1970s and 1980s, comparing continuous CTG monitoring with intermittent auscultation. Although these are widely cited, it is difficult to establish how they relate to current clinical practice, as the understanding of the physiology of fetal oxygenation and the relevance of newborn outcome measures, as well as CTG monitors and CTG interpretation, have evolved considerably since then. With these limitations in mind, they generally indicate that continuous CTG reduces neonatal seizures, increases intervention rates, and does not affect perinatal mortality and cerebral palsy rates. However, it is widely recognised that the trials were underpowered to detect differences in the latter two outcomes. There is a growing position within the scientific community that the evidence from these RCTs is weak and should be considered scientifically inconclusive. Poor interobserver agreement in CTG interpretation can be improved by the development of more consistent guidelines, such as those recently produced by the International Federation of Gynecology and Obstetrics (FIGO). It can also be overcome by computer analysis of CTGs, incorporating real-time alerts for healthcare professionals when characteristics associated with fetal hypoxia are detected. Three such systems have been developed and recently commercialized, and two RCTs were recently published, but their results were disappointing. Further development of the analysis algorithms is probably still warranted. CTG has been shown to have a high sensitivity and a limited specificity in predicting fetal hypoxia. To reduce false-positive rates, adjunctive technologies were introduced, such as fetal blood sampling and fetal ST waveform analysis. The latter has provided the most promising results in RCTs comparing it to CTG alone. The technique is widely available in Europe, but this has not occurred elsewhere. Despite the limited evidence from RCTs demonstrating that any form of intrapartum fetal monitoring improves clinical outcomes, several centres have reported important reductions in metabolic acidosis, hypoxic-ischemic encephalopathy, and intrapartum deaths over the last two decades. It may well be that RCTs are not the best way of evaluating the benefit of CTG, a diagnostic technique used in a complex environment where adequate clinical management plays a major role in avoiding adverse outcome and unnecessary intervention. The reduction in

intrapartum hypoxia rates reported in several European centres warrants a more detailed analysis, particularly regarding the importance of regular training in the use of available techniques and appropriate clinical management.

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LECT 3

THERAPEUTIC HYPOTHERMIA: REVISED INDICATIONS

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Therapeutic hypothermia (TH) has become the standard of care for newborns suffering moderate to severe hypoxic-ischemic neonatal encephalopathy (HIE). Meta-analysis of the randomized controlled trials (RCTs) concerning cooling has shown that TH significantly improves neurodevelopmental outcome at 18-24 months. The number needed to treat (NNT) for the primary outcome (death or disability) and secondary outcome (the number surviving with normal function) was 9 and 8, respectively. Although there were some differences in the method of cooling, all trials used a narrowly defined patient group in order to provide the best chance of detecting a treatment effect and to avoid potential adverse effects. Numerous exclusion criteria were thus used such as mild HIE, preterm infants, infants unable to be cooled by 6 hours of age. The main trials did not include infants with mild encephalopathy. Unfortunately, the patients who did not meet the entry criteria in the large RCTs were not followed-up, so we were unable to analyze the outcome of those screened but not treated. Many observational studies of cooled infants have reported outcomes that are

more favorable than those presented in the RCTs. It is possible that infants currently recruited have milder encephalopathy than those in the original trials. As TH is now widely used and the lack of other proven neuroprotective interventions has created opportunities to broaden the selection criteria beyond those used in the trials [1], this article reviews some novel clinical situations where TH may be considered for neuroprotection. It also highlights the precautions for the use of hypothermia outside the inclusion criteria retained by the principal RCTs.

Although TH improves outcomes for moderate and severe neonatal encephalopathy, whether this therapeutic benefit applies also to infants with mild neonatal encephalopathy is unclear. However, recent studies have documented evidence of injury among these infants. The grade of neonatal encephalopathy during the first hours of life may not discriminate adequately between infants with and without cerebral injury noted on MRI after TH. Walsh et al. [2] have shown that there is no difference in the rate of overall MRI abnormalities by grade of neonatal encephalopathy. Twenty-six infants (54%) with mild neonatal encephalopathy, 19 infants (54%) with moderate neonatal encephalopathy, and 3 infants (50%) with severe neonatal encephalopathy had an abnormal early MRI. Among the children who had an MRI, 66% presented neurological abnormalities. The Children's Hospital Neonatal Database reported that 59% of infants with mild neonatal encephalopathy from their network had an abnormality on MRI [3]. Additionally, in a recent cohort study, Gagne-Loranger et al. reported that, among 13 infants with mild neonatal encephalopathy who underwent TH, 31% (4/13) had MRI changes consistent with hypoxic-ischemic injury [4]. In addition to short-term neurologic outcomes, recent data from infants with mild neonatal encephalopathy have shown that 20% of infants with mild neonatal encephalopathy had an abnormal short-term outcome, including presence of seizures, abnormal neurologic examination at discharge, and feeding difficulties beyond the first week of life [5]. In addition to short-term neurologic outcomes, recent data from infants with mild neonatal encephalopathy have documented long-term neurodevelopmental morbidities in early childhood and at school age [6]. Infants with mild neonatal encephalopathy deserve greater focus and future investigation, with particular emphasis on the potential benefits of neuroprotective strategies. Important issues in considering application of TH

in mild HIE must be raised: RCT of TH in mild HIE would require a large population; effect size will differ; challenges in clinical diagnosis; the study may require longer follow-up for detection of any benefit. Risk versus benefit of therapy must be considered: separation of infant from mother; only a minority of these infants require ventilation and/or UVC; more than 20% of infant with mild HIE received some blood product.

The main RCTs have provided strong evidence for the use of TH for infants ≥ 36 weeks of gestation with HIE. An important clinical issue is whether TH should be provided for infants who are less than 36 weeks of gestation. An important aspect of extending TH to preterm infants will be safety. TH is associated with a broad spectrum of effects that may be more prominent when applied to preterm compared with term infants. In a recent study, Rao et al. reported a single center experience of TH in 31 preterm infants born at 34–35 weeks of gestation and 32 term infants treated [7]. Rewarming was initiated before completion of active cooling in 19% of the preterm infants and none of the term infants. Differences and trends were reported for variables assessed for safety: hyperglycemia and leukopenia were statistically higher among preterm compared with term infants. Coagulopathy and hypoglycemia tended to occur more commonly among preterm compared with term infants. MRI findings indicated a trend for more frequent and more severe brain injury among preterm compared with term infants. The findings of more deaths and potentially adverse events and trends for more brain injury detected by MRI among preterm infants are concerning. Extending TH to premature infants should only be performed in a research setting. A clinical trial is underway (ClinicalTrials.gov: NCT01793129). Pending the results of this trial, it is advisable to not extend the indications of hypothermia in infants of less than 35 weeks of gestation.

The recommendation to start cooling within 6 h of age before the secondary deterioration phase was based mainly on experimental trials. Experimental and human data strongly support the fact that TH is neuroprotective in term infants if initiated within the first 6 hours after birth. Such a short window does not allow clinicians to apply this treatment to all neonates who might benefit from it. In the cooling trials, TH was initiated at a median age of approximately 4 hours with little variability among the trials but with a trend favoring earlier cooling [8]. It remains unclear why there is a relatively prolonged period between delivery and

time to initiation of TH. Data from the French Register indicated that the late initiation of TH was the result of a long delay between admission and treatment time (nearly 8 hours). This delay was most likely secondary to the clinical and/or electrophysiological assessment of the newborn before starting TH [9]. Broader utilization of aEEG, which is simple to implement and interpret, should make it easier to obtain objective and earlier assessments of the neurologic prognosis and to select infants that could benefit from TH. Since many infants are outborn, training and education should focus on early identification of neonates at high risk for evolving brain injury and prompt cooling. There is no RCT with sufficient number of infants looking at a late cooling start to suggest whether late cooling is effective and safe or not. There is no evidence that TH is unsafe, at least within 9 hours after birth. After this delay, TH seems to be ineffective. Starting cooling late at 12 h or more increased neuronal injuries in the animal model. An RCT is currently assessing late-onset TH in infants with postnatal age between 6 and 24 hours (ClinicalTrials.gov: NCT00614744). An alternative strategy would be to extend the window of intervention of hypothermia. Strategies for extending the window of opportunity are being tested in experimental models. Pending the results of these studies, the recommendation therefore is still the same: start cooling within 6 h, or earlier if possible. Longer or deeper cooling to < 33.5°C and/or for > 72 hours has not been shown to be of benefit, and is harmful. A recent report from Shankaran et al. demonstrates that among term neonates with moderate or severe HIE, cooling for longer than 72 hours, cooling to lower than 33.5°C, or both did not reduce death or moderate or severe disability at 18 months of age [10]. So far there is a lack of data about other possible indications of TH. Additional research of the efficacy and safety of TH in conditions outside HIE is warranted. Carefully designed safety studies and large RCTs for these novel indications should be considered. We strongly caution that if TH is to be applied outside of a trial, clinicians should follow published trials protocols, ensure systematic follow-up of survivors and submit patient data to national registries.

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LECT 4

ANTIBIOTICS PRESCRIPTION IN NEONATAL INTENSIVE CARE UNIT

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Antibiotic overuse and subsequently infections caused by antibiotic resistant pathogens are a

worldwide problem that, according to the 2014 WHO report, “threatens the achievements of modern medicine”. In NICUs, antibiotics are the most frequently prescribed medicines. The use of broad-spectrum antibiotics has been linked to the emergence of multidrug resistant gram negative bacilli as well as invasive candidiasis and death. In addition to that, prolonged duration of antibiotics in premature babies has been linked to increased rates of necrotising enterocolitis. In 2011, the CDC has initiated a campaign to prevent antimicrobial resistance in healthcare settings with emphasis on antimicrobial stewardship (AMS) interventions. AMS is recognized as a critical patient safety and quality imperative, which aims at optimizing clinical outcomes while minimising the emergence of antimicrobial resistance and preserving the activity of the existing agents. Several AMS strategies in NICUs have been proposed and include one or more of the following aspects.

1. Improvement in the diagnosis of neonatal sepsis. The signs and symptoms of sepsis in infants are non-specific and may mimic the presentations of a non-infectious process but it is difficult not to treat with antibiotics for suspected sepsis when these symptoms appear. In addition, culture-negative sepsis is a common reason for antibiotic prescribing in NICUs. The use of biomarkers such as CRP, procalcitonin, interleukins 6 and 8, and of sampling with adequate blood volume for culture (at least one ml recommended from the American Academy of Pediatrics) could aid the earlier and accurate diagnosis of neonatal sepsis as well as the judicious use of antimicrobials.
2. Appropriate antimicrobial empiric cover for suspected sepsis based on local epidemiology. The latter requires continuous surveillance at a local and national level as both pathogens and their antimicrobial resistance patterns change over time. In NICUs, the use of cephalosporins should be avoided as their use increases the risk of invasive neonatal candidiasis and death. As for vancomycin, the introduction of an AMS strategy to use this antimicrobial only after isolation of a pathogen (*Coagulase Negative Staphylococci* most of the times), rather than empirically, did not lead to an increase in the morbidity or mortality of neonates.
3. Re-evaluation of empiric antimicrobials when specific pathogen are isolated and antibiogram is available. De-escalation to narrow spectrum and less toxic antimicrobials is strongly recommended.

4. Adequate dose and monitoring of therapeutic levels of certain antimicrobials such as gentamicin and vancomycin.
5. Rational use of antimicrobials for surgical prophylaxis and shorter duration.
6. Monitoring the use of antimicrobials. Recording DOTs (Days of Therapy) has proven to be a very useful AMS in children and can enable comparison between NICUs as well as measurement of outcomes of other AMS interventions.
7. Development of AMS team (consisting of neonatologists, paediatric infectious diseases specialists, pharmacists and infection control nurses) with primary responsibility for the continuous monitoring of appropriateness of prescribing aims to reduce antimicrobial abuse and misuse.

In conclusion, it is of paramount importance to prescribe antimicrobials wisely in NICUs and efforts should be made in this direction. Knowing how and why antimicrobials are utilised in the NICU setting is an essential element of AMS and offers the opportunity for quality improvement.

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LECT 5

CHORIOAMNIONITIS: FROM CLASSIC DATA TO METABOLOMICS

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Chorioamnionitis is a leading cause of preterm birth worldwide [1], affecting up to 50-70% of live births at 24-27 weeks of gestation. The term chorioamnionitis is used to refer to an intrauterine infection/inflammation occurring between the maternal tissues and the fetal membranes (chorio-decidual space) or in the fetal annexes (chorio-amniotic membranes, amniotic fluid, umbilical cord). Histological, microbiological, biochemical and clinical criteria are used to diagnose chorioamnionitis. Histological examination of the placenta constitutes the gold standard for diagnosis, however the results are available only several days after birth. In addition to histological criteria, chorioamnionitis can also be diagnosed on the basis of clinical criteria (presence of clinical signs of inflammation in the mother), microbiological findings (positive amniotic fluid culture or polymerase chain reaction detection of pathogens), and biochemical criteria (elevated cytokine levels in amniotic fluid, elevated maternal serum C-reactive protein concentration, presence of fetal fibronectin in cervical and vaginal secretions, etc.). Histology is the most sensitive method for diagnosing chorioamnionitis, and clinical examination the least sensitive. The prevalence of histological chorioamnionitis (HCA) decreases with increasing gestational age, ranging from 66% at 24 weeks of gestation to 16% at the end of the 34th week of gestation.

In recent decades, numerous studies have attempted to establish whether, and to what extent, intrauterine infection/inflammation might negatively affect the short- and long-term outcome of preterm infants. Despite the discrepancy across studies, most of them have reported an association between chorioamnionitis and neonatal adverse outcomes, such as death, early- and late-onset sepsis, bronchopulmonary dysplasia, necrotizing enterocolitis, and cerebral palsy. Recently, some authors hypothesized that there may be a stepwise increase in neonatal morbidities and mortality according to the stage or grade of HCA. Certainly, an early, reliable diagnosis of chorioamnionitis, before or immediately after birth, could improve the individualized care of the neonates.

In the last few years, omics technologies such as transcriptomics, proteomics and metabolomics have been applied to the field of intrauterine infection/inflammation and chorioamnionitis, main-

ly on amniotic fluid [2]. Metabolomics has been used by some authors to identify metabolic changes associated with early spontaneous preterm birth and to better understand the pathophysiologic mechanism of chorioamnionitis. In 2010, Romero et al. [3] reported that the presence of intra-amniotic infection/inflammation was associated with an altered amniotic fluid metabolite composition. In 2015, Dudzik et al. [4] demonstrated that amniotic fluid metabolomics analysis could identify women with and without chorioamnionitis.

Considering the results of the aforementioned studies, it should be hypothesized that metabolomics analysis of urines collected early after birth will be able to discriminate between neonates born to mothers with and without HCA. We have previously elucidated the great potential of metabolomics analysis on neonatal urine as a non-invasive, simple and fast tool to understand metabolic pathways involved in different pathological processes such as sepsis, congenital cytomegalovirus infection or intrauterine growth restriction [5]. The possibility of diagnosing HCA shortly after birth with a non-invasive neonatal biomarker might greatly improve the management of both HCA-exposed and not-exposed neonates. Furthermore, this particular technique could be useful to better understand the altered metabolic pathways associated with chorioamnionitis.

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LECT 6

FUNGAL INFECTIONS

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Invasive fungal infections (IFIs) have a significant impact in neonatal intensive care units (NICUs). They are an important cause of morbidity and mortality in very preterm (< 32 weeks) and in very low birth weight (VLBW) infants. IFIs, mainly those due to *Candida spp.*, can result in death and in short- and long-term morbidity, including adverse neurodevelopmental outcomes. Given the high mortality and morbidity associated with IFIs, and the difficulty in confirming a diagnosis, antifungal chemoprophylaxis is frequently used in very preterm or VLBW infants. Two broad chemoprophylactic strategies are employed in current clinical practice: 1. systemically-absorbed antifungal drugs (nystatin or miconazole) that achieve fungicidal concentrations in tissue, blood, cerebrospinal fluid and urine; 2. systemic antifungal agents, most commonly fluconazole or amphotericin B. The finding of a reduction in risk of IFIs in VLBW infants treated with oral/topical non-absorbed antifungal prophylaxis should be interpreted cautiously because of methodological weaknesses of available trials [1]. A recent *Cochrane* review reported evidence from some good-quality trials that giving a systemic antifungal drug regularly for the first 4 to 6 weeks after birth reduces the number of infants who develop severe infection; there is not yet any convincing evidence that death or disability rates are affected [2]. To date, antifungal prophylaxis with fluconazole is the recommended approach for neonates < 1,000 g and/or 27 weeks' gestation or less, mainly in NICUs with relatively high frequency of invasive candidiasis. First-line treatment of IFIs includes amphotericin B deoxycholate, fluconazole, or echinocandins. Intravenous micafungin, an echinocandin, is approved in the European Union for the treatment of invasive candidiasis in neonates. It has a broad spectrum of *in vitro* activity against clinically relevant isolates of *Candida spp.* (including fluconazole-resistant *C. glabrata* isolates), a low propensity for emergence of resistant isolates and a convenient once-daily regimen [3]. Choice of appropriate antifungal drug needs careful assessment of patient characteristics, epidemiology and drug pharmacokinetics. Ideally, antifungal drugs should target fungal biofilms, prevent or effectively treat end-organ localizations, be active

against fluconazole-resistant *Candida spp.*, and have reliable safety and tolerability profiles. To date, data on pharmacokinetic, schedule treatment and appropriate dosage of antifungal agents in neonates, mainly in premature, are still limited. Future strategies to reduce neonatal morbidity and mortality derived from IFIs include new echinocandins not yet approved for neonatal use (caspofungin or anidulafungin) and other adjuvant treatments as intravenous immunoglobulin, lactoferrin or probiotics. Guidance for prophylaxis and more importantly targeted antifungal treatment need to be further evaluated by large, multicenter, randomized controlled trials.

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LECT 7

METABOLOMICS IN SEPSIS: THE POWER OF PREDICTION

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Neonatal sepsis is one of the most severe neonatal pathologies due to its extremely high mortality rate (about one million neonatal deaths per year worldwide; around 25% of all causes of neonatal death), negative outcomes in case of survival, and difficult diagnosis and treatment of the disease [1]. The cause of neonatal sepsis is an infection, generally a bacterial or fungal infection acquired in the perinatal and/or postnatal period. Sepsis triggers a generalised systemic inflammatory response, which can lead to progressive multiorgan failure and consequent death of the patient. Regarding the physiopathology of neonatal sepsis, the first pathologic event is the injury of the intestinal mucosa, followed by endotoxemia and release of pro-inflammatory cytokines in the bloodstream. Autoptic specimens revealed a cascade of events taking place during sepsis: apoptosis of endothelial

cells, with subsequent endothelial cell injury and loss of endothelial integrity; diffuse oedema; intravascular clots; possible rupture of blood vessels; inflammation of the intestinal mucosa associated to a higher and abnormal permeability of the mucosa, which represents a portal of entry for bacteria [2]. These events finally result in the onset of diffuse thrombi with ischaemic damage in all organs, subsequent multiorgan failure and death. In this context, the intestinal microbiota plays a key role. The microbiota usually plays a protective role against pathogenic bacteria. In the case of sepsis, or another acute insult, such for example as asphyxia, it seems that 90% of the anaerobic bacteria that make up the intestinal microbiota is lost after the first 6 hours from onset. There is therefore a loss of the microbiota, which mechanism is unknown to date [3]. In these conditions, i.e. in the absence of competitors, pathogenic bacteria can replicate and synthesise proteases that damage the mucosal barrier, and consequently give rise to the chain of events, which may then be fatal to the patient. It could be envisaged that studying the modifications of the microbiota during sepsis in real time could maybe increase survival probability of patients. This is one of the ambitions of metabolomics (**Tab. 1**), one of the -omics sciences, which allows to study the instantaneous modifications of metabolism of an individual, both in health and disease, through the analysis of metabolites in biologic fluids such as blood, saliva, faeces and urine [4]. With this technology, it is also possible to distinguish metabolites of human and bacterial origin. Metabolomics could therefore be defined as the “Rosetta Stone” of microbiomics, and metabolites could be viewed as the secret language with which brain and our second brain (i.e. the intestine, and therefore the microbiota) talk to each other. A recent study in healthy volunteers demonstrated the presence of over 60 metabolites, which are modulated by intestinal bacteria, some exclusively, some together with eukaryote cells [5]. A search we conducted on PubMed about 1 year ago retrieved 107 papers on “metabolomics + sepsis”, and 14 papers on “metabolomics + sepsis + newborn”. 30 papers were well focused on the argument (24 in humans, 5 in rats, 1 in drosophila). The clinical studies involved 1,714 patients (with sepsis, SIRS), and 471 controls. The biofluids examined were serum in 12 studies; plasma in 12 studies; blood in 3 studies; urine in 3 studies; BALF in 1 study. The analysis was performed with Liquid Chromatography in 12 studies; Gas Chromatography-

Table 1 (LECT 7). Metabolomics ambitions in the diagnosis of sepsis.

- Diagnosis of the etiology
- Diagnosis of the severity
- Identification of therapy responders
- Identification of patients at risk of progression
- Identification of organ toxicity
- Advancing knowledge and influencing the “organ” microbiota

TABLE 2 (LECT 7). Main pathways involved in neonatal sepsis, according to metabolomics studies.

- Inflammation, hypoxia, oxydative stress
- Energy problem: reduced level of ATP and compensatory reaction to a major oxidation of fatty acids
- Increase in glucose turnover through glycolysis
- Redistribution of glucose consumption from the mitochondrial oxidative phosphorylation to other metabolic pathways, such as the production of lactate and the pentose phosphate pathway

Mass Spectrometry in 11 studies; Nuclear Magnetic Resonance in 11 studies. A few months after our search, studies have further significantly increased in number. Main pathways involved in neonatal sepsis, according to metabolomics studies, are presented in **Tab. 2**. In neonatology, the metabolome of both term and preterm newborns is already altered from 48 to 72 hours before the onset of signs and symptoms of sepsis (“not looking well, not feeding well, not breathing well”) [5-8]. On the basis of just about ten studies published in literature, the predictive power of metabolomics seems already extraordinary. Big data make it possible to predict the mortality of patients in the short or medium term with the current protocols. They also allow assigning some patients to more aggressive treatment, and others to less aggressive treatment, based on their fragility/resilience [9-10]. In the case of sepsis, and particularly neonatal sepsis, where the timing of treatment is even more crucial than in other age groups, it could be possible to achieve an earlier diagnosis and an individualised therapy with a reduction in mortality rate thanks to tailored medicine [11].

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LECT 8

PROBIOTICS IN OBSTETRICS AND GYNAECOLOGY

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INTRODUCTION

"Microbiota" is a term that was first introduced 10 years ago, substituting the term bacterial flora. It indicates the community of microorganisms (bacteria, protozoa, fungi and viruses) with which we share our everyday environment [1, 2] and without which humans wouldn't exist in the form

we recognise today. "Microbiota" has been defined as the human being's "sixth sense" [1, 2]. Human microbiota consists of 10 to 100 trillion symbiotic cells. One hundred thousand million of bacteria and billions of viruses live in or on each human body. For every cell in the human body there are 10 bacteria; for every bacterium there are 10 viruses and most of them are still unknown to medical scientific community. The "microbiome" represents the genes of our microbial symbionts (microbiota) and allows our body to enhance and multiply our genetic potential, controlling the relationship between genetic factors and environmental factors such as life style, nutrition and drug consumption [2, 3]. Every area of our body contains a specific microbiome, each one with the common aim of reaching "eubiosis", i.e. the balance between different species [4]. "Dysbiosis" is the breakdown of that balance, which can lead to a pathobiosis process involving one of more organs, manifesting itself with a variety of symptoms [5]. The most recent discoveries in molecular biology have introduced the new concept of "omics" science, including transcriptomics, proteomics and metabolomics. The implication of these concepts in medical practice may mean the ability to provide a preventive diagnosis, rather than just an early diagnosis. Identifying the conditions that could potentially impair eubiosis, thereby preventing with minimal medical intervention the onset of the pathobiosis process [6]. The interface between human tissue cells and the external environment consists of a barrier colonized by microorganisms which form the microbiota, dynamically exchanging information between our DNA and microorganism genes [7]. Vaginal microbiota, the first microbiota to be studied and known, is dominated by *Lactobacillus spp.*, including *L. crispatus*, *L. gasseri*, *L. jenseni* and *L. iners* [8, 9]. The predominance of *L. crispatus* indicates the ideal vaginal eubiosis status, expressing a perfect symbiosis between vaginal tissue and microbiota. Any factor causing an alteration of this equilibrium causes overgrowth of *L. crispatus* and activates the cytotoxic process in order to restore the eubiosis status. The presence of an imbalanced microbiota and the onset of pathobiosis are characterized by the substitution of *L. crispatus* with *L. gasseri* and *L. jenseni* and the prevalence of *Candida spp.* with alteration of pH. A switching of the process from spore to active form of *Candida spp.* represents a protection against the further penetration of the microorganisms through the cervical canal [10]. Further protection of the normal vaginal microbiota

is the dominance of *L. iner*, indicating the evolution towards pathobiosis. *L. iner* is able to survive in abnormal pH and to aggregate anaerobic bacteria resistant to metronidazole. The alteration of this last defense leads to a breakdown of the eubiosis and the prevalence of bacteria or viruses [11]. Bacterial vaginosis shows the gradual reduction of *Lactobacillus spp.* and at the same time an increased prevalence of anaerobic species. This is one of the most frequent gynaecological disorders among fertile age women, with an incidence of 29% among women between 14 and 49 years old [12, 13]. There is a widely understood correlation between bacterial vaginosis and sexually transmitted diseases such as HIV, HPV and HSV [14, 15] and this association can be explained by the *Lactobacillaceae spp.* imbalance. Dysbiosis condition can cause a predisposition to increased levels of bacteria or viruses, with uncontrolled expression of genetic information (DNA or RNA) [16] and atypical growth leading to preneoplastic or neoplastic lesions [17-25]. During pregnancy, placenta, embryo and fetus have their own microbiota, transmitted from the mother [18-20], hence a severe dysbiosis during pregnancy, either of oral, intestinal or vaginal origin, might cause a pathological process with increased risk of preterm birth [21-23]. The current pharmacological treatments, including bacteriostatic and bactericidal antibiotics, are not always effective in preventing a recurrent pathogenic event and represent the main cause of bacterial antibiotic resistance. WHO therefore recommends their limited use, also because they are not able to reconstitute the perfect eubiotic balance, and may provoke a predisposition to relapses or recurrence of diseases. Over the last few years new therapeutic strategies have been introduced, being used either as preventive tool, either alongside traditional agents or on their own in order to restore the eubiosis of the microbiota [10-17]. These include the use of probiotics, which can be generic, or targeted against a specific microorganism, aiming to induce a selective response.

MATERIALS AND METHODS

We conducted a prospective-observational study. 100 patients with vaginal dysbiosis were recruited and received a local and a systemic symbiotic agent. Patients were tested, prior to the treatment, with a bacterioscopic examination, on fresh preparations, and a vaginal swab looking at the presence of *Str. agalactiae*, *Mycoplasma spp.* (*M. hominis* and *M. urealyticum*) and fungi was taken. A PCR test for detection of *C. trachomatis* and Papilloma Virus

was also performed. The population of the study, with ages ranging from 17 to 67 years, received the treatment for an average time of 2.7 months (range 1-6). Patients were selected on the basis of the preliminary bacterioscopic examination; 58% of the cases demonstrated an alteration of the vaginal microbiota with mixed bacterial flora, 37% demonstrated bacterial vaginosis and 5% cytolysis from *Doderlein bacillus*. Within the group of patients studied, the percentage of co-infection was as follows: 25% *E. coli*, 6.2% *E. faecalis*, 4.1% *Str. agalactiae*, 22% *Mycoplasma spp.* (*M. hominis* and *U. urealyticum*), 6.2% *C. trachomatis*, and 21% showed positive PCR for HPV. Patients diagnosed with infection from *Mycoplasma spp.*, *Chlamydia spp.* and bacterial vaginosis received an antibiotic treatment according to the guidelines, plus a symbiotic agent. The remaining patients, affected from *E. coli*, *Enterococcus spp.* or *Str. agalactiae*, in absence of a positive urine culture, received the symbiotic agent only.

For 83% of the patients, a return to vaginal eubiosis, at the end of the protocol, was demonstrated by bacterioscopic examination (**Tab. 1**). A complete resolution was seen in patients affected with *C. trachomatis* and *Mycoplasma spp.* infection and in only 3% of patients with bacterial vaginosis, in which a mixed bacterial flora was demonstrated, with negative Amstel criteria.

CONCLUSIONS

The results of our study confirmed the increased prevalence of sexually transmitted diseases in patients diagnosed with bacterial vaginosis, which is consistent with an alteration of the vaginal microbiota, with reduction of *Lactobacilli* and increase of pathogenic microorganisms. The administration of targeted symbiotic agents, local or systemic, to patients with bacterial vaginosis leads to a reduction of the incidence of relapses and recurrence of disease. For patients with an *E. coli*, *Enterococcus spp.* or *Str. agalactiae* infection, there was therefore a significant reduction in the need of the use of antibiotics. With regards to the positivity of PCR for HPV, symbiotic agents, as demonstrated in a study not yet published [26], might reduce the timings for normalization of PCR in low-grade preneoplastic lesions. The role of vaginal mucosa as barrier against potential infections (barrier represented by the *Lactobacilli* responsible for the maintenance of an acid pH and the regulation of the interaction with potential pathogens) has already been widely investigated. New techniques are currently showing the role of

Table 1 (LECT 8). Study population and results.

Number of patients	100
Mean age	39.6 (range 17-67)
Bacterioscopic exam prior to treatment	
Mixed bacterial flora	58 (58%)
Bacterial vaginosis	37 (37%)
Cytolysis from <i>Doderlein bacillus</i>	5 (5%)
Mean treatment time	2.7 months (range 1-6)
Complete resolution on bacterioscopic exam	83 (83%)
Co-infections	
HPV	21 (21%)
<i>Mycoplasma spp.</i>	22 (22%)
<i>Chlamydia spp.</i>	6 (6%)
Common bacteria (<i>Enterococcus spp.</i>, <i>Streptococcus spp.</i>, <i>E. coli</i>)	18 (18%)

the mucosa as immunological mediator aiming at the maintenance of the vaginal microbiota. Further studies of molecular biology are needed regarding the indication of the probiotics in the clinical practice, with the aim of a personalised approach.

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LECT 9

LATE PRETERM INFANTS WITH RESPIRATORY DISTRESS SYNDROME: LIGHTS AND SHADOWS

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Late preterm infants (LPI: 34⁺⁰ to 36⁺⁶ weeks' gestation) are at increased risk for morbidities in the immediate newborn period including a higher rate of respiratory failure, and in particular of respiratory distress syndrome (RDS), transient tachypnea of the newborn (TTN) [1, 2]. Recently, we found that the occurrence of RDS in LPI is 6% [3], confirming previous findings by the Consortium on Safe Labor that reported an incidence of RDS of 5.2%, compared to 0.4% in infants born at 37-40 weeks'

gestation [2]. The pathogenesis of RDS in these patients is represented by the premature disruption of alveolar and surfactant system development. In fact, at birth LPIs are in the saccular phase of lung development when a process of septation matures saccules into alveoli with dramatic, weekly increments [4], along with the growth in length and diameter of new arteries and veins and with the expansion of the capillary network [5]. Obviously, during the saccular phase there is also the optimizing of surfactant system maturation characterized by the increase of surfactant synthesis but also by change in its composition, such as an increased content of phosphatidylglycerol that is more represented in the surfactant of term infants [4]. Moreover, the importance of surfactant proteins has been recently emphasized by Tsitoura et al., who demonstrated that some haplotypes of the surfactant protein A (SP-A1 6A, SP-A2 1A) independently increase the risk of RDS in LPI [6]. The exact molecular mechanisms underlying this association can be only speculated hypothesizing that these haplotypes might negatively affect the SP-A structure and function and predispose some LPIs to the development of RDS [6]. On the other hand, we have recently demonstrated in a large (n = 562) retrospective study that natural surfactant treatment significantly improves the respiratory function (i.e., FiO₂, PaO₂, and a/APO₂) in LPIs with RDS [3]. In our patients surfactant does not improve short-term outcomes, such as the duration of non-invasive respiratory support and of hospital stay, but this probably occurs because other factors such as the gestational age, occurrence of complications, and poor feeding play a relevant role [3]. On the other hand, McEvoy et al. [7] reported that healthy LPIs studied at term-corrected age have abnormal pulmonary function when compared with healthy term infants; thus, it would be interesting to evaluate if surfactant might beneficially affect pulmonary function in LPIs with RDS when compared with LPIs with surfactant untreated RDS. Another interesting pathogenetic factor of RDS in LPIs is that they are often born by cesarean section with a percentage ranging from 38% [2] to 81% [3] and do not benefit from the effect of uterine contractions in favoring lung fluid clearance and enhancing alveolar surfactant secretion secondarily to the increase of catecholamine and cortisol fetal increment [8]. This point raises the question of the opportunity of antenatal steroids treatment of LPIs for preventing respiratory failure when elective cesarean section is planned. A recent meta-analysis

by Berghella et al. concluded that antenatal steroids are effective in decreasing the respiratory morbidity in LPIs and that a “single course of corticosteroids can be considered for women at risk of imminent late premature delivery 34⁰-36⁶ weeks’ gestation” [2]. However, the question is still debated since ACOG recommends antenatal steroids for pregnant women between 34⁺0 weeks and 36⁺6 weeks of gestation at risk of preterm birth [10], while NICE suggests only of considering antenatal steroids in preterm deliveries at 34⁺0-35⁺6 weeks of gestation [11]. In summary, when we speak of RDS in LPIs, our knowledge on its pathogenesis and on surfactant effects in this population is increasing, but there is no standard approach to RDS treatment nor to its prevention with antenatal steroids.

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LECT 10

PRECISION VENTILATION IN NEWBORN

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INTRODUCTION

Medicine and in particular Neonatal Medicine is rapidly changing; in selecting the optimal therapy, the clinicians must consider not only the conclusions of RCTs and metanalysis but also the severity of the main disease, other concomitant or underlying diseases and, furthermore, the response of the patient to the initial therapy. We report our experience on precision High Frequency Oscillatory Ventilation (HFOV), during treatment of Respiratory Distress Syndrome (RDS) of preterm newborns.

MATERIALS AND METHODS

The Neonatal Intensive Care of MBBM Foundation-Monza and the Department of Bio-Engineering of Politecnico of Milan have been cooperating for more than a decade to improve the monitoring of HFOV; the aim is to optimize the ventilation considering not only the level of oxygenation, which represents the current standard of care, but also the hemodynamic effects of ventilation and lung mechanic properties, measured by Forced Oscillation Technology (FOT).

RESULTS AND CONCLUSION

In the first experimental study on rabbits [1], we demonstrated that Reactance (Xrs) can be accurately measured during HFOV without interrupting ventilation and/or connecting additional devices; Xrs might constitute a useful bedside tool for monitoring lung mechanics during HFOV. The aims of the subsequent animal study [2] were to characterize the relationship between mean airway pressure (PAW) and Xrs of the respiratory system, and to compare optimal PAW (P_{opt}) defined by Xrs, oxygenation, lung volume measured by Electric Impedance Tomography (VL), and tidal volume (VT) in preterm lambs. The PAW-Xrs and PAW-VT relationships were dome-shaped with a maximum at closing pressure + 6 cmH₂O, the same point as P_{opt} defined by VL. P_{opt} , as defined by oxygenation, was lower than the P_{opt} defined by Xrs, VL, or VT. We concluded that Xrs has the potential as a bedside tool for optimizing PAW during HFOV. Lastly, two studies on preterm infants were performed. The aim of the first [3] was to investigate the

relationship between PAW, lung mechanics, and right ventricular output during the recruitment manoeuvres in HFO ventilated preterm infants. At maximal distending pressure reached during the trial, oxygenation markedly improved, and Xrs and RVO decreased. These results suggest that Xrs and RVO are more sensitive than oxygenation to overdistension. In the second study [4], we investigated the effect of frequency on pressure cost of ventilation in newborns receiving HFOV: we hypothesized that ventilating at the resonant frequency of the respiratory system optimizes gas exchange while limiting the mechanical stress to the lung. The mean resonant frequency was 16.6 (\pm 3.5) Hz. The blood gases were unaffected by frequency. Damping of ΔP increased with frequency and with lung compliance. We concluded that there is no optimal frequency for gas exchange when DCO_2 is held constant. Greater attenuation of oscillatory pressure at higher frequencies offers more protection from barotrauma, especially in patients with poor compliance.

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LECT 11

PRECISION NON-INVASIVE VENTILATION IN NEWBORNS

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Non-invasive modes of ventilation are increasingly employed in preterm infants in order to reduce the intubation rate. Among these, nasal intermittent positive pressure ventilation (NIPPV) is a technique that provides continuous positive airway pressure (CPAP) plus superimposed ventilator mandatory breaths, and its popularity is increasing in NICUs as an alternative to NCPAP. NIPPV is identified as SNIPPV when the ventilator pressure waves are synchronized with the spontaneous efforts of the patient. A flow-sensor to detect spontaneous inspiratory flow has been developed by our group to trigger the ventilator (flow-trigger) [1]. Using this device we were able to demonstrate that flow-SNIPPV is more effective than conventional NCPAP in improving ventilation and in decreasing extubation failure in preterm infants who had been ventilated for respiratory distress syndrome (RDS) [2, 3]. Later we used flow-SNIPPV as the primary mode of ventilation, after surfactant replacement, reducing the need for mechanical ventilation and favorably affecting short-term morbidities of treated premature infants [4]. More recently we have also successfully applied flow-SNIPPV in the treatment of apnea of prematurity (AOP) [5]. This technique has also been successfully used as rescue therapy for infants failing on NCPAP [6]. The modern concept of "precision medicine" aims for the improvement of efficacy of flow-SNIPPV, first through continual enhancements in comfort and efficiency and secondly finding better ways to monitor the degree of ventilation, given that, due to leaks, it is impossible to measure expiratory tidal volume directly during non-invasive ventilation. Therefore, our main goals to further improve this technique are the development of very light and comfortable nasal prongs with an integrated flow sensor and the capacity to monitor the degree of lung ventilation. Different nasal interfaces are currently used to provide nasal respiratory support or ventilation, such as single nasopharyngeal tubes, short soft binasal prongs, or nasal masks, but these devices are not without adverse effects, above all nasal-mucosal trauma. The different shapes and properties of these interfaces may lead to varying impact on the pressure transmission to the lungs, which in turn may influence clinical outcomes. Moreover, in our ventilator expressly developed to perform flow-SNIPPV (Giulia®; GINEVRI srl, Albano Laziale, Rome, Italy), the flow-sensor is

currently placed above short binasal prongs and, even if it is very small and light, it is not completely comfortable for the patient and not easy for nurses to keep it stable and to maintain an adequate seal. Treatment with heated, humidified, high-flow nasal cannula (HFNC) is an increasingly popular means of noninvasive respiratory support and it has efficacy similar to that of CPAP when used as post-extubation support in neonates. However, this approach has several reported advantages over CPAP, including reduced rates of nasal trauma and reduced infant pain scores. Moreover, according to some surveys, this approach is preferred by parents and nursing staff [7, 8]. For these reasons our team has developed a new interface in order to perform flow-SNIPPV with prongs that have similar characteristics to the HFNC and with the flow-sensor placed far away from the infant's head, at the level of the Y-piece. The main objective of this preliminary study was to test the prototype of the new interface (Sync-flow Cannula®; GINEVRI srl, Albano Laziale, Rome, Italy) connected to a lung simulator by using it to determine the relation between set and delivered pressures and to measure the trigger's response time of the ventilator (**Fig. 1**). A Neonatal Active Lung Model simulator (NALM, S/N 014 by "Dr. Shaller Medizintechnik", Dresden) was programmed to mimic a VLBW infant with severely or moderately affected lungs (compliance 0.5 ml/cmH₂O with an FRC of 10 ml or compliance 1 ml/cmH₂O with an FRC of 15 ml; resistance 50-70 cmH₂O/l/sec). The respiratory rate was set at 60 breaths/min with an inspiratory time 0.33 sec and inspiratory efforts of -4/-5 H₂O. The Giulia® ventilator was set on SNIMV with the following parameters: flow 8 l/min; rate 30 breaths/minute; PIP +15/20/25 cmH₂O; PEEP +5 cmH₂O; Ti 0.35 sec; trigger level 0.1 or 0.2 l/min. The SNIMV mode was chosen in order to compare Vt variations during assisted and non-assisted breaths (**Fig. 2**). The prototype Sync-flow Cannula® was tested using a prong-size designed for VLBW infants (an external diameter of 2.5 mm and an internal diameter of 1.5 mm). The tubes connecting the prongs to the flow-sensor have a length of 270 mm and an inner diameter of 3.65 mm. The flow-sensor is a simple differential pressure transducer pneumotachograph, with an internal resistance given by a diaphragm with a central hole of 3 mm in diameter and 1 mm of depth. In order to mimic nasal leaks, the prongs were attached to the lung simulator by an artificial nose [9] with two separate connections, both with two small holes: in the first, the holes were each

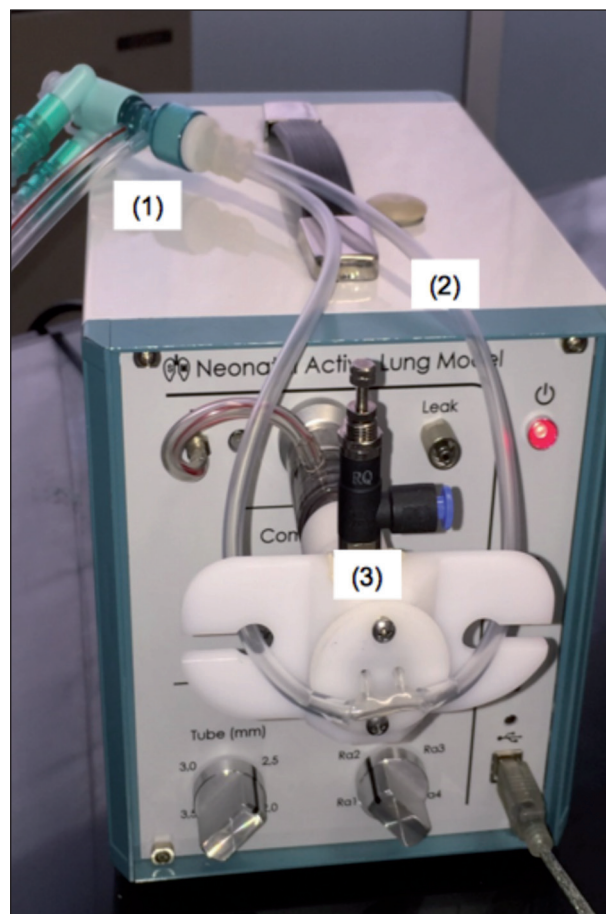


Figure 1 (LECT 11). In the picture are shown the test lung, the flow-sensor connected to the Y-piece (1), the prongs (2) and the artificial nose (3).

8% larger than the section of the prongs and in the second 16.5%. Anyway it must be underlined that, unlike HFNC that are recommended for use with 50-60% occlusion of the nares as the bulk of exhaled gas must occur through the non-occluded nostrils, with the Sync-flow Cannula® there is not this need because PIP and PEEP levels are controlled by the pressure valves of the ventilator. For each operating condition, we used the mean values from 10 breaths to calculate the response-time of the ventilator, the pressure drop between the Y-piece and the alveolar level, and the Vt increase between assisted non-assisted breaths. The response time we measured was typically in the range of 50-80 msec, and its length is influenced by the strength of the inspiratory effort and by the leaks. The alveolar pressure of the test lung, compared to the pressure at the level of the Y-piece, revealed a drop in the range of 15-30%, but the assisted breaths typically determined a significant increase of Vt of about 150-180% (**Fig. 2**). These data seem to confirm the efficacy of the new lighter and more comfortable Sync-flow Cannula®. The next step is to determine how to

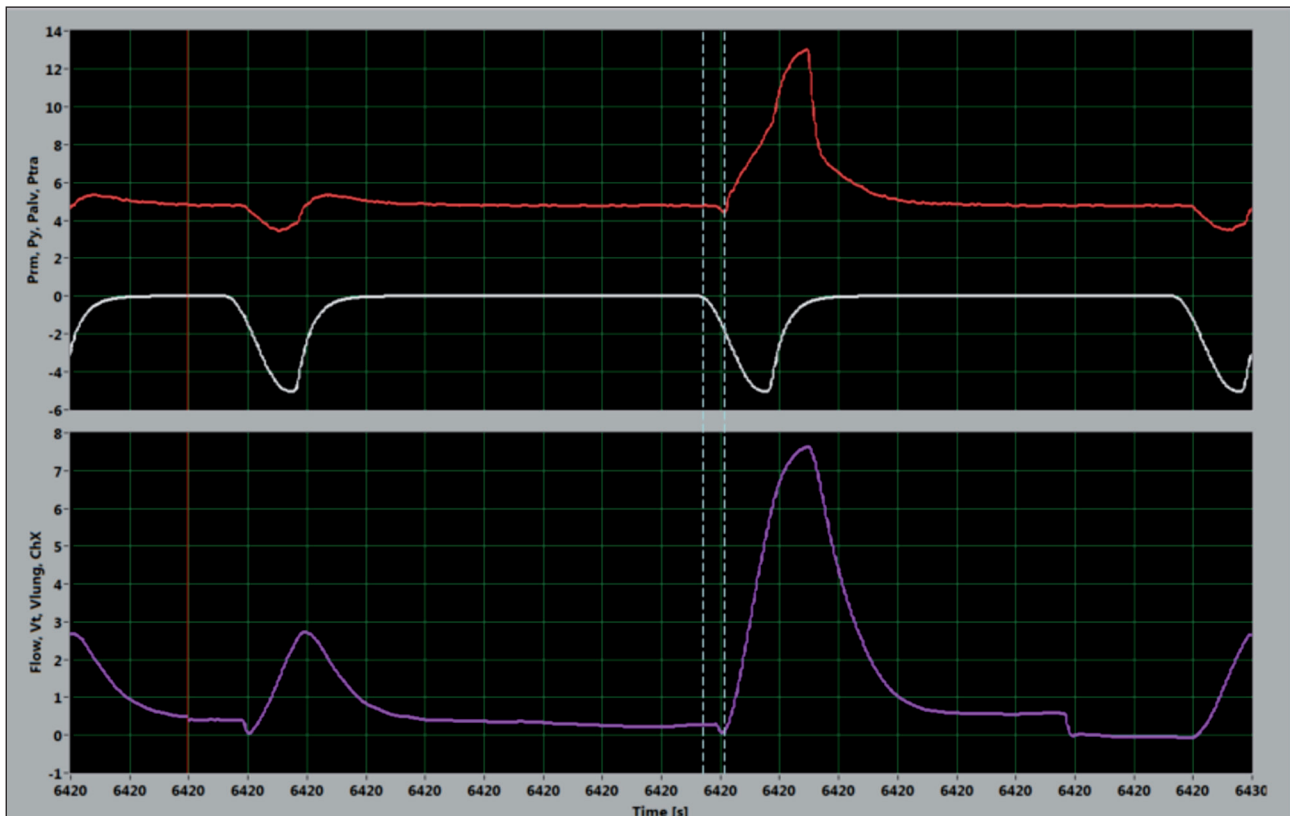


Figure 2 (LECT 11). Simulated flow-SNIMV of a VLBW infant who has a compliance of 1 ml/cmH₂O and a FRC of 15 ml. The “spontaneous” inspiratory effort (white line) produces a negative pressure of -4.5 H₂O and the response time of the ventilator (white dotted lines) is about 65 msec; every square are 200 msec. The alveolar pressure (red line) at the end of inspiration is 13 cmH₂O with a the PIP set on the ventilator of 15 cmH₂O and with 8% leaks at the level of the prongs. Tidal volume (violet line) increases from 3 ml (non-assisted breath) to 7.5 ml (assisted breath) with an increase of 150%.

monitor the degree of ventilation more effectively: electric impedance tomography (EIT) promises to be a useful tool to reach this goal [10].

DECLARATION OF INTEREST

C. Moretti is a consultant to GINEVRI Medical Technologies. GINEVRI Medical Technologies has not contributed any financial support for this paper or had any part in the authorship.

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LECT 12**BRONCHOPULMONARY DYSPLASIA: FROM NORTHWAY TO METABOLOMICS**

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Bronchopulmonary dysplasia (BPD) is one of the most common sequelae of premature birth and it is associated with significant morbidity and mortality. The term was coined by Northway et al. in 1967 to indicate a chronic pulmonary disease observed in preterm infants with respiratory distress syndrome (RDS) treated with high oxygen concentrations and mechanical ventilation. This condition, later named “old BPD”, was characterized, from a histopathological standpoint, by intense airway inflammation, heterogeneous lung injury marked by regions of atelectasia and others of hyperinflation, and parenchymal fibrosis [1-3]. With increasing survival of more immature infants, the definition of BPD has undergone several revisions. Currently, BPD is most often defined using the National Institute of Child Health and Human Development (NICHD) consensus criteria based upon oxygen requirement ≥ 28 days, gestational age (< 32 weeks – ≥ 32 weeks) and severity (mild, moderate, severe) depending on oxygen supplementation and/or respiratory support need at 36 weeks of postmenstrual age (PMA), or at 56 days of postnatal age for ≥ 32 weeks PMA. This definition was further refined by the use of an oxygen challenge test. Many advances in neonatal-perinatal medicine, such as prenatal steroids, surfactant replacement, and more gentle ventilation strategies, shifted the demographic characteristics of BPD to extremely preterm infants, born during the late canalicular or early saccular phase of lung development, with structurally immature lungs and underdeveloped pulmonary vasculature. The early disruption of normal lung development by preterm birth would evolve into aberrations in both alveolarization and pulmonary vasculogenesis. The latter may evolve in a significant pulmonary vascular disease (PVD), which in its serious form results in pulmonary hypertension (PH). These abnormalities have introduced the concept of “new BPD” which is histopathologically characterized by simplification of the parenchyma (larger but fewer alveoli with

decreased septation) and pulmonary vascular remodeling. The etiology of BPD is multifactorial: genetic predisposition and persistent inflammation due to exposure to antenatal and/or postnatal environmental factors contribute to lung injury and alter pre- and postnatal pulmonary growth. Preventive strategies such as prevention of infections and optimization of nutrition and ventilation have been proven to minimize lung injury; nevertheless, to date there are still no satisfactory therapies for BPD. Regenerative medicine might be an option. According to some preclinical results, stem cells administration appears as a promising therapeutic approach to improve the clinical outcome of BPD. It would be useful for prognosis and targeted therapeutic strategies to identify precociously those babies at higher risk for developing BPD. Currently, research has focused on identification of biomarkers to advance detection, to improve prognostic outcome, and to begin an appropriate treatment that can prevent later complications of BPD. In this regard, the “omics” sciences seem to be promising approaches to identify novel biomarkers and discover new evidence regarding lung disease. Metabolomics is the emerging field of “omics” which consists of the quantitative analysis of final products (named metabolites) of biochemical reactions released in fluids and biological tissues. This analysis has the ability to identify changes in metabolites caused by interaction between specific pathophysiological states, gene expression and environment. Actually, biomarkers were detected in several biofluids, including blood, urine, and bronchoalveolar lavage fluid. The application of clinical metabolomics in BPD has been described in literature and the results are promising [4, 5]. This information, combined with data from genetic and epigenetic studies, will contribute to the development of more effective diagnostic tools, the discovery of molecular pathways associated with the development and progression of the disease and the identification of new therapeutic targets. In conclusion, the goal of biomarkers identification should be to obtain tailored therapies for each individual patient, thereby reducing side effects and improving response to treatment. This field promises to improve the quality of clinical care by identifying the right treatment for the right patient at the right time.

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LECT 13

RESPIRATORY SYNCYTIAL VIRUS INFECTION: WHAT'S NEW?

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Respiratory syncytial virus (RSV) is the leading cause of acute bronchiolitis and one of the most common causes of infant viral death worldwide. It is an enveloped RNA virus belonging to the recently defined Pneumoviridae family, Orthopneumovirus genus [1]. The envelope of RSV is composed of the matrix protein, the small hydrophobic protein, the fusion protein (F) and the attachment glycoprotein (G). F and G proteins are in charge of the attachment of the virus to respiratory epithelial cells and the fusion of the viral envelope with the host membrane. They are crucial for virus infectivity and they stimulate the neutralizing antibody immune response by the host. Despite the global impact of RSV virus on human health, to date no antiviral agents are routinely used and the management of RSV infections is supportive, thus prevention plays an essential role. No vaccines against RSV are currently available, and prevention consists in environmental prophylaxis and passive immunization with a monoclonal antibody (mAb) – palivizumab – in high-risk children. Recent studies focused on understanding of RSV pathogenesis, diagnostics and surveillance in order to develop truly efficacious therapeutics and vaccines to control the burden of RSV disease. To date, two major RSV

subtypes (A and B) and multiple genotypes (11 RSV-A and 23 RSV-B) have been described, which can coexist during RSV epidemic season every year. The antigenic variability between the viral strains is determined by variations in the G protein. For this reason, most of the antibodies against the G protein are type specific, while antibodies targeted to F protein are cross-reactive for RSV A and B. The existence of different viral strains during local-community RSV epidemic season and their alternating infection incidences may play a role in the ability of RSV to infect previously exposed individuals and bypass preexisting immune responses. There are conflicting reports regarding the associations of different RSV groups and genotypes with severity of infection [2]. The herd immunity due to the recent RSV experience of a community may partially explain this controversial data, together with the different age range of the cohort studies, as older children are less likely to display severe illness. Moreover numerous environmental risk factors, such as overcrowding and hand smoke exposure, play a role in the clinical impact of RSV disease. The variations in viral load may also contribute to pathogenicity and severity of illness. Some studies show a correlation between higher viral load and greater disease severity, while others show none. These controversial data may depend on differences in study design and measurement methods. Moreover, higher viral load early in infection has been recently reported to protect against progression into RSV severe disease, possibly because of the development of a strong innate immune response leading to more rapid resolution of the disease. A number of promising options for the treatment and the prevention of RSV infection are currently objects of preclinical and clinical studies [3]. The well-conserved structure of F protein among different virus strains makes it an effective candidate for immunization strategies against RSV. Recent studies reported that the more potent neutralizing site is in the prefusion form of F, leading to the development of a new mAb, MEDI8897. This is a recombinant human IgG1 kappa mAb binding the prefusion form of F reported to have approximately 100-fold greater potency compared to palivizumab *in vitro*, and it is currently under clinical development. Maternal immunization against RSV is another promising strategy, as antibodies are transferred efficiently through the placenta. An ongoing phase III clinical trial is evaluating the effect of a recombinant F nanoparticle formulation vaccine administered

to pregnant women during the third-trimester is currently on phase III clinical trial. Moreover, approximately 60 RSV vaccines candidates are currently undergoing preclinical and clinical studies, including live attenuated, recombinant vector vaccines and viral-like particles. Regarding RSV antiviral strategies, the only licensed drug is aerosolized ribavirin but its use is limited due to its side effects. Thus, safer etiologic therapies are needed. ALX-0171 – a nanobody against the RSV F protein – and GS-5806 – an RSV fusion and entry inhibitor – are promising therapeutic options currently being tested in phase II clinical trials. Looking forward to efficacious RSV therapeutics and vaccines, clarifying the relation between viral factors and disease severity assumes a critical role. RSV surveillance is essential for detecting the emergence of new viral strains, predicting their clinical impact, selecting candidate strains for new vaccines and therapeutics and monitoring their effectiveness in the community.

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LECT 14

EMERGENCY IN OBSTETRICS, WORLDWIDE

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Emergency NGO is an independent Italian Non Governmental Organization, founded in 1994 to provide surgical treatment to civilian war victims in war-torn countries. During the years, the interventions were extended to address the needs of health among populations afflicted by poverty: from war surgery to general surgery, orthopaedics, cardio-surgery, internal medicine, paediatrics and maternal and neonatal health. The fundamental principles of Emergency action are the high quality of and the free-of-charge access to care. To codify a high-quality obstetric care in special

environments is a difficult challenge. Most of evidences available and guidelines published refer to high resource settings, meaning not only the availability of drugs, tools and human resources, but also the availability of reliable information (maternal history, laboratories evaluations, US scan) at the basis of clinical approaches and decisions (**Tab. 1**). Moreover, most of populations studied have small parity in average and expectation for a small number of total pregnancies, while low-income countries are characterized by high parity and high fertility rates: this creates specific problems as an increased incidence of some obstetric complications in pregnancy and labour and the necessity to consider the life-reproductive perspective in the management of single cases (especially about the mode of delivery). Last but not least, a lot of factors (local culture; scarcity of facilities; distrust in health facilities due to previous experiences; difficulty in transportation) cause often a significant delay in seeking for assistance. The foetus is usually not better known than the mother (**Tab. 1**).

Assessment of the well-being of unknown foetuses are big issues: Delphi Survey is an international survey still on-going, which has the aim to assess the best intra-partum monitoring in low resource settings. Once a consensus will be reached, the difficulty in individuating pre-labour risk factors (i.e., congenital abnormalities; intrauterine infections; foetal growth impairment) will continue to affect the diagnostic value of intra-partum foetal assessment. Our Maternity Hospital in Panjsher, Afghanistan, offers comprehensive maternal and neonatal care, at hospital and outpatient level, trying to overcome the delay in providing emergency care through a net of first aid posts spread in the catching area which give first assistance and prompt referral to the Main Centre. It was open in 2003, and experienced an overwhelming annual increase in activities: from a monthly number of deliveries less than 40 at the very beginning, to almost 700 deliveries per month currently. In this scenario, in our Project efforts have been done to contain the caesarean section (CS) rate. We consider it as a priority: for both maternal health (reducing the risks in current pregnancy – post-partum haemorrhage/ endometritis/infectious complications – and in subsequent ones – uterine rupture and related life-threatening consequences for both mother and foetus) and neonatal outcome (reducing the risk of respiratory distress mostly related to the uncertain gestational age). Potential complications

Tab. 1 (LECT 14). Factors affecting quality of care provided in perinatal medicine in low-income countries.

Maternal	Foetal
Lack of knowledge about own general data (age; menstrual period)	
Lack of knowledge about own health status (lack of documentation about previous diagnosis/therapies/surgery; current drugs in use)	
Difficult in collecting properly previous obstetric history (obstetric complications; indication to previous caesarean section; neonatal outcome)	Gestational age unknown
Scarce/absent antenatal checks	Morphologic evaluation (2 nd trimester US scan) not done
Lack of knowledge about dangerous signs in pregnancy (consequent delay in seeking for proper assistance)	Foetal growth not assessed
No standardized referral system (patients referred without any documentation of treatments already received in the referring facility)	Mother-to-foetus communicable diseases not assessed

in subsequent pregnancies are of special relevance in a population with high parity, difficulty in birth spacing and discontinuity in searching for assistance for pregnancy and delivery.

To obtain this goal the obstetric management follows two main directions:

1. humanized obstetrics practices (the Midwife as the main Skilled Birth Attendant; Evidence-Based (EB) application of birth technologies; EB induction of labour; use of partograph; free movement in labour; respect of the transitional period in labour; alternative positions at birth);
2. CS only for absolute indications.

About the point n° 2, the trial of labour after previous CS and for breech presentation demonstrated to reach very good outcomes in terms of successful vaginal birth and neonatal outcome (Apgar Score at 5'; need for resuscitation), with a significant reduction in terms of overall CS rate. Currently, the most challenging situations to be faced by the obstetrician (and by the neonatologist during the peri-partum assistance) are:

1. identification and management of post-term pregnancies;
2. recognition and management of the health status of foetus and newborn;
3. management of pregnancies and labours referred from other facilities with no documentations about treatments already provided;
4. management of patients arrived in hospital long time after critical events (rupture of membranes; antepartum bleeding; prolonged labour);
5. complications related to inappropriate use of drugs at home (i.e., open access to oxytocin);
6. widespread of inappropriate use of "birth technologies" in the Country, and easy access under payment (induction of labour included).

LECT 15

NEONATOLOGY IN AFGHANISTAN

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Emergency NGO provides free, high quality medical and surgical treatment to the victims of war, landmines and poverty, building and managing hospitals in countries with the worst mortality rates. In 2003 Emergency opened a Maternity Centre in Anabah to provide antenatal, gynaecological, obstetric and neonatal care to the population of the Valley and the surrounding provinces. The centre is the only specialized and free of charge in a huge area. In Afghanistan infant mortality rate is very high, the most recent data coming from the 2015 Demographic and Health Survey of the Afghan Ministry of Public Health, based on interviews of a sample of about 25,000 households, selected all around the country. According to this survey, infant mortality rate is 45 deaths per 1,000 live births, with a neonatal mortality of 22 deaths per 1,000 live births. These mortality estimates should be considered with caution, because they appear to be lower than the expected. Neonatal death, in particular, appears to be under reported, and based only on mother's report, because only about 50% of children under 2 years of age are registered with the civil authority. Only 9% of newborns receive a postnatal health check within two days of delivery, as recommended by WHO. More than 60% of women give birth at home without any qualified health assistance. In this scenery, Emergency

Maternity Hospital, in Panjshir, is a happy island. All births (the present average is 700 per months) are registered with the civil authority and postnatal health check is carried out by a doctor, generally within 6 hours after the delivery. Vitamin K and ocular prophylaxis are provided at birth and each one of the three delivery rooms and of the two obstetric operating theatres has a neonatal isolette. In the maternity department there is a Neonatal Intensive Care Unit with 10 incubators, each one with a pulse oximeters, oxygen availability and an infusion pump for parenteral feeding and/or I.V. therapy. Non-invasive ventilation with Bubble CPAP is also available, according to local staff level of knowledge and technology available in the Country. In 2016 the babies born alive were 6,230, but considering also the outborn newborns, the total newborn treated were 6,380, and of these the number of patients admitted in the Neonatal Unit was of 2,158. The newborns admitted presented one or more of the following medical problems: low birth weight, Apgar score < 7 at 5 minutes, meconium aspiration, PROM > 18 hours, maternal fever, hypoglycemia, respiratory distress, twins, congenital malformation, maternal diabetes, jaundice, convulsion, weight loss > 10%, neonatal infection, outborn, congenital syphilis. The number of deaths was 161, with a mortality rate of 2.5%. This rate is higher than the declared one of 2015 Health Survey, but it is very important to remember that in the survey the neonatal deaths were under reported (according to a WHO report neonatal mortality rate in the Country is 4%). Emergency has also activated a prenatal care program for women living in remote areas, who are assisted through the network of Emergency's First Aid Posts and Health Centres spread over the Panjshir Valley and the surrounding provinces. International and national midwives carry out periodical monitoring missions of pregnancies. Emergency NGO did a great job for neonatal care and for local staff training in particular for midwives training; from 2013 it is recognized by Afghan Ministry of Health and University as a training centre for residents in Gynecology and Paediatrics.

LECT 16

NEW PERSPECTIVES IN HUMAN MILK FORTIFIERS

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Very preterm newborns (gestational age lower than 32 weeks) and Very Low Birth Weight (VLBW) infants (birthweight lower than 1,500 grams) currently represent the majority of patients cared in Neonatal Intensive Care Units (NICU). The increase of the survival rate for these newborns, due to improvements in perinatal care, has opened new perspectives regarding their outcome and has a significant impact on their health status in adulthood. In these groups of infants, nutrition represents a fundamental factor not only for neonatal survival and short-term outcome, but also for long-term consequences and quality of life. The main issue is to ensure an adequate qualitative and quantitative nutrition, particularly in terms of protein intake, which is the main cause of post-natal growth deficit [1]. Human milk is the recommended food for all neonates including preterm infants [2]. Breast milk alone, however, does not meet the recommended nutritional needs in preterm infants [3]. The most common strategy is to cope with potential nutrient deficits by supplementing breast milk with additional nutrients (mainly proteins and minerals) to satisfy the special nutritional requirements of these infants [4]. At present commercially available fortifiers are based on bovine milk (BM), whose protein intake has raised concerns because of its association with allergies [5] and a possible role as a trigger of intestinal inflammation in preterm neonates [6]. In previous studies we observed that donkey milk (DM) was well tolerated in a group of highly problematic cow's milk allergic children [7]. Our hypothesis is that feeding preterm infants with HM supplemented with fortifiers derived from DM will improve the feeding tolerance. To confirm our hypothesis, we designed a randomized clinical trial to compare the use of DM-derived fortifiers with commercial BM-based fortifiers in infants with birthweight ≤ 1,500 g or gestational age < 32 weeks in terms of nutritional tolerance.

A total of 124 neonates were enrolled in Planned study (BF-arm n = 62; DF-arm n = 62). Further 32 infants were enrolled in Extended study (BF-arm n = 79, DF-arm n = 77). Our first results about DM-based fortifiers are promising. Compared to the bovine fortifiers, the DM-based fortifier seems to have a better feeding tolerance as well as some differences in metabolic outcome. We can therefore hypothesize that increasing the sample size we would observe a significant better feeding tolerance in DF-arm. Research is carried on with the evaluation of long-term clinical outcome and metabolomics in 2 groups.

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LECT 17

GUT PERTURBATION AND PROBIOTICS IN NEONATOLOGY

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Recent studies suggest that fetal colonization begins prior to birth [1]. Besides a possible prenatal transfer of maternal bacteria to fetus, other major determinants for neonatal gut colonization are mode of delivery, mode of feeding and perinatal antibiotic exposure. Generally, vaginally born infants are first colonized by bacteria from the maternal vagina, also including bacteria present in the maternal gut, while the gut microbiota of infants born by Cesarean (C)-section more often resembles maternal skin and oral microbiota, with delayed colonization of *Bacteroides*, and lower microbial diversity throughout the first 2 years of life. It has been suggested that the early gut colonization may have long-term medical consequences: indeed, C-section delivered babies seems to display higher incidence of celiac disease, obesity and asthma, with some implications on the maturation of the immune system, in terms of lower blood levels of T-helper cell-related chemokines, possibly due to the reduced gut colonization of *Bacteroides* genus. Perinatal antibiotic exposure is another major determinant of early gut microbial composition in newborns. Thanks to new molecular techniques currently available, we now have proof for antibiotic-induced intestinal dysbiosis, in turn associated with intestinal and plasma lipid profile alterations. Several studies have also demonstrated that antibiotic exposure in early infancy is associated with increased risk of developing overweight/obesity, as well as asthma, wheezing and inflammatory bowel disease later in life [2]. Finally, mode of feeding also plays an important role in influencing early intestinal microbiota. Breastfeeding is undoubtedly the best way to promote the healthy development of human offspring, modulating infant's early gut colonization both by human milk microbioma and by other unique nutritional components of human milk, such as oligosaccharides and lactoferrin, also known as prebiotic or bifidogenic factors. As a consequence, there are significant differences in the gut microbiota composition of breast-fed vs formula-fed infants. Several studies have pointed out that *Bifidobacteria*

are the most abundant organisms in breast-fed infant guts, whereas the gut microbiota of formula-fed infants is dominated by *Enterococci* and *Clostridia*, with more species diversity. In summary, neonatal early gut microbial colonization seems to be a crucial step at a critical age for modulating infant's healthy immunological, hormonal and metabolic development. Thus, according to the perinatal programming hypothesis, its perturbation might induce long-term negative effects. In this light, premature babies are particularly exposed to several risk factors such as gut immaturity, higher rates of maternal infections, perinatal antibiotics and other disturbing drugs and C-sections, as well as lower rates of breastfeeding. Overall, prematurity affects the microbiota as indicated by a reduced percentage of *Bacteroidaceae* during the first months of life and by a higher initial percentage of *Lactobacillaceae* in preterm infants compared with full term infants [3]. Perinatal antibiotics, including intrapartum antimicrobial prophylaxis, also affect the gut microbiota by increased *Enterobacteriaceae* organisms in the infants, with reduced *Bifidobacteriaceae* and *Lactobacilli* [4]. In the wake of this, the possible role and functions of early probiotics supplementation to modulate and reduce gut perturbations and dysbiosis has been extensively investigated. According to the definition of "live microorganisms that when administered in adequate amounts confer a health benefit on the host", probiotics promote microbial homeostasis rather than change its composition, through different mechanisms of action, such as competitive exclusion of pathogens, direct antagonism, gut barrier reinforcement and production of specific bioactives with immunological and endocrinological effects. Some of them, namely of *Lactobacilli*, *Bifidobacteria* and *Saccharomyces genera*, have been reported to prevent necrotizing enterocolitis, reduce time to full enteral feeding and mortality in very low birth weight infants [5], especially when associated to breastfeeding, as well as to reduce crying time in colicky babies and antibiotic associated diarrhea [6]. In contrast, probiotics supplementation is not linked to significant improvement of postnatal growth of preterm infants, as reported so far. Although the risk of sepsis may be an issue in some predisposed subjects, in general the use of probiotics can be considered safe also in premature babies. By the way, it's worth pointing out that clinical trial results from one probiotic strain in one population cannot be automatically generalized to other strains or to different populations and that the beneficial effects

reported in the vast majority of clinical trials refer to a very limited number of strains.

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LECT 18

NEAR INFRARED SPECTROSCOPY AND GUT FUNCTION IN PRETERM NEWBORNS

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The recent advances in neonatal care have significantly reduced neonatal mortality, but have also contributed to increase the survival rates of extremely and very preterm infants. The impairment of mesenteric perfusion and the subsequently lowered oxygen delivery to the gut are among the major issues underlying necrotizing enterocolitis (NEC), which is the most feared intestinal complication in premature neonates. By altering gut haemodynamics, patent ductus arteriosus (PDA) with mesenteric diastolic steal, indomethacin treatment for PDA closure, hemodynamic instability requiring inotropes and antenatal impairment of umbilical Doppler with evidence of absent or reversed end-diastolic flow velocity have also been associated with adverse intestinal outcomes in the preterm population. Doppler evaluation of superior mesenteric artery (SMA) blood flow has been widely adopted to investigate neonatal gut hemodynamics in several physiological and pathological conditions,

but it is subject to the following limitations: need for trained personnel, operator-dependency, inability to provide a continuous evaluation and to estimate tissue perfusion.

Near infrared spectroscopy (NIRS) is a non-invasive monitoring technique that, by detecting changes in oxygenated and deoxygenated hemoglobin levels within biological tissues, provides a continuous estimate of regional oxygenation saturation (rSO_2). The combination between rSO_2 and arterial oxygen saturation (SaO_2) monitoring allows the assessment of the fractional tissue oxygen extraction (FTOE: $[SaO_2 - rSO_2]/SaO_2$), which reflects the local balance between oxygen delivery and consumption. Over the last decade, a mounting body of evidence has supported the role of abdominal NIRS monitoring in shedding light on neonatal gut hemodynamics. Gut oxygenation response to enteral feeds in preterm infants has been investigated by several studies, showing a post-prandial rise in abdominal oxygen saturation ($ArSO_2$). While this pattern is typically observed after bolus feeds, continuous feeding has been associated with a significant $ArSO_2$ reduction, evident from 1 hour and 15 min onwards [1]. Although the possible underlying mechanisms still have to be thoroughly elucidated, a role for feeding-related differences in gut hormonal release has been proposed: the intermittent exposure of the intestinal mucosa to bolus feeds leads to cyclic surges of gastro-intestinal hormones with possible vasoactive effects, whereas this pattern has not been observed during continuous feeding. Abdominal NIRS monitoring has also been adopted to investigate $ArSO_2$ changes in preterm neonates with intestinal complications, such as NEC or feeding intolerance. Particularly, NEC development has been associated with persistently low $ArSO_2$, preceded or followed by very high $ArSO_2$ and periods of signal dropout; on the other hand, infants with feeding intolerance showed moderately lower $ArSO_2$ values compared to healthy controls, but maintained $ArSO_2$ variability over time [2]. The ratio between abdominal and cerebral tissue oxygenation, based on the evidence that cerebral autoregulation physiologically keeps cerebral blood flow and oxygen delivery almost constant, has also been proposed as possible marker of intestinal ischemia in neonates with acute abdomen. Furthermore, the combination between abdominal FTOE and intestinal fatty acid-binding protein levels has recently been reported to distinguish between medically vs. surgically treated NEC. On this basis, the possible role of NIRS as a predicting tool for intestinal outcome in preterm

infants has gained increasing interest, and particular attention has been paid to $ArSO_2$ at enteral feeding introduction. According to our recent findings [3], $ArSO_2$ values at first enteral feed were significantly lower in preterm infants who later developed feeding intolerance compared to those who did not, consistently with previous Doppler data from a similar cohort showing a post-prandial decrease in SMA blood flow after the first feed. On the other hand, a number of limitations for abdominal NIRS monitoring need to be acknowledged. First, a contribution from the different abdominal structures in determining $ArSO_2$ values cannot be ruled out. Moreover, the hollow gut structure and its physiological peristaltic movements can increase the risk of artifacts. Recent data, however, have shown a reliable correlation between $ArSO_2$ and neonatal intestinal peristalsis: high $ArSO_2$ values were found to be associated with normal or hyperactive gut motility, whereas lower values were seen in preterm infants with poorer motility, which is also observed in pathological gut conditions such as NEC.

In light of these overall findings, abdominal NIRS monitoring in the preterm population seems to bring useful information to evaluate gut hemodynamics in physiopathological conditions; however, further studies are needed to confirm and expand this promising but preliminary evidence.

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LECT 19

ALLERGY TO COW'S MILK PROTEIN

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INTRODUCTION

Cow's milk protein allergy (CMPA) is the most common food allergy in young children.

The World Allergy Organization definition for CMPA is “hypersensitivity reaction brought on by specific immunologic mechanisms to cow’s milk.” Incidence of CMPA during first year of life is estimated between 2% and 7.5%. Prevalence of CMPA in children 6 years or older is approximately 2% to 3% and 0.5% in breast-fed infants [1, 2]. It is helpful to differentiate between two immunological mechanisms of CMPA: IgE- and non-IgE-mediated. IgE-mediated CMPA is characterized by immediate onset (within minutes to 2 hours after allergen ingestion) and type 1 hypersensitivity. Non-IgE-mediated CMPA is a delayed reactions up to 48 hours or even 1 week following ingestion, type 4 hypersensitivity [1].

CLINICAL FEATURES

Clinical presentations of CMPA appear during the first few months of life, after the end of exclusive breastfeeding and the introduction of cow’s milk based formula into the diet. Sometimes symptoms may also occur with exclusive breastfeeding, if the mother ingest cow’s milk [2]. Symptoms and signs related to CMPA involve many different organ systems, particularly: skin (50-60%), respiratory (20-30%) and gastrointestinal tracts (50-60%) [3]. In IgE-mediated CMPA symptoms include oral pruritus, urticarial, rhino-conjunctivitis, angioedema of oropharynx, eczema, vomiting, and diarrhea. Reactions can vary from mild to life-threatening anaphylaxis, about 85% of symptoms are mild, 15% are severe such as stridor and wheezing and about 9% can develop into anaphylaxis within minutes to few hours after ingestion of cow’s milk. In non-IgE-mediated CMPA reactions symptoms are very nonspecific and include gastrointestinal (gastroesophageal reflux disease, malabsorption, constipation, bloody stool), skin (atopic dermatitis, urticarial, angioedema) and respiratory (wheezing, cough) symptoms; colic and food aversion [2]. Chronic iron-deficiency anemia may be the sole manifestation of CMPA in infant and children [3]. Other manifestations of non-IgE-mediated CMPA are allergic proctocolitis, allergic eosinophilic gastroenteritis, pulmonary hemosiderosis (Heiner’s syndrome) and food protein-induced enterocolitis syndrome (FPIES). The typical FPIES presentation is that of severe vomiting and diarrhea within two to four hours after ingestion of the offending allergen, causing profound dehydration, lethargy, and sometimes shock.

NATURAL HISTORY

The majority of children with CMPA achieves tolerance. In the experience of the taskforce

members, children with non-IgE-mediated CMPA tend to become tolerant more quickly than children with IgE-mediated CMPA. The prognostic indicators for the development of tolerance to milk in patients with IgE-mediated CMA include lower initial level of milk-specific IgE, faster rate of decline of milk-specific IgE level over time, and absence of concomitant allergic rhinitis or asthma. Children with a positive history of IgE-mediated CMPA usually have an increased risk of developing atopic diseases than those with non-IgE-mediated CMPA [1].

DIAGNOSIS AND MANAGEMENT

The gold standard for diagnosis of CMPA is elimination diets and challenge procedures [1]. Oral challenge should preferentially be performed in a setting where safety facilities are available because of the risk of anaphylaxis [3]. History and physical examination remain very important in diagnosing CMPA (**Fig. 1**); in fact, positive family history of atopy may represent an important risk factor. Skin prick test (SPT) and/or *in vitro* test for IgE are usually performed initially [3]. A limited number of clinical studies showed how higher concentrations of cow’s milk-specific IgE and larger skin test wheals correlate with an increased likelihood of a reaction upon ingestion. A cow’s milk-specific IgE level of ≥ 15 kUA/L in children ≥ 2 years and ≥ 5 kUA/L in children < 2 years of age is 90% predictive of a clinical reaction to ingested milk. Furthermore, if there is in a SPT a wheal diameter of 8 mm the likelihood is very high that the child will have a positive food challenge [1]. When the history and testing are not conclusive, the diagnostic procedure may include elimination of the suspected food for a limited period of time for infant or mother, followed by challenge (if the probability is higher) or reintroduction (if the probability is lower). The ranges of the duration of a diagnostic elimination diet range from 3 to 5 days in children with immediate clinical reactions, to 1 to 2 weeks in children with delayed clinical reactions and from 2 to 4 weeks in children with gastrointestinal symptoms. When no clinical improvement are obtained with milk-avoidance diet and no significant differences are noted with reintroduction (or challenge is negative), the food in question is not responsible for symptoms, it is unlikely to be CMPA and milk should be reintroduced. Since the only accepted treatment for food allergy is the complete avoidance of causative allergen, many authors find alternative treatments to change the natural course of food allergy and oral immunotherapy is one of the mostly investigated.

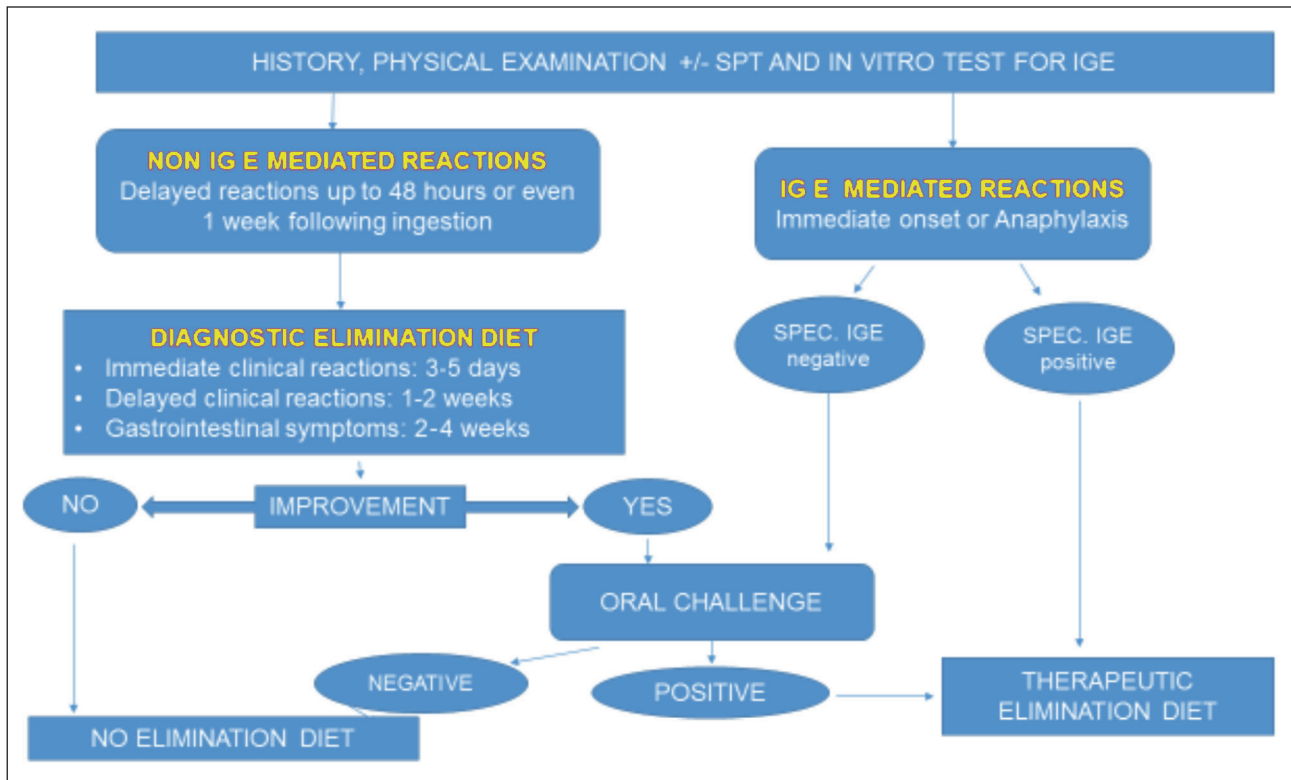


Figure 1 (LECT 19). Diagnosis and management in infants and children with symptoms suggestive of CMPA. Adapted from ESPGHAN GI Committee Practical Guidelines.

SPT: skin prick test.

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LECT 20

GLOMERULAR NUMBER IN LBW BABIES: NOTE FOR THE FUTURE. THE FAULT IS NOT IN OUR STARS BUT MAY BE IN OUR EMBRYOS

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Non-Communicable Diseases (NCD) such as cardiovascular disease, hypertension, type 2 diabetes, and obesity have replaced communicable diseases as the predominant causes of mortality worldwide. Genetic and environmental factors contribute to the development of NCD, and fetal development is an important modulator of NCD risk. Chronic Kidney Disease (CKD) is a major cause of hypertension and a major risk multiplier of cardiovascular diseases. Since the first observations by Barker that adults who were born with low birth weight (LBW) were at higher risk of premature cardiovascular death, increasingly epidemiologic and experimental evidence has highlighted the “programming” impact of intrauterine stresses on organ development and long-term organ function. Subsequently, Brenner and colleagues hypothesized that developmental programming in the kidney might reduce nephron number, which in turn later in life could contribute to hypertension through limiting sodium excretion because of decreased filtration surface area, and could increase the risk of CKD if further nephrons are lost through age or other injuries. Nephrogenesis continues until 36 weeks of gestation and no new nephrons develop after birth in term newborn infants. Therefore, a kidney with fewer nephrons, as in intrauterine growth-restricted

(IUGR), preterm and LBW infants, could have a reduced renal functional reserve capacity and be less able to withstand additional renal injury. Many factors may impact fetal kidney development: quality and quantity of nutrition received during fetal life, maternal diabetes, preeclampsia, infection, maternal exposure to nephrotoxic drugs, or toxic substances (alcohol, tobacco). Postnatal nutrition, neonatal exposure to nephrotoxic drugs and acute kidney injury (AKI) are also contributing factors to further nephron loss, enhancing susceptibility to CKD. The recently proposed consensus recommendations by the Low Birth Weight and Nephron Number Working Group emphasize the cooperation of different specialists, including obstetricians, neonatologists, pediatrics and adult nephrologists, with the common purpose of improving mother and child health, related not only to medical risk factors, but also to lifestyle, education, socio-economic status. Efforts should be made to improve maternal health before and during pregnancy, in order to improve fetal health. Meeting the current unmet needs would help to define the most cost-effective strategies and to optimize interventions eventually limiting or interrupting the developmental programming cycle of CKD later in life, especially in the poorest part of the world.

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LECT 21

VACCINE-PREVENTABLE DISEASES

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Immunization is the most beneficial and cost-effective disease-prevention measure. Due to vaccination, some diseases are eradicated and some other currently not endemic in Italy. The

incidence of most vaccine-preventable diseases of childhood has been significantly reduced in the last decades. Unfortunately pertussis, measles and varicella infections still circulate in Italy. Pertussis is a vaccine-preventable disease. Some countries with high immunization coverage against pertussis are experiencing a resurgence of the disease. Despite primary immunization, outbreaks have been recently reported also in Italy. We speculate that the epidemiology of this disease in Italy may be affected by the lack of clinical suspicion of the disease by clinicians. The consequence is a reduced prescription of laboratory examinations. Moreover, in order to start early treatment and prevent complications, the diagnosis of pertussis is sometimes reached without microbiological confirmation leading to possible misdiagnosis of the disease. Clinical manifestations may be different depending on age. Severe symptoms are common in young unvaccinated infants. In a study conducted at Bambino Gesù Children Hospital, Rome, Italy, out of 215 patients tested, 53 had a positive RT-PCR for *B. pertussis* (24.7%) [1]. The clinical suspicion of pertussis is not easy in infants because clinical manifestations of pertussis can overlap with several different diseases. Sometimes presentation may mimic a viral respiratory tract infection. The circulation among households could contribute to the transmission of the disease to infants too young to be vaccinated. The current risk for infants in the first months after birth and the crucial role of a pertussis booster in pregnancy may certainly be the key to address strategies to prevent infants' death. Measles still represents a serious public health problem worldwide. It is a highly infectious disease with severe complications. Although the vaccine was introduced in national vaccination schedules 20 years ago, several outbreaks have occurred because of insufficient vaccination coverage in Italy. Since January 2017, we are seeing a new outbreak of measles in Italy: 4,238 new cases were reported, of whom 27% were pediatric patients. Varicella is an acute, exanthematous, and highly infectious disease affecting virtually every child in the absence of vaccination programs. Varicella has mostly an uncomplicated course in early childhood. Nevertheless, it may result in severe complications. A common measure of varicella complications is the hospitalization of patients: most frequent and severe complications are bacterial infection, respiratory impairment, and neurological deficits [2]. Among neurological complications, acute cerebellitis is the most frequent manifestation. The phenomenon of

vaccine refusal could be associated with growing concerns regarding the safety of vaccines, encouraged by anti-vaccination movements, and highlights the importance of strongly reinforcing awareness on the safety and efficacy of measles vaccine [3]. It is necessary to identify susceptible individuals and consider undertaking catch-up immunization or supplementary immunization activities to close immunity gaps. Additionally measles immunization saves high costs for the national health care-system. It is mandatory to improve the knowledge and the scientific culture of health care professionals and all population for reaching adequate vaccination coverage for preventable diseases.

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LECT 22

WHAT IS NEW IN PATENT DUCTUS ARTERIOSUS TREATMENT?

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INTRODUCTION

Ductus Arteriosus (DA) is the shunt connecting pulmonary artery to aorta during fetal life. DA closure, usually occurring between 24-72 hours of life, in preterm infants can fail or be deferred. The resulting Patent Ductus Arteriosus (PDA) is a delicate situation, especially if persisting over time. On the 4th day of life, PDA is present in about 10% of infants between 30-37 wks of gestational age (GA), in 80% of those between 25-28 wks and in 90% of those born at 24 wks. From the 7th day of life, PDA in these categories reduces to about 2%, 65%, 87%. Short- and long-term conditions can complicate PDA (respiratory distress syndrome, prolonged need for assisted ventilation, pulmonary hemorrhage, bronchopulmonary dysplasia, necrotizing enterocolitis [NEC], renal damage, intraventricular hemorrhage, periventricular leuko-

malacia, cerebral palsy, death). Moreover, the “ductal steal” on systemic circulation can lead to brain, kidney and bowel hypoperfusion. Ductal closure can be pharmacologically or surgically performed. Strategies of treatment vary from prophylactic to early or delayed therapy and clinical practice is still heterogeneous. Treatment of hemodynamically significant PDA (hsPDA) seems today the most profitable strategy and medical approach with non-steroidal anti-inflammatory drugs (NSAIDs) is the first line therapy [1].

PATENT DUCTUS ARTERIOSUS TREATMENT

Indomethacin and ibuprofen show a closure rate of 70-85%. The first is associated with several side effects, such as renal function impairment until acute or chronic failure, oliguria, proteinuria, hyperkalemia, cerebral white matter damage, NEC, intestinal perforation, platelet dysfunction. Ibuprofen, showing a lower nephrotoxicity, now represents the drug of choice. Oral or intravenous paracetamol, since 2011, has been evaluated through many trials as effective as traditional NSAIDs, with fewer side effects (inconstant and low liver enzymes elevation has been described). Now, it is used in case of NSAIDs contraindications. Surgical PDA ligation, related to several complications, is reserved to patients showing medical consequences of a large hsPDA after the failure of two courses of medical treatment.

WHAT THE NURSE SHOULD KNOW

In the management of the newborn affected by PDA, the nurse contribution is very important to ensure an adequate patient care and to detect precociously signs of patient’s worsening or drug toxicity. Possible signs and symptoms of PDA must be known and recognized, such as fast and short breathing, increased breathing effort, active precordium, systolic murmur, occurrence of pulmonary edema, poor feeding, poor weight gain, excessive weight increase for fluids’ retention, bounding peripheral pulses, tachycardia, hepatomegaly. This kind of patient requires the standard NICU care, in addition to specific assistance measures. A close surveillance of vital signs, breath and heart rate, peripheral pulses and skin color must be carried out. Therapies must be accurately administered. Adequate rest, nutrition and oxygen if indicated have to be ensured.

Intravenous fluid administration must be carefully controlled to avoid an overload of circulatory system. The fluid balance has to be recorded, to detect a possible weight gain in case of edema (predisposing to heart failure). Urinary output monitoring is necessary. Patients treated with NSAIDs must be

monitored for oliguria, jaundice, gastrointestinal bleeding or diarrhea; those treated with paracetamol, for hepatotoxicity. Signs of heart failure must be rapidly assessed, such as restlessness, fatigue, rapid and short breathing, retractions, tachycardia, extra-heart sounds, peri-orbital edema, hepatomegaly, oliguria. An increased oxygen requirement could indicate the occurrence of pulmonary edema. Feed tolerance and weight gain must be daily evaluated; the mother must be encouraged to provide breast milk to feed the newborn. A psychosocial assessment, including patient's habits, family compliance and awareness of disease, promotes a good therapeutic alliance. After an eventual surgical ligation, post-surgery homeostasis and peacefulness have to be maintained to avoid complications [2].

CONCLUSIONS

Recent data mostly support paracetamol efficacy in PDA closure; however, more studies are necessary to clarify its safety and long-term outcomes, before considering it as first choice drug. The goal of PDA treatment would be an individualized therapy, based on each patient's features, which can result effective and with the lowest side effects. In the next future metabolomics could be useful in PDA management; it can detect, at birth and in a non-invasive way, the successive condition of PDA, also predicting the individual therapeutic response. Different metabolic profiles have been described comparing ibuprofen responders to non-responders; these are preliminary results of the studies of Fanos et al. and Castell Miñana et al. [3, 4] on small preterm groups and should be confirmed through several studies.

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LECT 23

NEW PROSPECTS FOR PEDIATRIC RESPIRATORY MEDICINE FROM METABOLOMICS

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Metabolomics is a comprehensive approach that enables all the metabolites in a biological sample to be identified and quantified. Metabolic characterization identifies a set of metabolites that reflect enzyme expression and activity, and include the building blocks and breakdown products of the DNA, RNA, proteins, and cellular components. Metabolomics is affected by several factors unrelated to the genome, such as interactions with commensal microorganisms, nutritional factors, environmental agents, and any exposure to drugs or toxic substances resulting in discordance between genotype and phenotype. The metabolomic approach thus promises to enable states of disease to be detected, patients to be stratified on the basis of their biochemical profiles, and the progression of disease to be monitored. Metabolomic analyses may also be able to orient the choice of therapy, identify responders and predict toxicity, paving the way to more customized therapies. Being a very informative technique that can be applied to samples collected noninvasively, metabolomics has considerable appeal for the study of pediatric and neonatal respiratory diseases [1, 2]. We will discuss some of the results of our metabolomic investigations on asthma, prematurity, bronchopulmonary dysplasia (BPD), and recurrent respiratory infections (RRI). Asthma and its phenotypes can hardly be investigated by concentrating on a given biomarker because there are so many cellular and molecular mechanisms involved, and they interact in complex ways. That is why new, integrated systems approaches are needed. Metabolomic analysis has lately been applied to respiratory diseases by studying an individual's exhaled breath condensate (EBC) [3], a biofluid that is collected noninvasively by cooling the air expired during tidal breathing. We used metabolomics to analyze the EBC of children with more or less severe asthma [4] in an attempt to discriminate between their clinical phenotypes, and to establish whether severe asthma has a characteristic biochemical-metabolic fingerprint. The study proved that metabolomic profiling of EBC can clearly distinguish between different biochemical-metabolic profiles in asthmatic children, fully discriminating the severe asthma phenotype from milder forms.

BPD is the main cause of respiratory morbidity in babies born prematurely. After the acute phase of the disease, the respiratory symptoms of survivors of BPD often improve during early childhood, but many such children have airflow limitations that persist into adolescence and adulthood. We report the results of two studies based on the metabolic analysis of EBC in one [5], and amniotic fluid (AF) in the other [6]. We recruited 20 consecutive survivors of BPD who were routinely followed up, and a group of age- and sex-matched healthy adolescents. The metabolomic analysis on their EBC was able to distinguish cases of BPD from healthy individuals, suggesting that the lung of survivors of BPD is characterized by long-term metabolic abnormalities. The search for putative biomarkers pointed to a role for an altered surfactant composition, which may persist far beyond infancy [5]. In a second study, we examined whether an unbiased metabolomic analysis of AF can be used to investigate the risk of spontaneous preterm delivery and the onset of BPD in the offspring. Preterm delivery is a major challenge in the field of obstetrics and neonatology. Preterm newborn are at higher risk of both short- and long-term pathological outcomes, BPD being one of the major concerns. This study suggested that metabolic profiling of the AF will be able to predict cases of spontaneous preterm birth and fetuses at risk of BPD, supporting the hypothesis that some prenatal metabolic disruption plays a key part in the pathogenesis of preterm delivery and the onset of BPD [6]. The term recurrent respiratory infections (RRI) covers a broad set of conditions that can be involved in the pathogenesis of asthma and chronic obstructive pulmonary disease. In a pilot study, we applied metabolomics to the analysis of urine samples from children with RRI (as compared with those obtained from healthy peers), before and after a 3-month period of treatment with a synthetic immunostimulant, pidotimod [7]. Data modeling generated a robust model capable of distinguishing between the urine samples from the children with RRI and those of healthy controls, and suggesting that the former have a dysregulated metabolic profile. After pidotimod treatment, the metabolic profile of the children with RRI was partially restored to normal, except for the persistence of some microbiome-related variables. In conclusion, metabolomics can be considered one of the core disciplines of systems biology with the potential to reveal new metabolic pathways, prompt the formulation of new pathogenic hypotheses, and enable the identification of new treatment targets.

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LECT 24

NEW BIOMARKERS AND “OMICS” IN PRE-ECLAMPSIA

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Every day, approximately 830 women around the world die from preventable pregnancy or childbirth-related complications [1] and more than 99% of these deaths occur in low-resource settings, i.e. low-income countries [2]. Pre-eclampsia (PE), a hypertensive disorder of pregnancy affecting 3-5% of all pregnancies [3] and marked by hypertension in conjunction with proteinuria, is among the leading causes of maternal and perinatal death worldwide [4]. About 12% of all maternal deaths worldwide is caused by eclampsia; however, this mortality rate varies consistently among countries, being significantly lower in several high-income nations [5]. The worldwide perinatal deaths attributable to PE and eclampsia can be condensed into three data: 18 late fetal deaths (≥ 28 weeks of gestation) per 1,000 births; 8 early neonatal deaths (≤ 7 days of life) per 1,000 births; 3 late neonatal deaths (between 7 and 28 days of life) per 1,000 births [6]. The placenta is the exclusive cause of the disease; accordingly,

the therapeutic treatment for PE continues merely to be delivering the baby, even preterm when the severity of the disease becomes at risk for both the mother and the fetus. The delay in diagnosing PE can elicit potentially life-threatening complications including eclampsia, HELLP syndrome (hemolysis, elevated liver enzymes and low platelets), placental abruption, disseminated intravascular coagulation, stroke, multiorgan dysfunction. In most cases, PE leads to serious health problems later in the life of both mothers and their children; indeed, they could develop hypertension, type-2 diabetes, ischemic heart disease, stroke, venous thromboembolism, and death from any cause [7]. Early-onset PE is associated with an even greater risk of future cardiovascular disease (CV). It is largely assumed that eclampsia was mentioned in the ancient Egyptian, Chinese, Indian, and Greek medical literature; however, no historical document supporting this thesis has been found. The term eclampsia was firstly coined in 1739 by Boissier de Sauvages, who intended it to distinguish eclampsia-derived convulsions from epilepsy. The name was derived from the Greek word *exlampsiēz* meaning lightning, shining, brilliance and reflecting the sudden onset of convulsions in pregnant women. In 1843 John Lever and James Y. Simpson simultaneously discovered the presence of albumin in the urine of pregnant women with puerperal convulsions and ultimately in 1897 Louis H. Vaquez and Pierre-André Nobecourt demonstrated the presence of hypertension. Later, the additional term PE was assumed to identify the state preceding eclampsia. In 1901, the early prevention of PE was designed as a crucial goal in prenatal care. In 2014 the International Society for the Study of Hypertension in Pregnancy (ISSHP) has changed the diagnostic criteria for PE by defining the disease as the recognition of *de-novo* hypertension after the 20th week of gestation combined with a urine protein excretion rate > 300 mg/day and co-existing fetal and maternal multi-organ dysfunctions: neurologic disturbances such as seizures and headache; kidney disease; pulmonary edema; intrauterine growth restriction; utero-placental impairment; thrombocytopenia; coagulation abnormalities. According to gestational age at diagnosis or delivery, PE has been classified as early (< 34 weeks) or late (≥ 34 weeks). The former is uncommon (prevalence 0.38% or 12% of all PE), while the latter represents the majority of all cases of PE (prevalence 2.72% or 88% of all PE). The endothelium is the target of the disease being implicate in the pathogenesis of both hypertension and proteinuria. Peripheral vasoconstriction and

decreased arterial compliance characterize the hypertension of PE, while a pathognomonic kidney injury known as glomerular endotheliosis is associated with proteinuria.

Basically, genetic and environmental factors are the main etiological factors involved in the pathways leading to defective deep placentation; unfortunately, the pathogenic mechanisms of PE have been not exhaustively elucidated [8]. The leading hypothesis is that the disease is primarily due to the failure of physiological transformation of the spiral arteries, which in turn leads to the utero-placenta ischemia and hypoxia together with impaired fetal growth. Other factors may be involved in triggering placental dysfunction including epigenetic changes, inflammation, oxidative stress, deficient heme oxygenase expression, altered natural killer cell signaling, and deficient catechol-O-methyl transferase. Placenta infarcts coexist with sclerotic narrowing of arteries and arterioles enhancing the inadequate remodeling of the uterine spiral arterioles. Concomitantly, placenta angiogenesis becomes inadequate because of the blockade of the pseudovasculogenesis, i.e. the property of cytotrophoblast cells to lose their epithelial phenotype and to differentiate in endothelial-like phenotype leading to maternal vascular re-modelling. Consequently, cytotrophoblasts adopting an endothelial phenotype express adhesion molecules classically found on the surface of endothelial cells. The multi-organ failure in PE is triggered by several soluble factors released by the diseased placenta into maternal circulation; these factors mediate endothelial dysfunction (glomerular endotheliosis, increased vascular permeability, intravascular inflammation) and a systemic inflammatory response resulting in tissue hypoperfusion and ultimately in end-organ damage. The diagnosis of PE is affected by a number of pitfalls and in most cases it is overdue. Firstly, clinical features as well as the course of the disease are highly variable between subjects. Secondly, signs and symptoms are nonspecific, including hypertension, proteinuria, platelets count less than 100,000 cells/ μ L, increased serum transaminase levels, headache, and persistent visual disturbances. Thirdly, most of the mentioned symptoms as well as any others including dyspnea and epigastric and/or chest pain do not adequately predict adverse maternal outcomes [3]. In addition, it is still unclear whether or not proteinuria may be included for the diagnosis of PE. Some authors claim that the rationale for omitting proteinuria lies on the evidence that in many circumstances PE

manifest itself before glomerular capillary endotheliosis becomes severe enough to induce proteinuria [9]. Interestingly, PE adverse clinical outcomes consisting of severe hypertension and premature delivery are more likely in women without proteinuria; in addition, when present the severity of proteinuria has limited prognostic value [9]. The low accuracy in diagnosing PE in conjunction with the potential severity of the disease have pushed to the early identification of women at risk to develop PE for monitoring the course of their gestation and avoiding a sudden onset of the disease. High-risk pregnant women include those with preexisting hypertension, chronic kidney disease (CKD), diabetes, and previous PE. Unfortunately, neither specific set of symptoms nor reliable tests can predict PE accurately. On one hand, women considered at high risk may be over-managed, leading to increased costs of monitoring (e.g. unnecessary hospital admission) and greater uneasiness; on the other hand, this approach may miss women recognized as subjects at lower risk. Thus, there is the need to accurately predict PE, especially by using tests with a very high negative predictive value (NPV). Over the last decade, several scientific bodies and working groups have tested the diagnostic accuracy of emerging candidate biomarkers with the aim to better predict and detect PE. Obviously, biomarkers of angiogenesis were extensively investigated and two candidate biomarkers received the greatest interest: a soluble form of the vascular endothelial growth factor (VEGF) called FMS-like tyrosine kinase-1 (sFlt-1, an antagonist of proangiogenic proteins) and the circulating placental growth factor (PlGF). In PE, the former is increased while the latter is decreased. Changes in the circulating levels of these biomarkers are detectable prior to the onset of PE. Notably, the increased risk of PE is better recognizable by a high ratio of sFlt-1 to PlGF rather than either biomarker alone. At least four commercially available methods for measuring sFlt-1 and PlGF can be routinely implemented in clinical laboratories; two out of four have been recently recommended by the National Institute for Health and Care Excellence (NICE) to rule out PE in women between the 20th and 35th week of gestation [10]. A large observational study have reported that a sFlt-1-to-PlGF ratio ≤ 38 (obtained by using the Elecsys® immunoassay, Roche Diagnostics, Bern, Switzerland) rules out PE, eclampsia, and HELLP syndrome within the next week with a negative predictive value of 99.3% (95% confidence interval [CI] 97.9-99.9) [11]; these

findings have been subsequently confirmed by several published studies. The most important advantages derived from the measurement of angiogenic and antiangiogenic factors in maternal plasma are: (a) identification of the majority of women who will develop early PE; (b) positive correlation between the magnitude of their plasma concentration and the severity of the disease; (c) high prognostic value for maternal and perinatal complications [12]. In addition, at least four published studies performed in different settings have demonstrated considerable costs saving when these biomarkers are used in clinical practice. However, the analysis of the literature suggests the need of further clinical trials to establish definitively the clinical reliability of these tests [13]. Various studies investigating candidate biomarkers for predicting PE, such as inhibin A, activin A, placental protein 13, soluble endoglin, pentraxin 3, P selectin, neither provided encouraging results nor were subsequently corroborated. New perspectives emerge from the measurement of cell-free fetal DNA, probably originating from apoptotic trophoblasts: preliminary results showed that this test can accurately predict PE before 20 weeks of gestation. Similarly, the quantification of cell-free mRNAs encoding relevant proteins seems to be hopeful. Recently, a number of case-control studies using transcriptomics, proteomics and metabolomics approaches have published, even in the first trimester. In particular, metabolomics studies contribute to discover the molecular phenotype correlated with PE and to diagnose PE at a very early stage [14]. Despite their heterogeneity in samples size and type (plasma, serum, urine, placental tissue), analytical methods, clinical settings (early-onset or late-onset PE), and statistical approaches, metabolomics studies allow the early identification of metabolic pathways altered by the individual risk of PE long before the onset of the disease as well as the identification of emerging candidate biomarkers of PE. Most of metabolomics studies have found significant abnormalities in pathways involved in the lipid, oxidative stress and energy metabolisms, suggesting that the attack of reactive oxygen species (ROS), and oxidative stress may be considered the most important contributing factor to the pathogenesis of late PE. Other studies have also found aberrant changes in the taurine, glutamate and phospholipid metabolism. Interestingly, branched chain amino acids (valine, leucine and isoleucine) and propionic acid pathways were found altered in PE, confirming the association between PE with insulin resistance

and metabolic syndrome; indeed, branched chain amino acids are known markers of insulin resistance. Robust evidences designated hippurate and stearyl carnitine as novel metabolomics biomarkers for the prediction of PE. Further potential sensitive and specific candidate biomarkers for PE diagnosis and prognosis have been proposed, including taurine, proline betaine and proline. Finally, one study investigated the metabolic profile of the placenta tissue, pointing out a highly significant altered metabolic state in PE compared to controls as well as between severe and non-severe PE. Despite the encouraging results published in the literature, they should be confirmed by further large prospective cohort studies.

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LECT 25

MATERNAL MILK: IMMUNOMICS AND MICROBIOMICS

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Several scientific evidences established that breastfeeding provides short and long-term protection against a myriad of health outcomes, such as allergic diseases, diabetes, metabolic disorders. These beneficial effects are mediated, at least in part, by components that are specific and unique of the human milk (HM). About these components extensive research has not yet been conducted because of the wide variability in concentrations among women and the wide number of components present in the samples. Furthermore, the large variety of immune active components that are present are at varying concentrations among mothers and among different times of lactation in the same subjects. Whilst HM provides macronutrients, micronutrients and vitamins essential for appropriate growth and development of the infant, it also includes a multitude of bioactive factors including immunoglobulins (IgA), cells (lymphocytes, neutrophils, etc.), cytokines, growth factors, and antimicrobial proteins that promote protection from infections as well as maturation of gut immunity [1]. The standard approach in HM research is to study one or two components, for instance PUFA, nano-vesicles, immunologically active molecules or particular pathways such as Th1/Th2 cytokines. Also soluble receptors and growth factors have been studied in isolation. It is likely that no single molecule but rather the synergistic combination of multiple molecules may influence the immunological outcomes developing later in life in the offspring. Therefore, extrapolation of results from studies is often limited [2]. In a

study from our group, multiple active immune molecules at two stages of lactation, in colostrum and in mature milk, have been evaluated. In order to explore associations of these components of HM and infant health outcomes, appropriate statistics such as models were employed. Principle component analysis (PCA), which allows pattern identification and clusters data and assesses if any trends are present, has been employed. Aim was to measure multiple immune components in HM and to explore physiological patterns (“lactotypes”), and determine associations between lactotypes and infant immunological outcomes (atopy, eczema, early wheezing). Although more extensive studies are required to understand the factors that determine HM lactotypes, we were able to identify particular lactotypes that are associated with infant immune outcomes such as cough/wheeze.

Metabolomics or the study of metabolites, could be useful to clarify the complex interactions of HM constituents, and to understand the physiological state of HM in various stages of lactation. Metabolomics, together with other “omics” arena such as proteomics and glycomics and genomic methods, can enable us to better understand this complex and dynamic relationship. Several complementary analytical platforms such as nuclear magnetic resonance, capillary electrophoresis, liquid or gas chromatography coupled with mass spectrometry have been proposed to characterize the HM components. In a recent study more than 700 metabolites have been identified in HM.

Furthermore, the development of the immune system is implicated in incidence of allergy and autoimmune disease as well as metabolic syndrome and has recently been linked to gut maturation and type of gut colonization early in life. HM is able to provide, immediately after birth, several factors, including also microorganisms other than growth factors, oligosaccharides, useful for a correct early infant gut colonization. This colonization should be based on different arrays of microbial diversity, characteristic that for the moment the formula-based milk cannot provide.

In the recent years the development of cultivation-independent techniques, such as bacterial DNA extraction and 16S gene sequencing, for the study of bacterial population of different biological samples allowed a deeper analysis of bacterial diversity. Therefore, the presence of a HM microbiome has been not only confirmed, but also better characterized for the presence of a variety of microbes and their associated genes and antigens

transmitted to the infant during breastfeeding. These data show that microbiota of HM is a dynamic, and complex, ecosystem with different bacterial networks among different populations containing diverse microbial hubs and central nodes [3]. These hubs and nodes are probably influenced by several factors such as genetic predisposition, diet and ethnic characteristics, use of antibiotics, perinatal infections, etc. Being dynamic, HM microbiota deeply changes during the transition from colostrum to mature milk. Furthermore, in our study a greater abundance of anaerobic intestinal bacteria in mature milk compared with colostrum samples has been observed [3]. In conclusion, different factors can contribute to modulate the HM composition. Out of them, probably specific foods or food supplements may represent a more direct and sustainable strategy to protect newborns from the onset of several infections and diseases, promoting the growth of probiotic and beneficial bacteria with a protective role for the host.

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LECT 26

NUTRIMETABOLOMICS OF MILK: COLOSTRUM AND MORE

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Metabolomics is able to provide a snapshot of the metabolome, the complete set of metabolites produced by an organism, a mirror that reflects the physiological, developmental and pathological state of a biological system. The application of

metabolomics in neonatology offers an approach to investigate the complex relationship between nutrition and child's health. The characterization of the metabolome of the maternal milk compared to that of formula milk allows to understand how every nutrients affects the neonates metabolism, and could offer the possibility to intervene in the diet composition according to their nutritional needs [1]. The first study ever performed on the metabolome of maternal milk was carried out by Cesare Marincola et al., in which the composition of the maternal milk was examined through the first month of lactation of preterm infants with low birth weight. For the purpose of comparison, formula milks were analyzed as well [2]. The results showed that maternal milk contains higher quantity of lactose compared to formula milk, which is, however, more rich in maltose. It was also found that there are differences in milk composition depending on the gestational age and the metabolic profile of the milk changes even after birth, in particular in the carbohydrates composition. Furthermore, there is a quantitative and qualitative difference in the fatty acids profile, indeed, their concentrations are higher in maternal milk compared to formula milk [2]. Longini et al. [3] also compared the maternal milk composition with regard to formula milk. It has been established that the lactose concentration is higher in maternal milk, while maltose and galactose 1-phosphate are higher in formula milk. The time-related variations of the maternal milk metabolome was investigated by Villaseñor et al. The concentration of several metabolites increases after birth, such as the palmitoleic acid, oleic acid, phosphoglycerides; while others decreases, such as phospholipids, cholesterol, alpha-tocopherol [4]. Another study, performed by Urbaniak et al. [5], investigated the effect of chemotherapy on milk of women to whom a chemotherapy regimen was administered to treat Hodgkin's lymphoma. It was observed a decrease in *Bifidobacterium spp.* and CONS (*coagulase-negative Staphylococci*) in women that received the chemotherapy regimen, with a subsequent alteration of the neonate intestinal microbiome. Recently, particular attention is given to the metabolomics analysis of the oligosaccharides of the milk (HMO), the third most abundant component after the lactose and fatty acids. Over 200 HMO have been described and it is well known that each woman synthesizes a different set of oligosaccharides. In fact, the HMO composition depends on the expression of some glycosyltransferases. Two genes, crucial for the determination of the oligosaccharides profile that the

mother produces, are: the Secretory genes (Se) and the gene of the Lewis blood group (Le) [6]. In the study of Spevacek et al. [7] several milk metabolites of women who delivered at term were measured. 65 metabolites were identified by NMR spectroscopy. Metabolites were classified as sugars, amino acids and derivatives, fatty acids, vitamins, nucleotides and others. In particular, the different oligosaccharide composition of maternal milk implies the presence of different maternal phenotypes that regulate the concentration of sugars. In the study by Praticò et al., NMR spectroscopy showed 3 different profiles of breast milk. The first group was compatible with the Se+/Le+ phenotype, the second with Se-/Le+ and the third with Se+/Le- [8, 9]. Thus, the metabolic profile could be characterized through the identification of 3 different classes on the base of the oligosaccharides profile. This approach may provide a powerful tool for the evaluation of the effects of the patho-physiological conditions of the mothers on the milk and the influence of the diet on milk metabolome. Spevacek et al. compared the breast milk of mothers who delivered at term and preterm, identifying 69 main metabolites. 10 of 15 at term mothers and 10 of 13 preterm mothers were classified as Secretors. HMO have different functions, in particular oligosaccharides of Secretory phenotype protect against infants diarrhea thanks to their specific anti-adhesive and bifidogenic functions and promote the maturation of the intestinal tract in preterm neonates. The reduction of oligosaccharides in 1-2-fucosylated is related to an increased risk of neonatal pathologies, thus these oligosaccharides are fundamental for the protection against several infective pathologies. They can represent a component of the innate immunity, by which the mother can protect the infant against environmental pathogens. The future of research seems to involve metabolomics and milk microbiota. Maybe, thanks to the integration of these two fields, significant progresses could be made in the understanding of the composition and functions of maternal milk [9].

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LECT 27

AUTISM FROM CONVENTIONAL DIAGNOSIS TO METABOLOMICS: URINARY METABOLOMICS PROFILE IN A POPULATION OF CHILDREN WITH AUTISM SPECTRUM DISORDERS

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INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impairment in social-communication skills, and restricted and repetitive interests. Epidemiological studies show a recent increase in worldwide prevalence rates of ASD, currently estimated over 1% with a 4:1 M/F ratio. Despite the progress in understanding the neurobiology of ASD, the causes remain still unknown. A complex relationship between genetic, epigenetic and environmental

factors contributes to ASD etiopathogenesis and is responsible of the clinical phenotypic heterogeneity [1]. Many epigenetic factors, such as maternal infection, environmental toxins exposure, have been associated with ASD. Currently, the diagnosis is still clinical, based on standardized neuropsychological assessment; no valid and specific biomarkers for ASD have been identified yet. Metabolomics provides a direct functional read-out of the phenotype by the detection, identification, and quantification of as many metabolites as analytically possible in biological fluids with the aim to search variations that can be used to discriminate between comparative samples [2]. Recent evidences show a different urinary metabolomics profile between ASD children and their unaffected siblings [3].

MATERIALS AND METHODS

We enrolled 105 children, aged 18 m-11 y: 38 ASD, 35 siblings, 32 typical developing children without ASD familiarity. Morning urine samples were collected from all the participants, GC-MS quantification of urinary metabolites, multivariate statistical and standardized neuropsychological assessment were performed.

RESULTS

Our findings show two distinct urinary metabolomics profiles in the ASD population compared to their unaffected siblings. The loading plot analysis reveals the different clusterization among the two groups. The main metabolic perturbations include high concentrations of mammalian-microbial metabolites, alteration of tryptophan pathway and low levels of uric acid (**Fig. 1**). Our study suggests a potential role of these pathways (mitochondrial dysfunction; antioxidant status; gastrointestinal dysbiosis) in the etiopathogenesis of ASD comorbidity (epilepsy, gastrointestinal disorders, sleep disruption).

CONCLUSIONS

Preliminary clinical evidence shows that ASD children have a distinct urinary metabolomics profile compared to their unaffected siblings. These findings can provide a clinician with a possible biomarker, representing metabolomics a network-based approach. Metabolomics, as emerging tools of network medicine, offers a platform to explore the molecular complexity of ASD and the molecular relationships among apparently distinct (patho) phenotypes related to external perturbations.

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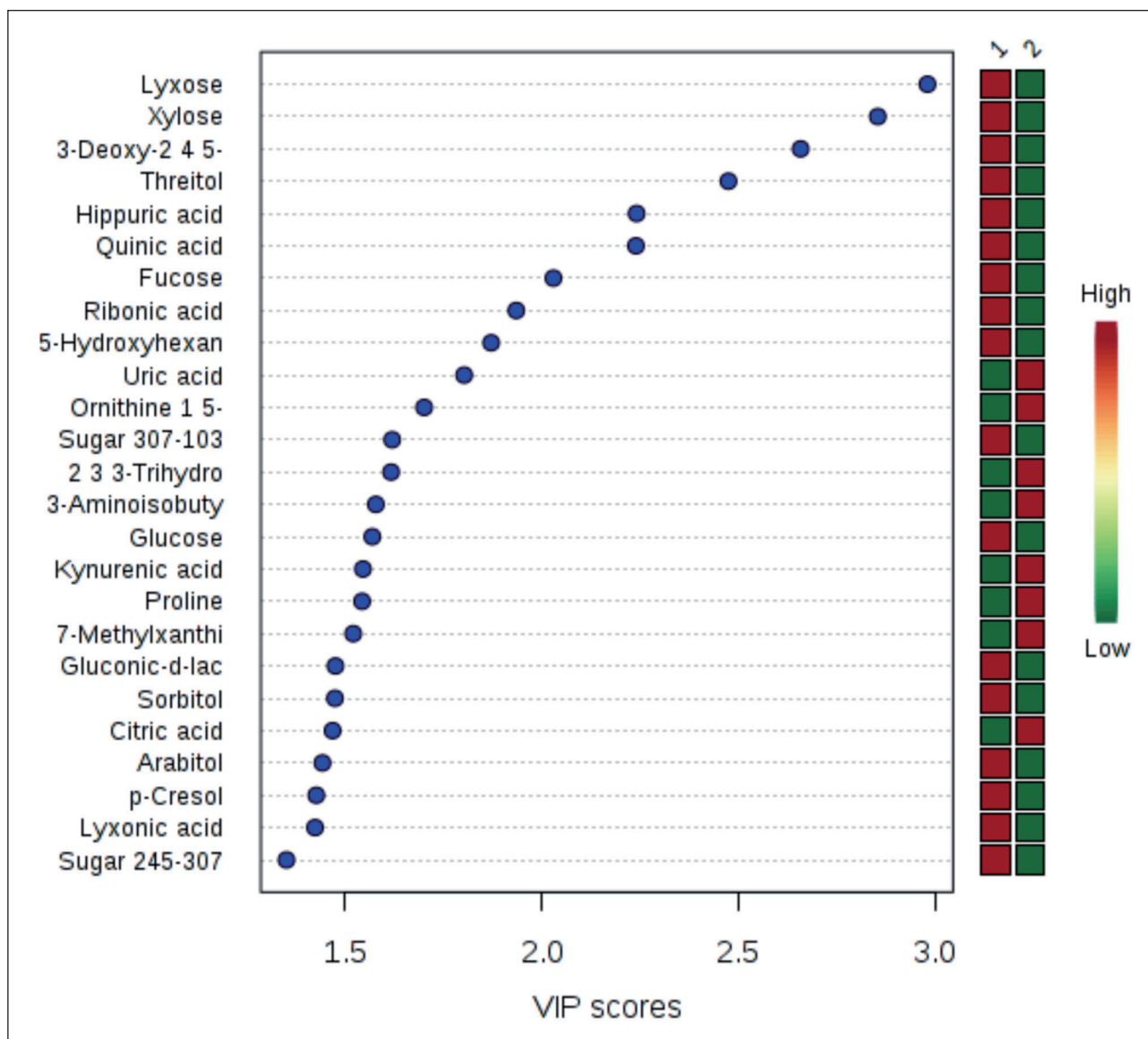


Figure 1 (LECT 27). The 25 metabolites more discriminant among ASD children (Class 1) and unaffected siblings (Class 2).

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LECT 28

PLACENTAL BIOMARKERS

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INTRODUCTION

Some placental (Pl) substances are of extreme interest in clinical practice for assessing the development of pregnancy (Pr). Since the 1st trimester of Pr, it is possible to assay in the maternal blood some parameters that allow for screening of fetal conditions and, in particular, fetal chromosomal anomalies. By adding to these parameters the evaluation of anamnestic and biophysical parameters (mean arterial pressure and flow of uterine arteries) it is also possible to perform a precocious screening of preeclampsia (PE). Aspirin (Asp) treatment more

than placebo administered from 11 to 14 weeks of Pr until 36 weeks of Pr is capable of improving the PI blood perfusion and preventing the PE [1]. Another substance in the maternal bloodstream is an estrogen, estetrol (E4), secreted by the fetal liver exclusively during Pr. The E4 passes in the amniotic fluid and in the maternal blood (MB). Although the increase of E4 levels in the MB during the Pr, for follow-up and survey of Pr pathology E4 levels were not suitable due to the large intra- and inter-individual variation of plasma levels [2]. However, its physiologic role in Pr has been investigated. The studies so far carried out demonstrate that E4 exerts an important role in the regulation of the fibrinolytic protein system in endothelial cells, showing a key action in the vascular system, with potential implications for the local control of blood clotting and for vascular remodelling [3]. A recent study in cell cultures of rat's hippocampus demonstrates that E4 exerts an antioxidative action mostly dependent on estrogen receptor (ER) α (ER α) and ER β [4]. The same study demonstrates that E4 exerts an important effect on neurogenesis and possibly promyelinating activities through its link with ER β [4]. In the context of placental biomarkers, our focus was on early markers of PE.

MATERIAL AND METHODS

Precocious screening of PE was performed in 700 women at the 1st trimester of Pr [1]. Asp treatment was started in at risk of PE subjects from the screening up to the 36th week of Pr.

RESULTS

The test was capable of detecting 58 women at risk for PE. The Asp treatment from the screening up to the 36th week of Pr impeded in these subjects the occurrence of PE.

CONCLUSIONS

The Asp treatment in selected women at risk of PE since the 1st trimester of Pr reduces the occurrence of this pathology. However, only an adequate screening can indicate women susceptible to treatment. Further studies are needed to clarify the exact role of E4 in the protection of endothelium and brain during the fetal life.

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LECT 29

BIOMARKERS IN NEONATAL SEPSIS

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Neonatal sepsis (NS) is one of the leading causes of neonatal morbidity and mortality, particularly in preterm infants. Early diagnosis and treatment is of vital importance in NS in order to decrease mortality. It is difficult to distinguish the clinical findings of NS, especially for early-onset NS (EONS) during the initial period of sepsis, from non-infectious causes because there are no specific signs or symptoms in EONS. Obtaining a positive blood culture is considered a definitive diagnostic tool in sepsis, but this is a time-consuming procedure. To avoid the unnecessary treatment of uninfected patients, an early, sensitive, and specific laboratory test would be helpful to guide clinicians in deciding whether or not to start administering antibiotics. C-reactive protein (CRP), white blood cell count, absolute neutrophil count, and immature/total neutrophil ratio are the most widely used tests in the diagnosis of NS. Many biomarkers can be used in NS. CRP is frequently used in the diagnosis of EONS. The delayed CRP elevation at the onset of infection showed that it should not be used alone in the diagnosis of sepsis and in deciding to begin antibiotherapy. A single determination is often not helpful in determining infection, serial CRP measurements may be more useful in evaluating the efficacy of treatment. Procalcitonin (PCT) as a biomarker may also be used to monitor the activity and the prognosis of severe bacterial infections, but it has some limitations in the diagnosis of NS. In EONS, PCT measurements at birth may initially be normal; serial PCT measurements at 24 h of age may be more helpful for an early diagnosis. We found that during the first 24 h of life PCT is a more sensitive marker of infection than CRP. Because the dynamics of PCT and CRP are time-dependent, serial PCT and CRP measurements at birth and at

24 h of age may be more helpful for the diagnosis of EONS. Urinary Neutrophil Gelatinase-Associated Lipocalin (uNGAL) may be helpful as an early marker of nosocomial infections in newborns. We found that uNGAL has higher sensitivity, specificity, and positive and negative predictive values than CRP and PCT in the diagnosis of late sepsis in premature newborns. Furthermore, contrary to other blood biomarkers, uNGAL prevents unnecessary phlebotomies; it may be an ideal biomarker in NS. But we need further studies to confirm these results for NS cases. Serum amyloid A (SAA) seems to be a better marker than CRP and has a better prognostic value during the first 24 h after NS onset. Various cytokines have also been studied, although they have limitations such as their inconsistent cutoff values, delay in getting results, and lack of availability for routine measurements in clinical settings. According to a recently published study, neutrophil CD64 marker in EONS had sensitivity, specificity and a negative predictive value of 100%, 68%, and 100%, respectively.

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LECT 30

ACUTE KIDNEY INJURY IN NEWBORNS

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Acute kidney injury (AKI) is the sudden decline in renal function that leads to derangements in fluid balance, electrolytes and waste products, occurring in 16-70% of neonatal populations. Newborns, especially preterm babies, are at risk for AKI due to low glomerular filtration rate, immature tubular functions and many life-threatening conditions that might result in AKI, like perinatal asphyxia, major surgery, sepsis or congenital heart disease. The drugs used to treat these disorders might themselves lead to or exaggerate AKI. The definition of AKI is problematic in newborns since serum creatinine (sCr), the most commonly used marker of renal injury, is dependent on maternal levels in the first day of life and there is a 48-72 hours of delay in the rise in sCr after a major insult. Urine output, which also is an important clinical sign in AKI, might not be helpful since many cases of AKI in newborns are non-oliguric. There is a need to identify novel and practical biomarkers that can be detected either in the blood and/or urine to diagnose AKI in newborns. Some of these biomarkers are: urinary neutrophil gelatinase-associated lipocalin, kidney injury molecule-1, cystatin-c, matrix metalloproteinase-8, osteopontin, uromodulin, interleukin 18, epidermal growth factor, trefoil factor 3, β 2 microglobulin, interleukin-18. Urinary metabolomic profile is another promising method (**Tab. 1**). The inclusion of these biomarkers in neonatal AKI classifications can increase their accuracy. In this lecture, I will go through the diagnostic criteria and use of biomarkers in neonatal AKI.

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Table 1 (LECT 30). Biomarkers in acute kidney injury (AKI).

Study	Biomarker	Comment
Du, 2011	Urinary KIM-1, NGAL, β 2MG, IL-18, osteopontin	232 children admitted to ED KIM-1, NGAL and β 2MG predict 25-50% decrease in Cr clearance
Zappitelli, 2011	Plasma Cyst-C	228 children with cardiac surgery Postop Cyst-C predicts PICU stay
Buelow, 2012	Urinary NGAL, IL-18	20 children with cardiac surgery Urinary NGAL, IL-18 predicts AKI
Genç, 2013	Urinary KIM-1	48 preterms Serial KIM-1 predicts AKI
Basu, 2014	Plasma NGAL, MMP-8, Ela-2	214 septic children in PICU Biomarker+ Risk scoring
Mc Caffrey, 2015	Plasma NGAL, Cyst-C, Urinary NGAL, KIM-1	49 children in PICU Plasma NGAL predicts AKI Cyst-C shows rise in sCr
Westhoff, 2015	Urinary TIMP-2, IGFBP-7	133 children Both biomarkers predict AKI
Sweetman, 2016	Urinary albumin, NGAL, β 2MG, Cyst-C, osteopontin, uromodulin	Babies with NE, KDIGO Day2 Cyst-C predicted AKI
Askenazi, 2016	14 urinary biomarkers: albumin, Cyst-C, NGAL, KIM-1, EGF, uromodulin, osteopontin, clusterin, α GST, VEGF, TFF3, β 2MG	113 VLBWs, urine in DOL 1-4 Lower urinary EGF and uromodulin; higher Cyst-C, NGAL, osteopontin, clusterin, α GST levels in babies with AKI
Hanna, 2016	Urinary NGAL, EGF	45 preterms, 25 with AKI NGAL higher, EGF lower in AKI
Oncel, 2016	Urinary NGAL, NTN-1, NHE3, IL-18	45 babies with perinatal asphyxia NGAL, NTN-1, NHE3, and IL-18 higher in DOL 1 in AKI
Elmas, 2017	Urinary NGAL, Cyst-C, KIM-1	64 critically ill preterms Higher urinary NGAL in DOL 1, 3, 7 in babies with AKI
Mercier, 2017	Urinary metabolomics	\leq 31 weeks and/or \leq 1,200 g babies Urinary hippurate & homovalinate predicted AKI

ED: Emergency Department, PICU: Pediatric Intensive Care Unit, NE: neonatal encephalopathy, DOL: day of life, TFF3: trefoil factor 3.

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LECT 31

BIOMARKERS IN CARDIOLOGY

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A “biomarker” or “biological marker”, refers to a large number of indicators of a biological state which can be measured with accuracy and reproducibility. By definition, biomarkers are an objective and quantifiable measures of biological or pathogenic processes or response to pharmacological therapy and may do not necessarily correspond with subjective patient’s perception of his/her own health [1]. In literature have been reported many definitions of biomarker, which often overlap considerably. The first use of this word stated in the 50s [2]. A more modern definition was released in 1998, when the National Institutes of Health

Biomarkers Definitions Working Group defined it as “a characteristic that is objectively measured and evaluated as an indicator of normal biological or pathogenic processes, or pharmacologic responses to a therapeutic intervention” [3]. Three years later the International Programme on Chemical Safety of the World Health Organization (WHO), together with the United Nations and the International Labor Organization, defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence and outcome of a disease” [4]. A broader definition takes into account not only the incidence and outcomes of a disease, but also the effects of therapies, interventions, nutrition and even environmental exposure to pollution. In this respect, the WHO has stated that a correct definition of biomarker includes “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction” [5]. Medical signs are as old as medical practice itself, but they are also vague and sometimes contradictory. Conversely, biomarkers are objective, quantifiable and reproducible in laboratory setting. Their use is somewhat new, and they need to be developed and refined further. In medicine their aim is usually determining a disease state or predicting relevant clinical endpoints. Many biomarkers have been proposed in Cardiology, the most important and used of whom are troponins and brain natriuretic peptides. Cardiac troponins are proteins involved in the contractile system of cardiac cells, which in turn is primarily controlled by changes in intracellular calcium concentration. In general, when calcium rises, the heart contracts, whilst when calcium drops, the heart relaxes. Furthermore, troponins are also markers of myocardial necrosis, with their blood concentrations exactly representative, in subjects with normal renal function, of degree of cardiac injury [1, 6, 7].

Specifically, certain subtypes of troponin (cTnI, i.e. cardiac I and cTnT, i.e. cardiac T) are very sensitive and specific markers of myocardial damage. Increased troponins concentration mean myocytes death, since these molecules are released into the blood after cardiac damage. Troponins can be detected in patient’s blood 3-6 hours after onset of the chest pain, reaching peak level within 16-30 hours. Furthermore, raised cTnI and cTnT blood levels can be detected even 5-8 days after

onset of myocardial infarction [8-10]. Non cardiac conditions inducing an increase in troponin levels (differential diagnosis) include sepsis, gastrointestinal bleeding, chemotherapy, ascending aorta dissection, chronic obstructive pulmonary disease, pulmonary hypertension, hemorrhagic stroke, end-stage renal disease, strenuous endurance physical exercise, inflammatory muscle diseases and pre-eclampsia. All of them are not primary heart diseases, but they exert indirect effects on heart muscle [11]. Troponins are useful diagnostic tools even in Neonatology, for example in the medical condition known as asphyxia. The latter results from lack of oxygen to a newborn during his/her birth that lasts long enough to cause ischemia to brain and other organs, heart included [12, 13]. As a consequence of asphyxia, electrocardiogram alterations, represented by T waves inversion and pathological Q waves as well as increase in troponins concentration similar to that observed in adults suffering from myocardial infarction, have been described [14]. There are also several literature reports showing good correlation among troponins raise and echo-derived markers of myocardial dysfunction [15]. However, troponins dosage in Neonatology still remains a research tool. More studies are needed to explore their role in prognosis and in monitoring response to treatment in case of newborns’ cardiac involvement. For example, in preterm infants the effect of inotropes on myocardial function needs further studies and troponins may play a crucial role in this setting [16]. On the other hand, brain natriuretic peptide (BNP) is a peptide released from stretched left ventricular myocardial cells and related to volume overload (i.e. in congestive heart failure with or without symptoms). Even a BNP fragment, named NT-proBNP, is able to predict cardiovascular adverse events and cardiac death in patients with acute or chronic congestive heart failure [1, 17]. The higher is NT-proBNP, the worse is patient’s prognosis [18]. BNP induces a decrease in blood pressure due to a drop in systemic vascular resistance (afterload) as well as a decrease in cardiac output due to a decrease in central venous pressure (preload) caused by the reduction in blood volume that follows natriuresis and diuresis [18]. In Neonatology the biomarker NT-proBNP is a useful tool in monitoring persistent patency of ductus arteriosus as well [12, 13]. In fact, in preterm infants the latter can lead to significant hemodynamic consequences, depending on the magnitude of the shunting, which in turn is determined by three major interrelated factors: 1) length, diameter and morphology of the ductus,

which may affect blood flow; 2) the pressure gradient between aorta and pulmonary artery; 3) finally, the pulmonary and systemic vascular resistance [19]. A small or moderate ductal left-to-right shunt leads to an increase in pulmonary blood flow, whilst in case of a large shunt, left atrial and left ventricular enlargement may develop [20]. As a result of a recent meta-analysis, sensitivity and specificity for BNP were 88% and 92%, respectively, and for NT-proBNP 90% and 84%, respectively [21]. NT-proBNP is usually measured at day 3, and its serial measurements may be useful in assessing the clinical course of patent ductus arteriosus [22]. Based on these premises, brain natriuretic peptides clinical utility would be as a method of triaging cases of suspected significant patent ductus arteriosus, in order to decrease the need for echocardiograms, and to monitor treatment response [23]. As previously mentioned, other in depth studies are needed to better clarify the role of the already existing biomarkers in Cardiology as well as to identify others more specific and sensitive. In this respect, a new exciting era in medicine would be represented by metabolomics, one of the new “omics” sciences enabling creation of a photograph of the metabolic state of an individual exposed to different environmental factors and diseases. Its application in Cardiology may allow understanding the metabolic shifts that occur even before the clinical manifestation of pathologies, in order to find possible predictive biomarkers, which are earlier and more sensitive than those available at moment [24, 25]. In fact, in the last decade medicine has been changed in depth. Its goal for the next future is to be really personalized, perspective, predictive, preventive, precise, participatory, patient-centric, psychocognitive, postgenomic and public. In one word, a tailored or individualized medicine [26]. In this respect, even the possible future applications of stem cells, which are potentially able to produce a sufficient number of myocardial cells to repair a damaged heart, is hypothetically endless: a tailored regenerative medicine with less ethical problems and better outcomes for patients may be developed [27, 28].

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LECT 32

QUANTIFICATION IN NEONATAL URINE AND PROFICIENCY TESTING COMPARED TO NON-NMR METHODS

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Urinary analysis has been a challenge over many years due to the complexity of metabolic and ion composition and its rapid changes over time. Starting from neonates through childhood and adults, ionic matrix is changing very substantially and influences the analysis. For NMR (Nuclear Magnetic Resonance) Spectroscopy this means for example changes in chemical shift going from neonates to adults. This means changes in the overlap of signals to be quantified and needs to be validated independently for the 2 age ranges. Since NMR is an important tool in metabolomics analysis and is completely quantitative over the complete dynamic range of signals, which are typically spanning more than 5 orders of magnitude in difference, an automatic quantification procedure is of high relevance. First task to achieve this is to work under strict Standard

Operation Procedures (SOPs) under one field strength, based on the highest reproducibility of NMR this ensures data comparability independent of the instrument and the laboratory. This also allows remote analysis concepts to be applied. Using data created under such SOPs knowledge bases are developed for the 2 age ranges mentioned. These knowledge bases describe the metabolite signals to be quantified in details and contains rules like describing chemical shift ranges, signal multiplicity and overlap. Based on wet spiking according to DIN-Norm, a first idea can be created what the LOD for NMR looks like. However due to the complexity and changes in composition, wet spiking alone cannot solve the problem alone. A system of numerical spiking was developed, where metabolite signals from a pure compound spectrum are added to all urine spectra available for LOD determination. Numerical signal addition is done under changing intensity of the signals added and positional changes. Such more than 300,000 spectra are created, which are used for LOD determination. Based on this an automatic quantification of 150 compounds in neonate and adult urine was developed, that has highest precision and sensitivity towards metabolites to be quantified. To further ensure correctness of results, comparison to non-NMR methods was applied on a large number of spectra. Data thereof are presented. In addition, participation in ERNDIM (European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism) proficiency testing was executed over 2 years to confirm the urine analysis results. Using NMR resulted in more than 95% correct analysis as is shown in the presentation. New results will be demonstrated, using additional 2-dimensional results obtained during the screening process. This substantially reduces overlap and such also leads to much improved LODs and opens the way to quantification of many more metabolites in urine. In addition it is also shown, how the methodology is applied to plasma/serum analysis.

LECT 33

ENHANCING IMMUNE RESPONSES IN CHILDREN WITH RECURRENT RESPIRATORY INFECTIONS

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Acute respiratory infections (ARTIs) are one of the most common childhood illnesses. ARTIs incidence peaks are in the first 4 years of life, especially in children attending daycare. ARTIs usually involve the upper respiratory tract [1]. The majority of children with ARTIs involving the upper airways are otherwise healthy, just a minority are affected by an underlying immunological or non-immunological disease. A tendency toward hyporesponsive immune responses in early life, characterized by both reduced innate and adaptive immune responses, plays an important role in ARTIs recurrence in the first 6 years of life [1]. Over the last decades intensive investigations have been carried out on immunostimulants (IS). IS are defined as biologically active substances from natural or synthetic origin with different chemical characteristics and mechanisms of action. Among the available IS, probiotics, bacterial lysates, synthesis molecules and vitamins have been judged interesting for their *in vitro* and *in vivo* immunomodulatory properties [2]. IS have been shown to stimulate the immune system such as phagocytosis, complement, T- and B-lymphocytes, secretory IgA and cytokines [2]. Over the last years the mechanism of action of bacterial lysates has been explored. After an initial hypothesis of their exclusive action on adaptive immunity, the most recent data have shown that bacterial lysate also enhances innate immunity (dendritic and NK cells). Preclinical studies suggest that bacterial lysates stimulate regulatory T cells (Treg) and decrease the Th2 responses [3]. Pidotimod has an immunomodulatory activity on both innate and adaptive immune responses. Pidotimod induces dendritic cell (DC) maturation, upregulates the expression of HLA-DR and co-stimulatory molecules CD83 and CD86, stimulates DCs to release pro-inflammatory molecules, driving T cell proliferation and differentiation towards a Th1 phenotype, enhances natural killer cell functions, inhibits thymocyte apoptosis, and promotes phagocytosis [4, 5]. Modulation of the immune system is one of the most important mechanisms of action of probiotics underlying the beneficial effects on human health. Probiotics enhance both innate (NK, macrophages, granulocytes, dendritic cells, epithelial cells) and adaptive immunity (Th1, Th2, Th17, Treg, B lymphocytes) [6]. Vitamin D and lactoferrin showed interesting immunomodulatory properties [7, 8]. However the *in vitro* activity does not always result in an *in vivo* effect.

The most recent *Cochrane* meta-analysis reported a reduction of ARTIs following the administration of IS up to 40% [7]. However, some bias and limitations of these studies were reported such as heterogeneity in studied populations, the variable duration of the interventions and the poor statistical analysis [9]. A meta-analysis and review about probiotics and bacterial lysate found these IS to be better than placebo in preventing acute ARTIs. However, more trials are needed to confirm this conclusion. We recently explored *in vitro* and *in vivo* effect of Pidotimod in pediatric population. In children with Down syndrome, Pidotimod upregulates genes involved in the activation of innate immune responses and antimicrobial activity. Moreover, when administered with a virosomal influenza vaccine, Pidotimod potentiated the beneficial effect of the immunization, possibly resulting in a greater activity of both innate and adaptive immune responses [10]. We also performed a double-blinded randomized placebo-controlled trial study to assess the efficacy of Pidotimod in a population of 3-year-old healthy children, who just entered kindergarten [11]. Children were randomized to receive either Pidotimod 400 mg per os or placebo twice daily for the last 10 days of each month for 6 months. The incidence rate ratio for respiratory infections was 0.78 (95% CI 0.53 to 1.15, $p = 0.211$) for Pidotimod vs. placebo. The corresponding risk ratio for antibiotic usage was 0.56 (95% CI 0.27 to 1.16, $p = 0.120$). In this trial, Pidotimod showed some potential as a means for reducing antibiotic usage in these children.

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LECT 34

OBSTETRICAL MANAGEMENT IN TWIN PREGNANCIES

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Multiple pregnancies (MP) are defined as the pregnancies with two or more fetuses. It may result from fertilization of two or more oocytes, or from one zygote, which splits and forms two embryos. The incidence of MP has risen in the last 30 years due to pregnancies in an advanced maternal age, the use of fertility drugs for induction of ovulation and the increasing use of assisted reproduction techniques (ART). In developed countries, 1-3% of all pregnancies are obtained by ART and up to 24% of successful *in vitro* fertilization procedures result in MP. Other factors include family history of twinning, maternal height and weight, previous history of twin delivery and diet.

MP are associated with a broad range of possible complications for both the mother and the fetuses. MP have increased risks of miscarriage, anemia, hypertensive disorders, polyhydramnios, gestational diabetes, fetal malpresentation and postpartum hemorrhage. Newborns from MP are at significantly higher risks of early death, respiratory distress syndrome, intraventricular hemorrhage, sepsis and low birth weight, disabilities related to prematurity. The appropriate management of these complications involves an early detection to reduce the risk of adverse outcome. MP need more monitoring than women with singleton pregnancies. Gestational age, chronicity and screen for Down's syndrome should

be offered during a first trimester ultrasound scan. A correct estimated gestational age in MP avoids the risk of estimating it from a fetus with early growth pathology. The chorionicity is determined by the number of placental masses, the lambda or T-sign, membrane thickness and the discordant fetal sex. Risks depend partly on the chorionicity and amnionicity of the pregnancy. Monochorionic (MC) monoamniotic twin pregnancies, MC monoamniotic triplet pregnancies, MC diamniotic triplet pregnancies, dichorionic (DC) diamniotic triplet pregnancies should be referred to a tertiary level fetal medicine centre. In twin and triplet pregnancies there is a greater likelihood of Down's syndrome. The false positive rate of screening tests is higher in twin pregnancies and there is a greater likelihood of complications of invasive testing [1]. During the second and third trimester, twin pregnancies should be monitored to early recognize signs of twin-to-twin transfusion syndrome (TTTS), intrauterine growth restriction (IUGR) and risk of preterm birth. TTTS is a severe complication of MC twin pregnancies, characterized by the development of unbalanced chronic blood transfer from one twin to the other through placental anastomoses. Prevalence is 10-15% of all MC twins. Diagnostic monitoring with ultrasound for TTTS should start from 16 weeks and should be repeated monitoring fortnightly until 24 weeks. The presence of possible early signs of TTTS needs to be monitored weekly to allow time to intervene if needed [2]. Twin pregnancies have a higher risk of an IUGR fetus, but growth charts specific to twin and triplet pregnancies according to chorionicity of the pregnancy are not available. Diagnosis of clinically significant is now based on estimated fetal weight discordance (25% or greater difference) using biometric parameters at each ultrasound scan from 20 weeks. Research should evaluate clinical outcomes associated with growth velocity and trajectories. Women with twin pregnancies have a higher risk of spontaneous preterm birth. About 60% of twin pregnancies result in spontaneous birth before 37 gestational weeks (gw), about 75% of triplet pregnancies before 35 gw. Spontaneous preterm birth and elective preterm birth are associated with an increased risk of admission to a special care baby unit. Existing evidences for the effectiveness of cervical cerclage and tocolytics are limited. Beta mimetics seem to have an effective role, but no randomised controlled trials were identified. Data should also be reported separately according to different chorionicities and to different gestational ages at birth. Continuing

uncomplicated twin pregnancies beyond 38 gw and uncomplicated triplet pregnancies beyond 36 gw increases the risk of fetal death. Elective birth should be offered from 37 gw in uncomplicated DC twin pregnancies. In uncomplicated MC twin pregnancies elective birth should be offered from 36 gw after a course of antenatal corticosteroids because it does not appear to be associated with an increased risk of serious adverse outcomes [3]. In triplet pregnancies elective birth should be offered from 35 gw, after a course of antenatal corticosteroids. Evidence suggests a consistently higher fetal death rate in MC twin pregnancies than in DC twin pregnancies. It is uncertain whether elective birth in MC twin pregnancies at 1 week earlier than recommended (from 35 gw) would reduce fetal death rates significantly without increasing adverse neonatal outcomes significantly.

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LECT 35

THE PATHOLOGIST AND TWINS

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Twins arise from the division of a single zygote or from separate fertilized ova. The ones from the division of one zygote are named monozygotic, those from multiple ovulation dizygotic. The latest is more frequent accounting of 70% of twin pregnancies, while the monozygotic one is 30%. Monozygous twins usually are genetically and phenotypically identical, while dizygous ones show the same differences seen in brothers and sisters. The zygosity is strictly associated with the development of the placenta. The type of placentation is referred as chorionicity, as well the type of conception is related with zygosity. Monozygotic pregnancies show a type of placentation according to the timing of division of the zygote. A division within 3 days develops

dichorionic, between 3 and 9 days diamniotic and monochorionic, after 9 monoamniotic and monochorionic placenta. In dizygotic twins, each zygote has its own placenta, amnion, chorion, and circulation. Thus, dizygotic pregnancies lead to a dichorionic placenta. The sites of implantation of the blastocyst give the final appearance of the dichorionic placenta: distant sites are more likely to result in separate placentas, whereas closed site in a single fused placental mass with separate circulations. The twin pathological placentas need a complete macroscopic and microscopic examination [1]. Reference values for fetal weight at birth, placental weight, and fetal/placental weight ratio (FPR), as the placental functional efficiency index in monochorionic and dichorionic twins, have been established [2]. The main objectives of the pathologist in twin's placenta examination are: a) the determination of chorionicity and amnionicity; b) the analysis of choriovascular anastomoses; c) the description of any residual anastomoses and laser coagulation in the laser-treated placenta for twin-to-twin transfusion syndrome (TTTS). Uncomplicated, term dichorionic twin placentas can be routinely stored after the macroscopic examination. The identification of the cord and the owning twin is mandatory and should be performed for instance by labeling at the time of delivery. The identification of differences between twin placentas may explain many twin conditions such as birth weight discordance, especially in the context of dichorionic twinning and the twins' outcome differences. The vascular injection studies can be useful in selected cases of monochorionicity. In these cases, the fixation should be avoided. The microscopic examination of twin placentas is important in order to confirm the type of chorionicity, by studying the layers of the membrane between the gestational rooms. Prematurity is a major risk in twin pregnancies. In addition, monochorionic twin pregnancies have a specific set of complications, as chronic and acute TTTS, twin reversed arterial perfusion syndrome (TRAP), twin anemia-polycythemia sequence (TAPS), malformations, and inter-twin growth discordance. Although the diagnosis of TTTS is a clinical one and based on ultrasound criteria, the pathologist may pronounce whether the findings in placental examination are consistent with or suggestive of TTTS, especially in special context as unexplained poor pregnancy outcome [1]. The microscopic findings of chronic TTTS are nonspecific, thus the histologic features

of TTTS placenta are variable. The features of discordant villous maturation, including villous accelerated maturation with dilated and congested vessels and large and edematous (immature) or small and atrophic (hyper-mature) villi, peripheral cord insertion, smaller placental sharing, increased number of immature erythroid precursors, amnion nodosum may be seen. The differential diagnosis of TTTS consists of discordant severe IUGR, discordant structural fetal anomalies, chromosomal anomalies, and TRAP sequence [3]. A vanished twin is a possibility that should be taken in account in all placentas (twin and singleton): the appearance may vary ranging from an amorphous fibrous plaque within the membranes to a papyraceus fetus. The radiography and the histologic examination can endorse the hypothesis [1]. In conclusion, the pathophysiology of twin gestations and placenta may give such valuable data. Thus, despite limitations of pathology placental examination, a detailed report of placental findings should be warranted in order to explain the clinical outcome and twins complications.

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LECT 36

TWINS: THE POWER OF EPIGENETICS

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INTRODUCTION

The conception and birth in human beings are planned in single. Spontaneous multiple births occur with a prevalence of 1 to 80. In terms of global conceptions, the prevalence seems to be ten times higher, due to the high rate of spontaneous embryonic and fetal losses among twins. Nowadays, in relation to the widespread availability of assisted reproductive technology (ART), twin pregnancy rates have increased by 50%. Mostly they are

dizygotic (DZ), resulting from fertilization of two oocytes by two sperms. The monozygotic (MZ) ones are approximately 30% and depend on the early postzygotic division of a single embryo, result of a single oocyte fertilized by a single sperm.

Twins are a model of biologic variation in humans useful to understand some phenomena occurring during human development. While DZ twins are genetically distinct as siblings, MZ twins have the same genome. Recent evidences confirm that in MZ twins some genes may be active only in one twin in relation to a different epigenetic modulation [1]. One of the epigenetic mechanism peculiar of twins is the “Polar Body”, where sperms fertilize both the ovum and the second polar body. The resulting twins share the same genes from the mother but different genes from the father, with a monozygosity limited to maternal lineage and total of 75% of genes in common. Other varieties are represented by MZ twins with chromosomal mosaicism or discordant for monogenic diseases or pathological conditions transmitted through a mendelian pattern. These conditions depend on epigenetic changes that arise in one embryo. This discrepancy can be the consequence of mutations in regulatory genes, abnormal imprinting pattern or unbalanced inactivation of the X chromosome [2].

CONGENITAL MALFORMATIONS AND DEVELOPMENTAL PROFILES

The risk of congenital defects in twin pregnancies changes in relation to zygosity. In MZ pregnancies the risk of chromosomal abnormalities is identical for both twins while in DZ pregnancy each fetus has an individual risk of being affected by chromosomal abnormalities. In twin pregnancies, compared to singleton ones, the relative risk of congenital malformations for DZ is 1.17 (95% CI, 1.04 to 1.173) and for MZ 1.25 (95% CI, 1:21 to 1:28). Discordant congenital defects as well as phenotypic discordance in MZ twins may be also related to epigenetic events acting in a different way in the couple of twins. In addition, the phenotype of twin newborns with a genetic syndrome in which epigenetic abnormalities are determinant, such as Prader-Willi and Beckwith-Wiedemann, may be discordant between MZ twins. Developmental outcomes of twins are closely related to complications occurred in pregnancy, gestational age, birth weight and perinatal pathology. Following the death of a fetus, the risk of fetal death of the co-twin is three times greater (12%). Premature birth and fetal growth restriction in more frequent than singletons and may be associated due to abnormalities of chorionicity

and peculiarity of twin anatomic asset (vessels anastomosis determining a twin-twin transfusion syndrome or a twin-twin disruption sequence in the case of the death of one twin and spread of cytotoxic components in the vascular bed of the surviving co-twin). In preterm twins neurological sequelae are more frequent than singleton preterm. Monochorionic twins, especially if born prematurely and showing fetal growth restriction, are at high-risk not only of major neurological sequelae such as cerebral palsy and neurosensory deficits, but also of “minor” neurodevelopmental sequelae, potentially leading to future integration difficulties. Recent studies showed that different epigenetic changes in MZ twins may play a role in such developmental profile [3]. The role of pediatricians is crucial for the long-term management of such disabilities.

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LECT 37

MANAGEMENT OF PERINATAL LOSS OF TWINS

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The death of a twin during pregnancy or in the neonatal period causes contradictory and painful feelings that counteract the mourning process and make it difficult for the parents to care for the survived twin. Having to simultaneously deal with the grief over the loss of a child and the joy of a new birth, the parents undergo a kind of “emotional splitting” where, if you mourn the death of a child, you feel yourself taking something out of the surviving baby; vice versa, you feel guilty about being happy when should weep for the death of the one who is gone. Parents may find themselves crying more than one child, in the case of multiple pregnancies, even at a distance of days, and at the same time hope for the survival of others [1, 2]. When the babies are admitted in NICUs due to their very low gestational age, the parents experience an anticipatory grief due at first to their separation

from the child and then to the difficulty of taking the first rituals of parenthood. If one of the twins dies, a doubly critical and contradictory situation arises where the mourning for the dead child joins the anticipatory grief also linked to the fear of losing the other child. At this stage, it is important that parents perceive the health providers’ willingness to listen to them and feel themselves free to express their conflicting feelings [2]. The dead child may seem like a fantasy, but he has to become real through the narrative of his life and his memories: having memories as well as pictures of the baby, possibly together with the brother, will help the grieving process, where having nothing will increase the feeling of emptiness. A real memory box, filled with objects that remind the child, and a virtual one, filled with memories that parents will be able to access whenever they need, will help them overcome grieving, whether this occurs shortly after childbirth, or after days or weeks since birth [1]. Although any loss requires individualized approach, family should be involved in the care of the child close to and after death, with rituals that are part of normal parental activity. Bathing the baby, dressing it, talking to him, bringing him toys and holding him can be the only opportunity for them to play the role of parents. The care of the environment in which parents have to process mourning deserves attention: placing the surviving twin and his parents close to parents whose twins are both alive deals to a painful discomfort and should be avoided, although this may be difficult in open-space NICUs [3]. A further pain is felt if health professionals forget that the surviving baby has a twin who is dead. The surviving twin is still a twin, the surviving triplets are still triplets. Placing a small sign, as a butterfly, in the incubator can help them not to forget it [3]. When we talk about perinatal loss of twins, one last interesting consideration, which most complicates the mourning process, is the further mourning of the mother losing the condition of being a mother of two or more twins. A woman who has been the mother of two or more twins continues to feel like that. The pride she felt pregnant with more children and the pain to be the mother of one or two if they were three are other contradictory feelings that dominate this type of mourning [3]. **Tab. 1** lists some suggestions for health providers to manage the loss of twin in NICUs.

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Table 1 (LECT 37). Some suggestions for health providers to manage the loss of a twin in NICUs (from Pectora et al.[1], modified).

What not to say:
<ul style="list-style-type: none"> • You couldn't take care of all. • At least it happened before you felt strong affection to him. • It's better so, he would have a severe disability. • How could you have managed a so sick baby? • Stop being sad, you have to be strong for the other. • Fortunately it was a twin, otherwise your pain would have been much worse.
What to say:
<ul style="list-style-type: none"> • It must be really difficult for you. • I don't know what to say. • I'm so sorry that Ann and Mary died. • I'm happy that Ann e Mary are alive, but I feel pain for Paul's death.
What to do:
<ul style="list-style-type: none"> • Collect pictures of twins together, even after the death of one of them. • Take hand and footprints of both children together. • Avoid placing the surviving twin and his parents close to parents whose twins are both alive. • Don't forget: the surviving twin is still a twin; the surviving twin's mother will always be mother of twins. • Place a small sign in the incubator of surviving twin to remember he has a dead twin: parents feel pain if health professionals forget it. • Speak of both children calling them by name. • Encourage parents to tell the story of the baby who is gone: this helps the mourning process.

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LECT 38

NEUROPSYCHIATRIC PROBLEMS IN TWINS

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INTRODUCTION

Taking good care of twins means to deal with more complicated relationships among parents-sons-environment and with a hard differentiation of consciousness resulting in bad relationship performances, in rigid behaviour and in twin complementarity with the development of more complex personalities [1]. The possibility of genetic risk occurring in expressing some psychiatric disorders, particularly depression, hyperactivity, in monozygotic twins is higher than in sons of the total

population. Some environmental factors may act via epigenetic mechanisms such as DNA methylation to dysregulate neurodevelopmental processes [2].

MATERIALS AND METHODS

We have studied two male monozygotic twin pairs, one pair is seven years and six months old, the other pair is sixteen years old. Both of the pairs were affected by social impairment and neuropsychiatric disorders. They were fostered with the Social Services and they were referred to the Juvenile Court of Florence during the period 2016-17. In addition we have analysed the family drawings, Corman test, of five twin pairs attending the fifth year of a Tuscan primary school, followed by a pediatrician. These children don't suffer by neuropsychiatric symptoms.

RESULTS

The seven years and six months old pair was affected by behaviour disorders and signs of neglect. They got better with their emotional relationships and with their suppressed aggressive behaviours when they were entrusted to two different families while staying in regular contact between them. They met their parent during the weekends. The dyslexic twin was making very good progress on his schoolwork.

The sixteen years old pair was affected by cognitive disorders. They became more peaceful and more

organized after their integration into a residential home with an individual project that provides coming in their family during the weekends.

The analysed drawings show rigid and stereotyped traits of human figures of Corman test (**Fig. 1**). These data could be related to some relational behaviour.

CONCLUSIONS

Concerning emotional intrafamiliar relationship, rigid and immature patterns arise among analysed cases. Although the management of the psychological and psychiatric issues is more complex, the best changes of development for the twins are interventions focused on the single subject. Sometimes integration into different families is necessary so that every twin can develop the best opportunities for his growth.

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LECT 39

SELF-REPORTED AGGRESSIVE BEHAVIOR IN HUMANS AND BIOMARKERS: A FOCUS ON LIPIDS AND METHYLATION

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Large individual differences have been observed in aggressive behavior, with twin-family studies

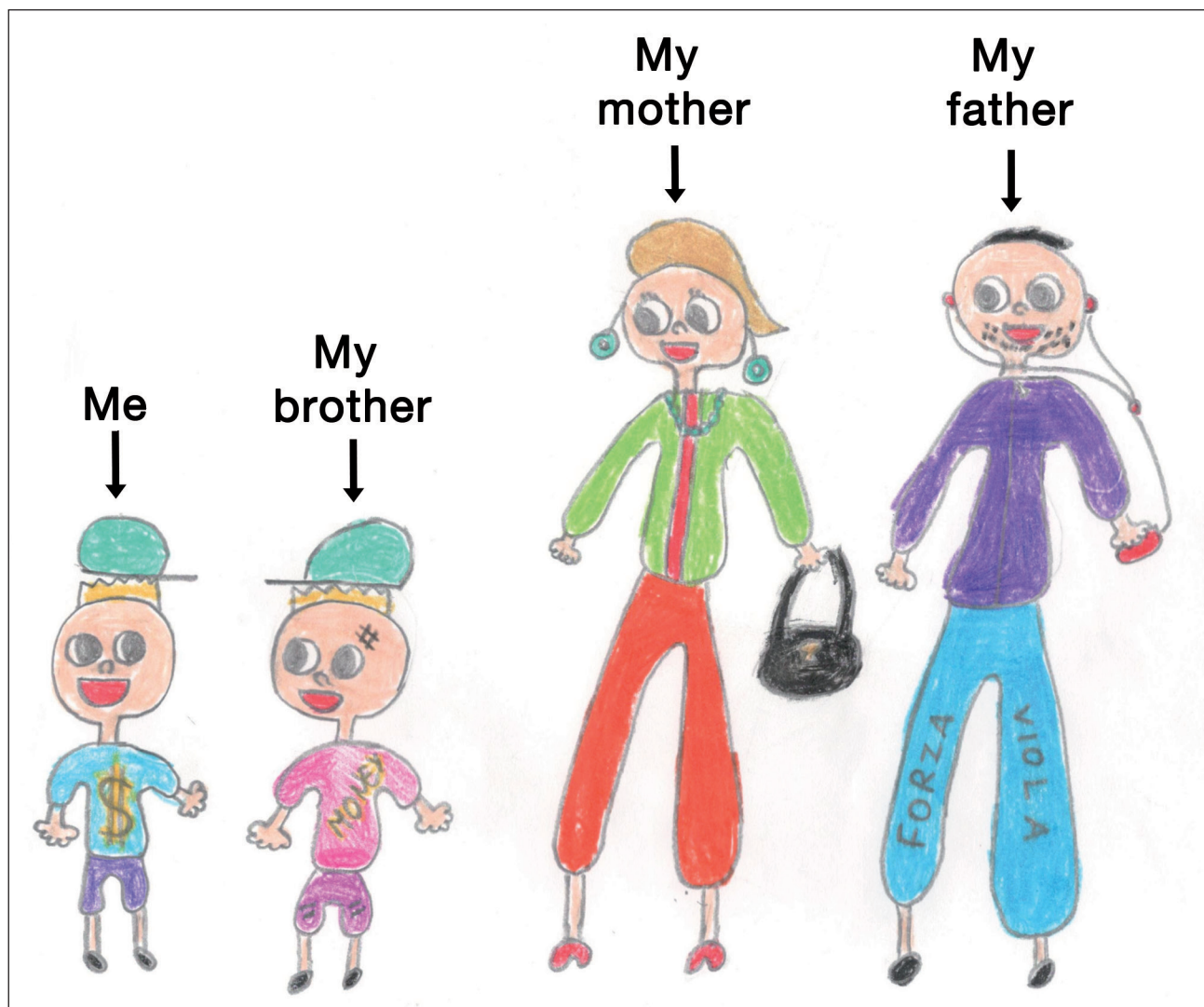


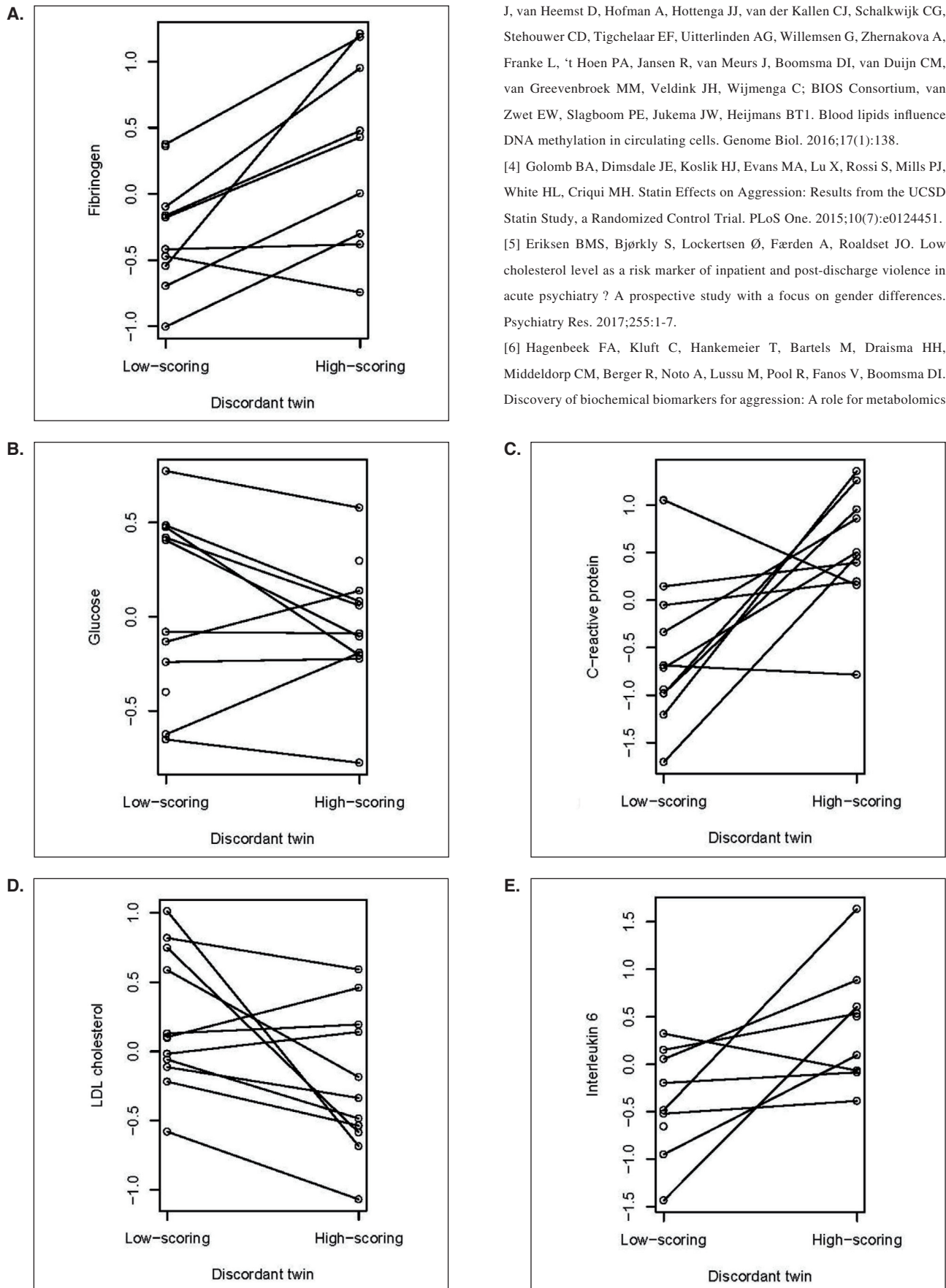
Figure 1 (LECT 38). Corman test of an eleven years old schoolboy.

reporting that approximately half of the phenotypic variance in aggression is explained by genetic factors [1], with the other half of the phenotypic variation ascribable to environmental influences. Epigenetic mechanisms mediate the effect of the environment and genome on complex traits. Recently, a large Epigenome-Wide Association Study (EWAS) [2] observed suggestive differential methylation for 8 CpG sites and identified 3 additional suggestive markers in a discordant monozygotic (MZ) twin design. The differential DNA methylation may be the consequence of variation in blood lipid levels as shown by Dekkers et al. [3] in a Mendelian randomization analysis. Thus, the association of epigenetic markers with aggression may be mediated by variation in lipid levels. Serum or plasma levels of lipids in humans have been explored as potential biomarkers of different types of aggressive behavior, such as violence, self-harm and suicidal behavior. An increase in aggressive behaviors tends to be associated with lower levels of total cholesterol and HDL cholesterol. Especially in participants with low baseline aggression, lowering cholesterol levels by statins decreased aggression in men but generally increased aggression in women [4]. Additionally, Eriksen et al. [5] observed an association of low HDL on violence in men post-discharge from an acute psychiatric ward. The first aim of the current study is to investigate if lipid CpGs as reported by Dekkers et al. [3] are enriched in the aggression EWAS [2]. In the enrichment analysis the test statistics of the aggression EWAS were regressed on a variable indicating whether a CpG was significantly associated with lipids. Our second aim was to test for associations between aggression and lipids, as well as between aggression and glucose, and inflammatory markers, as Hagenbeek et al. [6] have argued that these should be studied simultaneously. Data on self-reported aggression were available for around 5,600 persons from a population-based cohort for whom six lipid, six glucose metabolism, and five inflammation markers were measured after overnight fasting. Demographic and lifestyle variables included sex, age (mean age at blood sampling = 41 years, SD = 13.9, females = 66.4%), body mass index and smoking status, and were included in the statistical analyses as covariates. The association of biomarkers and aggressive behavior was tested by generalized estimation equation (GEE) models, which corrected for family resemblance, and in the discordant MZ twin design by paired t-tests on residual biomarker levels adjusted for the covariates. Results showed

that CpGs associated with lipid levels were not significantly enriched in the aggression EWAS and GEE analyses resulted in non-significant findings. However, in the MZ pairs discordant for aggression (n = 31 pairs) we observed a trend towards significant differences for glucose (mean difference = -0.27, p = 0.003) and fibrinogen (mean difference = 0.43, p = 0.01), indicating that the twin who scored lower on aggression had lower glucose levels and higher fibrinogen levels. Repeating the analyses on only extremely discordant MZ twins (n = 12 pairs) indicated that the results for fibrinogen could be explained by these extremely discordant pairs (mean difference = 0.67, p = 0.008; **Fig. 1A**), while the association with glucose disappeared completely (mean difference = -0.16, p = 0.19; **Fig. 1B**). The analysis on extremely discordant MZ pairs also resulted in marginally statistically significant findings for the association between aggression and C-reactive protein (CRP; mean difference = 1.08, p = 0.016; **Fig. 1C**), LDL cholesterol (mean difference = -0.45, p = 0.038; **Fig. 1D**) and interleukin-6 (IL-6; mean difference = 0.78, p = 0.045; **Fig. 1E**). In the current study, we combined multiple statistical methods to elucidate the role of biomarkers in adult aggression. While we did not find evidence for enrichment of lipid CpGs in the aggression EWAS, analyses in MZ twins discordant for aggression identified marginally significant within-pair differences for cytokines. Previously, elevated cytokine levels have been observed in adult patients with intermitted explosive disorder [7] and in children with severe affective and behavioral dysregulation [8]. Similarly, childhood physical aggression in males has been associated with differential DNA methylation in cytokine regulatory regions [9]. Taken together, these results suggest that the previously reported association of DNA methylation with aggression might be mediated by variation in cytokine levels. A next step will be to examine the role of biomarkers in aggression across the lifespan, including childhood.

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Figure 1 (LECT 39). Biomarker levels in extremely aggression-discordant MZ twins. The residual fibrinogen (A), glucose (B), C-reactive protein (C), LDL cholesterol (D) and interleukin 6 (E) levels, adjusted for covariates, are plotted for low- and high-scoring twins of 12 extremely discordant MZ twin pairs. The biomarker levels of co-twins are connected by lines.

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LECT 40

SOFT TISSUE TUMORS IN THE NEONATAL AND PEDIATRIC SETTING

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Soft tissue tumors represent a very large and heterogeneous group of tumors, the majority of which is derived from supporting tissues like fat, blood/lymphatic vessels, fibrous tissue, nerves, smooth and skeletal muscle. When compared to soft tissue tumors in adults, there are some notable differences [1]. In the perinatal/pediatric age group they not only present as benign or malignant neoplasms, but also as hamartomas (e.g. smooth muscle hamartoma), choristomas (e.g. neuromuscular choristoma) and malformations (e.g. many vascular lesions) [2]. In very young children many soft tissue lesions show an attempt to recapitulate the developmental phases of a particular organ, the embryology is never far away. Fetal rhabdomyoma, embryonal rhabdomyosarcoma and lipoblastoma are examples of tumors that strongly resemble fetal muscle or fat [3]. Some soft tissue tumors virtually exclusively occur in this age group (e.g. fibrous hamartoma of infancy, infantile digital fibromatosis, congenital/infantile fibrosarcoma) [4]. When comparing the incidence of the different categories of soft tissue tumors in young children versus adults, there are striking differences. Vascular, (myo)fibroblastic and skeletal muscle tumors are much more frequent in children than adults, the opposite is true for adipocytic tumors [5]. When looking to the group of sarcomas in young children, more than half of them correspond to embryonal rhabdomyosarcomas, congenital/

infantile fibrosarcoma is the second most frequent sarcoma (10-20%). Overall, sarcomas represent 7% to 10% of childhood (≤ 15 yrs) cancers, almost half occur in children younger than 5 years old. Based on the current WHO classification, the following tumors are typically or frequently seen in young children. This list presented in **Tab. 1** is obviously not exhaustive and the entities in *Italic* will be discussed in detail at the meeting.

Table 1 (LECT 40). List of the most important soft tissue tumors in the neonatal and pediatric setting, based on the current WHO classification. Entities in *italic* will be discussed in detail at the meeting.

<p>I. (Myo)fibroblastic tumors</p> <p>Cranial fasciitis <i>Fibrous hamartoma of infancy</i> [1] Fibromatosis colli Juvenile hyaline fibromatosis Inclusion body fibromatosis Gardner fibroma Calcifying fibrous tumor Fibrous umbilical polyp Desmoid type fibromatosis Lipofibromatosis Giant cell fibroblastoma <i>Congenital/infantile fibrosarcoma</i> [2]</p>
<p>II. Fatty tumors</p> <p><i>Lipoblastoma</i> [3]</p>
<p>III. Skeletal muscle tumors</p> <p>Fetal rhabdomyoma <i>Embryonal rhabdomyosarcoma</i> [4] <i>Spindle cell rhabdomyosarcoma</i> [5] Malignant ectomesenchymoma</p>
<p>IV. Smooth muscle tumors</p> <p>Congenital smooth muscle hamartoma <i>EBV driven smooth muscle tumor</i> [6]</p>
<p>V. Pericytic tumors</p> <p>Infantile myofibroma(tosis)</p>
<p>VI. Neurogenic tumors</p> <p>Neuromuscular choristoma Congenital granular cell tumor</p>
<p>VII. Vascular tumors</p> <p><i>Congenital hemangioma</i> [7] Infantile capillary hemangioma Lymphangioma Papillary intralymphatic angioendothelioma (Dabska tumor) <i>Kaposiform hemangioendothelioma</i> [8] Endemic Kaposi sarcoma</p>
<p>VIII. Tumors of uncertain differentiation</p> <p>Primitive myxoid mesenchymal tumor of infancy Extrarenal rhabdoid tumor Ewing sarcoma</p>

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LECT 41

PLACENTAL SOFT TISSUE TUMORS

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INTRODUCTION

Placental tumors are very rare, they are mainly vascular tumours and are usually found incidentally. In most cases, placental tumors are asymptomatic. Occasionally, when they are large or multiple, they can result in poor outcomes for both the fetus and the mother [1]. Since placental vascular tumors are perfused by the fetal circulation, they may represent a significant impediment to fetal cardiac activity or may sequester platelets, giving rise to fetal thrombocytopenia. Vascular shunting caused by placental tumors may also cause fetal high-output cardiac failure, preterm delivery and hydrops fetalis

[2, 3]. The aim of this work was to describe three tumors occurring in the human placenta, which showed peculiar and histological patterns.

CASE REPORTS

1. A 38-year-old woman underwent preterm delivery at 33 weeks, following the precocious detachment of the placenta. At macroscopy, the placenta weighed 312 g, measuring 14 x 13.5 x 3 cm. A large hematoma, 4 x 1 cm in size, was detected on the inner surface of the chorionic plate. On the cut surface of the chorionic plate, a roundish area, red in color, 0.7 x 0.5 cm in size, was detected. At histology, the lesion appeared formed by proliferating vascular channels, irregular in shape, embedded in a collagenous stroma and surrounded by p57+ cytotrophoblasts and hCG+ syncytiotrophoblastic cells, suggestive for the diagnosis of placental chorangioma.
2. A 32-year-old woman underwent delivery at 38 weeks of gestation. At macroscopy, the placenta weighed 360 g. No pathological changes were observed. On cut sections of the chorionic plate, a round area with a diameter of 8 mm, reddish in color, was sampled. At histology, the lesion was characterized by the proliferation of CD31+ and CD34+ epithelioid cells, occasionally arranged around a small lumen and surrounded by Alpha-SMA+ spindle cells, suggestive for the diagnosis of juvenile capillary hemangioma (Fig. 1).
3. A 38-year-old woman, affected by Hashimoto thyroiditis, presented during gestation with hypertension and preeclampsia, leading to preterm delivery at 28 weeks of gestation. The placenta weighed 179 g, 14 x 10 x 2 cm in size. At macroscopy, on cut surface of the chorionic plate, multiple infarcts were detected. At histology, in one of the samples of the chorionic plate, a small proliferative lesion, 0.3 x 0.4 cm in size was found. The lesion was characterized by epithelioid large cells, with a abundant cytoplasm. No clear vascular lumina were detected. At high power, some lumen-like rudimentary structures were detected inside the cytoplasm of tumor cells, a picture suggestive for the diagnosis of epithelioid hemangioendothelioma.

CONCLUSIONS

Our work confirms that the majority of placental tumors are soft tissue tumors and, in particular, they are vascular in nature. Even though the diagnosis of chorangioma should always be first considered in the differential diagnosis of placental tumors, the

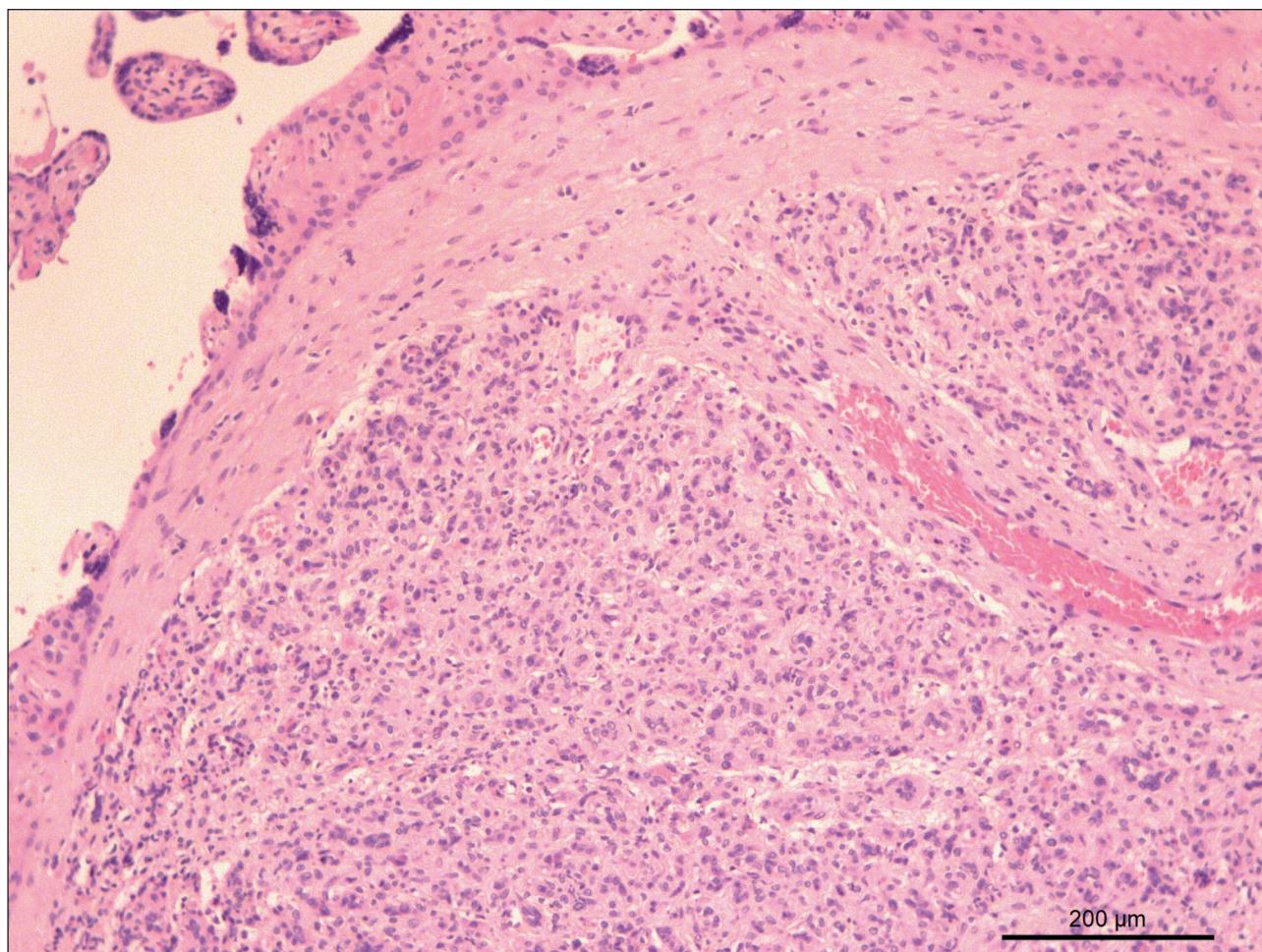


Figure 1 (LECT 41). Juvenile capillary hemangioma.

cases here described evidence that other vascular neoplasms, including juvenile hemangioma and epithelioid hemangioendothelioma (EHE), a soft tissue tumor with intermediate malignant potential, may occur in the placenta. Regarding the previously reported extreme rarity of placental tumors, we may hypothesize that an accurate macroscopic and histological analysis of the entire chorionic plate might lead to an increase in the frequency of placental tumors.

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LECT 42

DO OBSTETRICIANS REALLY KNOW PLACENTA?

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The placenta is a fundamental temporary organ and its most important task is to transfer oxygen and nutrients from the mother to the fetus. Probably the obstetricians keep in mind only this definition and their attention to placenta is limited to some important medical aspects of its function and some obstetrical emergencies. The development of placenta during pregnancy is generally well known. The placenta progressively and temporarily assumes the role of the fetal lungs (gaseous exchange), gastrointestinal tract (uptake of nutrients), and kidneys (regulation of fluid volume and elimination of waste metabolites) while these organs are developing. Clinical follow-up of the placenta is often part of the routine ultrasound evaluation of a normal pregnancy (**Fig. 1**). In fact, recognition of both normal placental anatomy and anatomical variations is important for evidencing

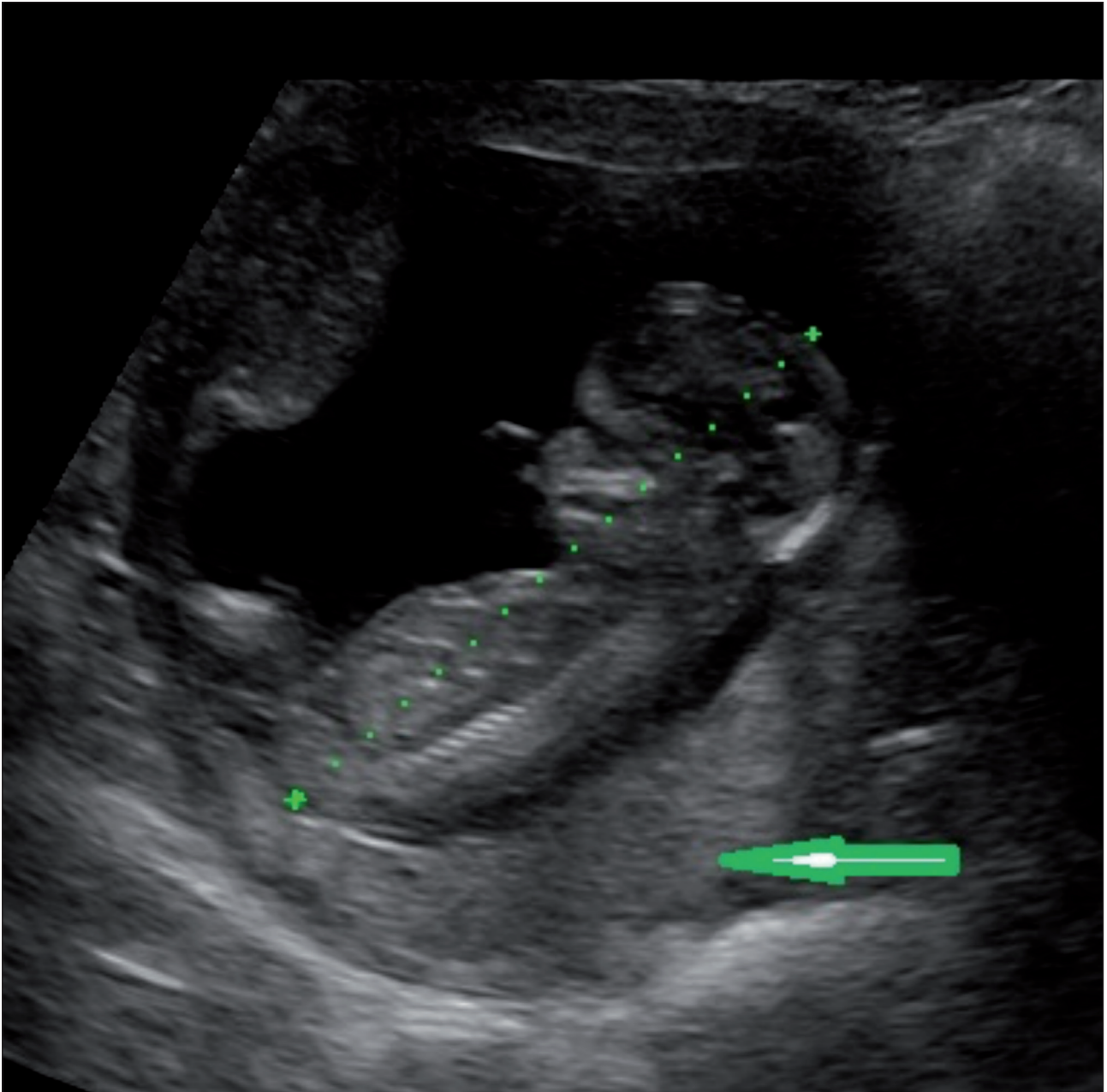


Figure 1 (LECT 42). Normal placenta (arrow) at first trimester ultrasound scan.

significant abnormalities. Except for very expert sonographers, some ecographic signs are not well known and often misinterpreted. Among these: the chorionic bump in the first trimester, the marginal sinus, the sub placental hypoechoic zone in the second and third trimester, calcifications at the placental-myometrial junction and cotyledon calcifications after 30 weeks. Recognition of succenturiate lobe is important because may be associated with vasa previa. Another important diagnosis to perform is morbidly adherent placenta. An ultrasound diagnosis often missed is velamentous umbilical cord insertion. Generally, the ultrasound signs of different placentation in

twin pregnancies are well known. Obstetricians well know the role of abnormal placentation as the necessary component for the genesis of a very important pregnancy disease: preeclampsia. It is still not widely diffuse the understanding of new data on the two clinical subtypes of preeclampsia, early onset, late onset and associated or not associated with fetal growth restriction. Clinical signs and management of obstetrical emergencies like abruptio placentae or postpartum hemorrhage (PPH) are constantly under the attention of obstetricians as they continue to remain the leading causes of global maternal mortality and morbidity. Indeed many other functions and role of the

placenta are little known to many clinicians, for instance, the important endocrine function of the placenta. One of the major functions of the human placenta is the capacity to synthesize important hormones and other mediators, as this placental endocrine function is crucial for gestational success [1]. The release of placental hormones into maternal circulation has been the target of intense research into their potential as biomarkers for predicting and diagnosing pregnancy related diseases. Other than the classical protein (human chorionic gonadotropin [hCG] and human placental lactogen [hPL]) and steroid hormones (progesterone, estradiol, estriol and estrone), it synthesizes hypothalamic-like neurohormones and neuropeptides [2]. Moreover, growth factors and cytokines are also produced in human placental cells and are able to influence the endocrine function [3]. Cytokines are glycoproteins produced by a large variety of cells especially of the immune system. Hormonal-releasing properties of cytokines on hypothalamus, pituitary gland or on the other endocrine glands have been described. Moreover, placental cytokines may be involved in the immunoregulation of the maternal-fetal interface. It is suggested to be important for fetal survival with cytokines being the mediator of this phenomenon. Most of the characteristics and functions of the placenta presented below are known only to researchers:

- a. although placental growth and function is relatively similar between males and females, when exposed to an *in utero* stressor, sexually dimorphic phenotypes become apparent. Collectively the current literature suggests that distinct sexually dimorphic responses can occur in some situations;
- b. intriguing recent data showed the intrinsic hematopoietic potential and appearance of hematopoietic cells in human placenta;
- c. prenatal development is a particularly vulnerable period in life when tissues are rapidly developing and are susceptible to shifts in programming. There is the hypothesis that selective pressures on the human placenta may favor evolutionary determination of future health of the offspring.

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LECT 43

ULTRASOUND AND PLACENTA

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There are numerous fields where ultrasound is useful for diagnosing of placenta anomalies (structural and/or functional), thus helping to improve maternal-fetal wellbeing. In the last decade, the increment of the Cesarean sections caused an increase of placenta invasion anomalies. Placenta accreta is becoming a prevalent risk factor of post partum haemorrhage developing maternal mortality and morbidity. Furthermore, a new pathological entity such as "scar pregnancy" (implantation of the gestational sac on the Cesarean section scar) seems to be a new risk factor for maternal haemorrhage in the first trimester of pregnancy. In recent years, new different parameters have been proposed in order to improve the detection rate of placenta invasion anomalies. The absence of Clear Space (a hypoechoic zone of demarcation between the placenta and the myometrium) is a diagnostic factor of accrete placenta in all (100%) affected women [1]. Bladder line interruption is proposed as a diagnostic factor with sensitivity of 90% and specificity of 80% [2]. The observation of more than six placenta lacunae, irregularly shaped, that involve a large part of the placenta, together with a turbulent flow and an elevated systolic peak at the Color Doppler scan are associated to high detection of Cesarean hysterectomy [2]. In addition, the 3D power Doppler ultrasound study is a useful instrument for evidentiating the irregular vascularization concerning all of the placental volume, which strongly suggests placental percreta, especially if associated to the vascularization of the uterine-bladder interface with appositive predictive value 97%. The scar pregnancy is a new pathological entity determined by the nesting of the gestational sac on the scar of a previous Cesarean section. Accrete and percreta placentas increase the incidence of maternal complication due to massive haemorrhage and uterine rupture. The diagnosis can be made as early as the first trimester of pregnancy (within the 8th gestational week) by observing a low implantation of the gestational sac

which is adherent to the hysterotomic scar with a thin or absent myometrial layer between the sac and the bladder. Given the important complications that such an anomaly can determine, it is useful to perform an early diagnosis performing early ultrasound scan (before the 8th week of gestation) on all pregnant women at high risk (those who have had a Cesarean section delivery) [3].

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LECT 44

MALE AND FEMALE PLACENTA: A REVOLUTION?

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INTRODUCTION

Is there a sex of the placenta? This is the title of an intriguing article that puts some doubts regarding the assumption that the placenta, traditionally considered as an asexual organ, might show functional and/or structural differences according with the fetal gender [1]. The widely accepted “asexual” theory of the placenta contrasts with a simple fact: trophoblasts, one of the most important cell component of every placenta, do not originate from the mother, taking origin from the embryo and reflecting fetal sex as either XX or XY. The different karyotype is at the basis of the new revolutionary theory, according to which major differences in placental biochemistry, function, and signaling might exist between male and female placentas. Recently, it has been proposed that gender differences in the placenta may produce sex-specific placental signals to the developing brain, ending with a major risk for neurodevelopmental diseases in males, including autism, mental retardation, stuttering, dyslexia, and attention deficit/hyper-

activity disorder (ADHD) [2]. Moreover, gender-related differences in placenta structure and function might help to explain some important differences between male and female fetuses: a) male fetuses have a higher risk for peri- and postnatal mortality and are generally bigger than female [3]; b) male fetuses in pregnancy diseases have poorer outcomes than female fetuses [4]; c) male newborns are more likely to develop hypertension, diabetes mellitus, or metabolic syndrome [5]. The aim of this study was to verify the presence of structural changes, in particular regarding trophoblast cells, in male and female human placentas at different gestational ages.

MATERIALS AND METHODS

Ten placentas, ranging from 19 up to 38 weeks of gestation, five of male fetuses and five of females, were utilized in this study. A sample from the chorionic plate was formalin-fixed and paraffin-embedded. Tissue sections were stained with H&E and immunostained for p57, a typical marker of cytotrophoblasts.

RESULTS

At histology, no significant differences were found between male and female placentas of the same gestational age. At immunohistochemistry, significant differences were detected between male and female placentas, regarding the number of p57-reactive cytotrophoblastic cells in the terminal villi (**Fig. 1**).

CONCLUSIONS

Our preliminary data demonstrates, to the best of our knowledge, for the first time that placentas of male and female fetuses show significant differences regarding their cellular components. In particular, in this study, we observed major gender-related differences regarding the number of cytotrophoblasts in the developing terminal villi. Given the key role of placenta in fetal growth and development, and in particular in exchange of nutrients, oxygen, hormones, cytokines and waste products between the mother and fetus, the finding of significant differences in cell structure, related to the gender of the fetus, might have important effects on our knowledge of both maternal and fetal physiology. Further immunohistochemical studies will clarify if gender-related differences exist in the human placenta regarding the multiple other cell types that give rise to the complex architecture of the human placenta.

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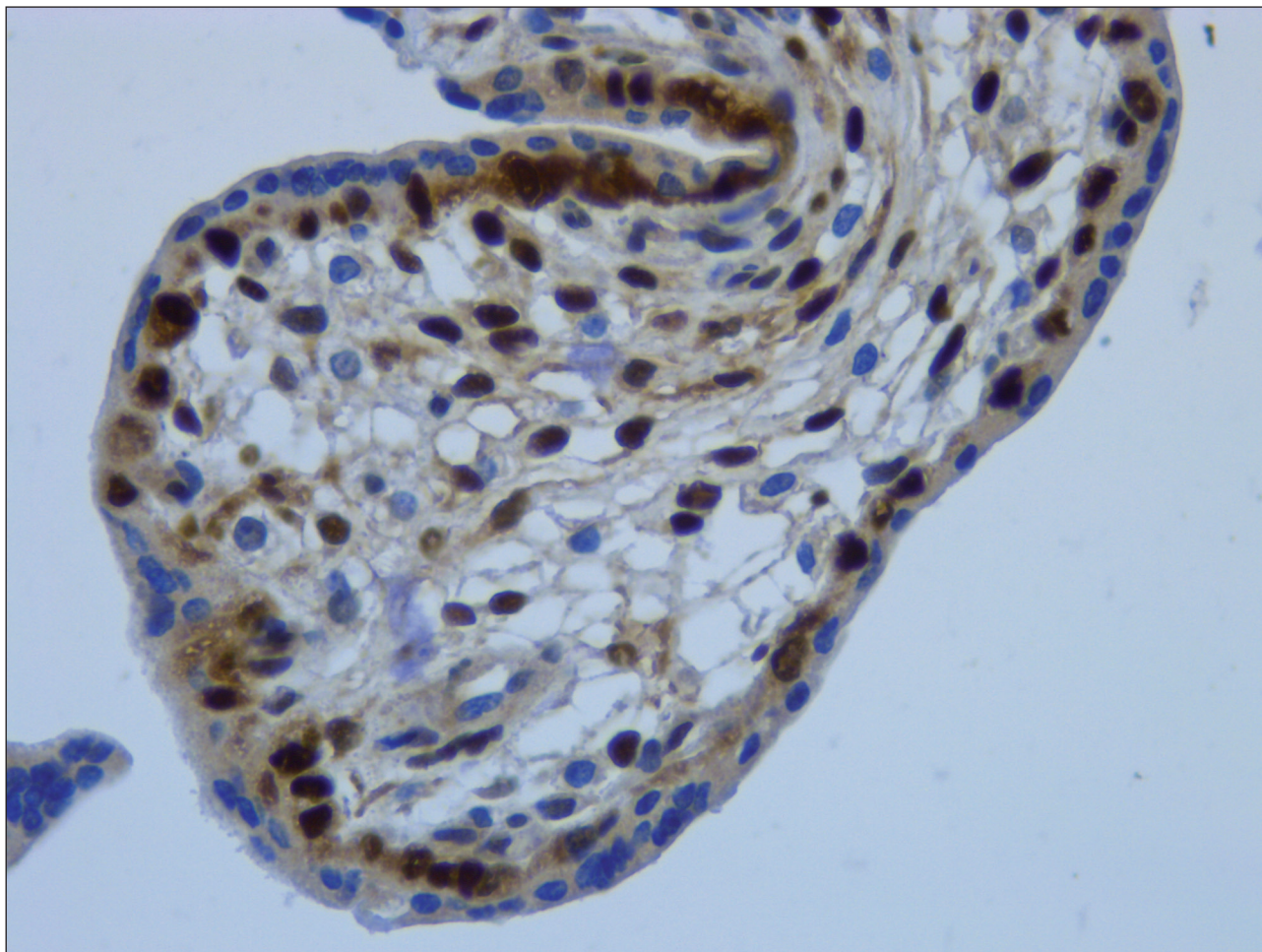


Figure 1 (LECT 44). p57-reactive cytotrophoblastic cells in terminal villus.

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LECT 45

DO NEONATOLOGISTS REALLY KNOW PLACENTA?

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Pregnancy is a critical period of plasticity whereby fetal development may be influenced by inherited

genetic profile as well as by external stimuli. The placenta plays a crucial role in developmental plasticity, and its proper function is central to the health of both the mother and the fetus. A critical component of normal placental function is the adequate development of its vascular network. A disturbance in the development of placental vasculature leads to placental insufficiency, resulting in adverse uterine conditions and complications of pregnancy such as gestational hypertension, intrauterine growth restriction, preeclampsia, preterm delivery, or miscarriage. Intrauterine hypoxia or infections/inflammation are associated with alterations in vasculogenesis and in metabolic activity of the placenta, free radical generation, oxidative stress (OS) and autophagy [1-3]. Oxidative damage and the associated mitochondrial dysfunction may result in energy depletion, accumulation of cytotoxic mediators, oxidation of lipids, proteins, polysaccharides and cell death. Disturbance in the normal redox state of fetal organs tissue contributes to changes in fetal

development [4]. OS is identified as a common feature of adverse conditions in pregnancy, such as: preeclampsia, hypertension, diabetes, smoking, infection or inflammation and also obesity and maternal malnutrition [5, 6]. OS in pregnancy may also enhance the risks of premature rupture of amniotic membranes that is mediated by excessive or undamped peroxidation of amniotic epithelium and chorioamniotic collagen [7]. Adverse *in utero* environment is associated with both short-term complications, including altered fetal growth, increased perinatal morbidity and long-lasting effects on offspring's subsequent health (Fig. 1). Changing developmental signals or placental adaptation occurring in response to an altered maternal environment may be the general underlying mechanisms that link altered placental function to "fetal programming" [8]. Fetal programming occurs when the normal pattern of fetal development is disrupted by an abnormal stimulus or an "insult" during intrauterine life, which leads to adaptations by the fetus to allow its survival, but which finally result in permanent structural and physiological changes with long-term consequences in adulthood. In sight of this theory, cardiovascular and metabolic diseases in adult life can be traced back to their intrauterine origins [5, 8]. Epigenetics also integrates microRNAs (miRNAs) due to their capability to

affect the methylation machinery and the expression of proteins involved in histone modifications. In turn, the expression of certain miRNAs is controlled by DNA methylation and chromatin modifications. Prominent miRNAs are also regulated by OS and vice versa; they act in a redox sensitive manner, thus modifying their functioning due to the cellular redox state or disease-associated OS condition [9]. As a combinatorial approach, these mechanisms may then determine gene expression and the resultant phenotype, thus setting an "*in utero* programming". Therefore, because the placenta is the organ that supports fetal growth and development, and is a diary of intrauterine life, placental examination can be a useful tool associated with timing of tissue damage and infant prognosis. In this context, precise pathological analysis of the placenta is indispensable, but in fact the clinical utility of analysing placenta is underestimated and the overall quality of placental pathology reporting is highly variable [10, 11]. Clear benefits of examining placentas include the early diagnosis of treatable conditions in both the mother and the newborns, clarification of the underlying etiology of adverse pregnancy outcomes, estimation of recurrence risk, and guidance for the management of future pregnancies. In order to realize these benefits and get the most out of histologic examination, it is

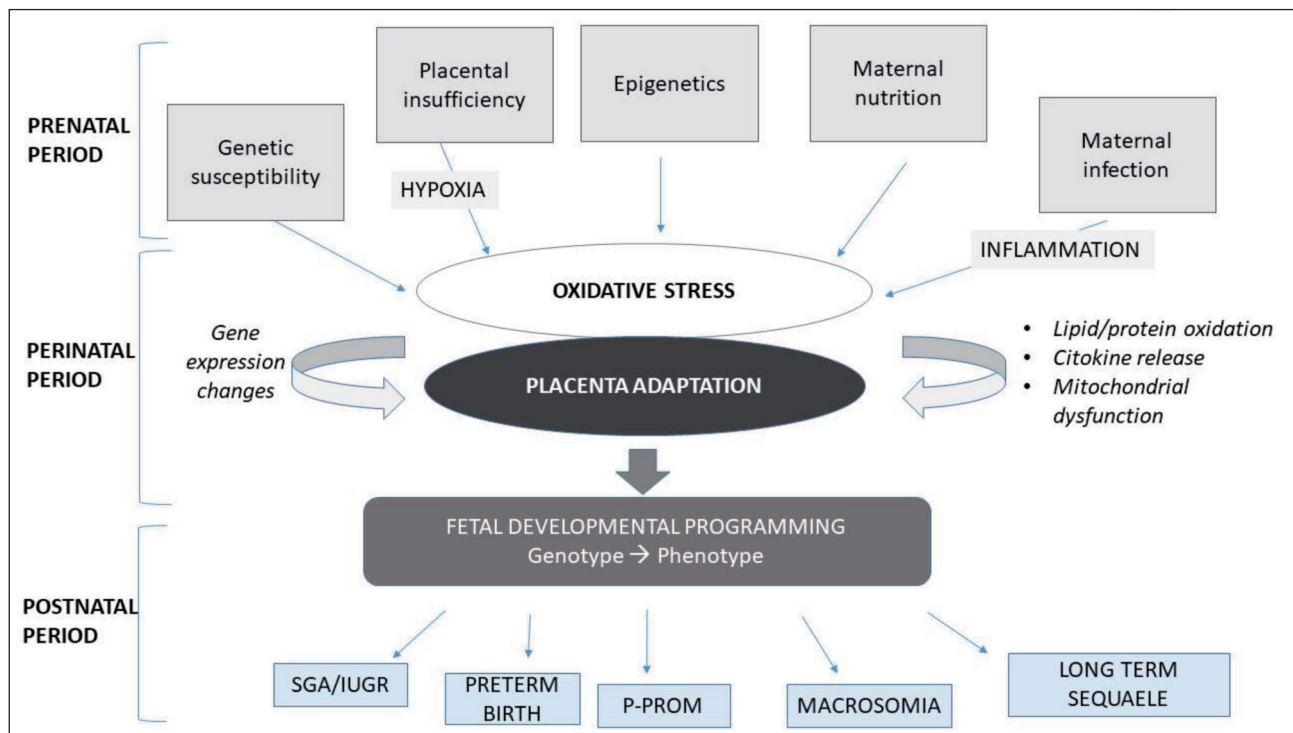


Figure 1 (LECT 45). Association between oxidative stress (OS) and placental susceptibility in adverse utero environment. OS-related conditions induce alteration to both genotype and phenotype and are associated with short-term complications and long-lasting effects on offspring's subsequent health.

fundamental for clinicians to understand the range and implications of placental lesions [11].

Further investigations of the interrelationships of maternal-fetal interface at different gestational stages will lead to an improved understanding of the mechanisms by which alterations in their interactions contribute to placental pathology, disorders of pregnancy, and preterm delivery. With this increased knowledge, novel protocols can then be developed to improve pregnancy outcomes.

Gaining mechanistic insight into the cellular and molecular mediators associated with complications of human pregnancy will be essential for developing successful intervention/prevention strategies. The additional benefits of preventing pregnancy complications extend beyond the immediate protection afforded to mother and child such that the risk of developing adult-onset diseases as a result of fetal programming may be ameliorated.

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LECT 46

S100B EXPRESSION IN PLACENTA

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INTRODUCTION

S100B is present in various damaged tissues. In clinical practice, S100B is assessed as a very reliable biochemical marker of cerebral damage, a useful tool to identify newborns at higher risk of neonatal death or full-term infants at risk of hypoxic-ischemic encephalopathy. Results in high-risk pregnancies demonstrated that S100B concentration increased in amniotic fluid and in cord blood of fetuses with brain damage [1]. A strong positive correlation exists between amniotic fluid S100B and erythropoietin concentrations in pregnancies at high risk for chronic fetal hypoxia. This suggests that chronic fetal hypoxia increases the intrauterine release of S100B [2]. Higher serum concentrations of S100B in preterm fetuses in comparison with fetuses at term could involve blood-brain barrier permeability and cerebral circulation, which could be different in preterm and term fetuses. The aim of this study was to verify, by immunohistochemistry, the presence of reactivity for S100B in human placentas at different gestational ages.

MATERIALS AND METHODS

Ten placentas, ranging from 12 up to 38 weeks of gestation, were utilized in this study. At macroscopy and at histology, all placentas were characterized by the absence of significant pathological findings. Tissue samples were formalin-fixed and routinely processed. 5-micron-thick sections were stained with H&E and immunostained with an anti-S100 B antibody.

RESULTS

S100B positivity were detected in all placenta specimens analyzed and was mainly detected both in the stroma of terminal villi and in the stroma of anchoring villi and, occasionally, in the intervillous spaces. At morphology, S100B-positive cells were

large, epithelioid with an abundant cytoplasm and an oval nucleus, showing finely granular chromatin and the amount changed from one case to the next: they were abundant during the early stages of gestation (**Fig. 1**), below 20 weeks, decreased during middle gestation, around week 30 and their number decreased significantly in late gestation, after 37 weeks.

CONCLUSIONS

Our preliminary data suggest that S100B-positive cells represent an important component of the stromal villous cells in the human placenta, in absence of dendritic appearance, suggesting the different nature of the cells here described. The high number of S100B-positive cells detected in placenta villi in this study and the finding of significant changes in the amount of S100B-reactive placental stromal cells during gestation suggest a possible relevant role of these cell types, particularly in the early phases of gestation. The absence of S100B released

into the villous stroma, might be related to the absence of severe pathological changes in all the ten placentas here analyzed. Further studies are needed to clarify if S100B extracellular expression might occur in placenta in pathological conditions and, in particular, in hypoxic placentas.

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LECT 47

ACUTE CHORIOAMNIONITIS AND NEONATAL OUTCOME

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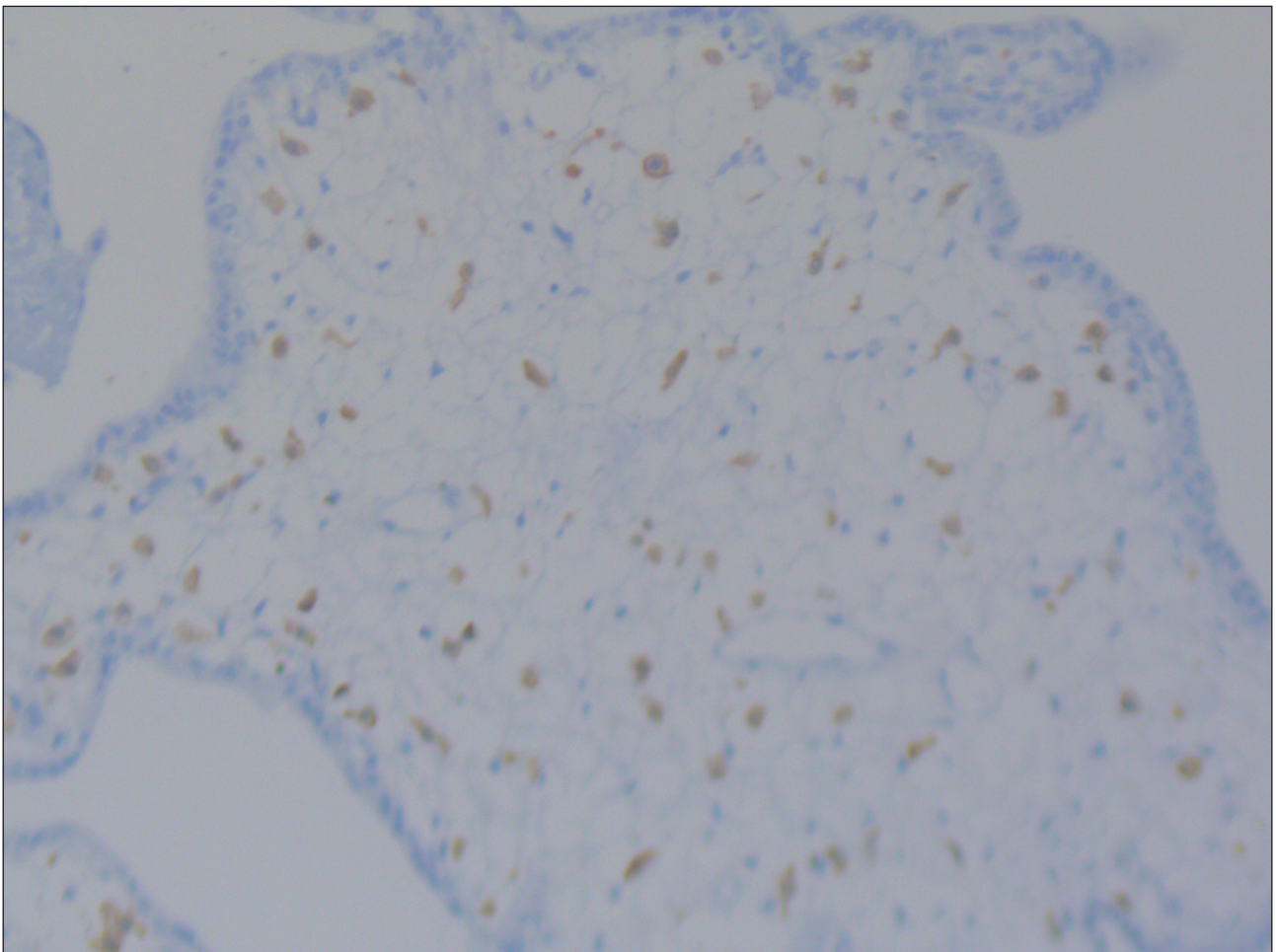


Figure 1 (LECT 46). At morphology, S100B-positive cells were large, epithelioid with an abundant cytoplasm and oval nucleus, showing finely granular chromatin and the amount changed from one case to the next: they were abundant during the early stages of gestation below 20 weeks, decreased during middle gestation, around week 30 and their number decreased significantly in late gestation, after 37 weeks.

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INTRODUCTION

The human placenta is composed of placental disc, chorioamniotic membranes (CAMs) and umbilical cord. Although in continuity with maternal decidua, the CAMs are of fetal origin. Therefore, from the uterine mucosa (decidua) to the amniotic cavity we can distinguish four layers: decidua, cellular chorion, fibrous chorion and amnion. Histologic acute chorioamnionitis (HAC) is defined by the infiltration of neutrophils into the CAMs and represents a maternal inflammatory response [1]. In fact neutrophils, normally absent in the CAMs, migrate from the decidual vessels into the CAMs, therefore they are always of maternal origin [2]. HAC is absolute expression of intra-amniotic inflammation, but not always of intra-amniotic infection. In fact, not only microorganisms, but also danger signals released by cell death or cells under stress conditions can stimulate the production of neutrophil chemokines. These chemotactic stimuli induce the migration of neutrophils from the decidual vessels into the CAMs. The prevalence of HAC increases with the decreasing gestational age at birth, and the frequency of HAC is higher in patients with preterm premature rupture of membranes, preterm labor and spontaneous labor [3]. A correlation between HAC and neonatal morbidity and mortality has been hypothesized. HAC is associated with low Apgar score, bronchopulmonary dysplasia, patent ductus arteriosus, necrotizing enterocolitis, intraventricular hemorrhage, neurodevelopmental sequelae, neonatal sepsis and higher mortality rates [4]. The aim of this study is to evaluate the correlation between HAC and neonatal outcome.

PATIENTS AND METHODS

We selected 11 placentas with histologic diagnosis of HAC: 2 cases with stage 1 and grade 1; 1 case with stage 2 and grade 1; 4 cases with stage 2 and grade 2; 1 case with stage 3 and grade 1, and 3 cases with stage 3 and grade 2. The grading and staging system utilized was that reported by Redline et al. in 2003 [1]. The stage refers to anatomical layers infiltrated by neutrophils: stage 1 when the presence of neutrophils is limited to the subchorionic space or cellular

chorion, not extending into fibrous chorion, stage 2 characterized by neutrophils in the fibrous chorion and/or amnion and stage 3 defined by necrotizing chorioamnionitis, with karyorrhexis, of the chorion and amnion. The grade refers to the intensity of neutrophils infiltration: grade 1 (mild-moderate) when there are scattered or small clusters of neutrophils and grade 2 (severe) with three or more microabscesses (at least 10 neutrophils x 20 cells) or a continuous band of confluent neutrophils (**Fig. 1**). For each case, data regarding maternal diseases, pregnancy pathologies, delivery data and neonatal health status at birth were obtained, including maternal infection, oligoanhydramnios, intrauterine growth restriction (IUGR) and Apgar score at 1 minute after birth.

RESULTS

We found 5 cases of IUGR (5/11 = 45.5%) with different stages and grades on histologic examination. In 5 cases we had oligoanhydramnios (3/11 = 27.3%) also with different stages and grades on histologic examination. In 6 neonates the Apgar score at 1 minute was < 7 (6/11 = 54.5%), but even in this case we didn't find correlations with stages and grades of HAC on histologic examination. Finally, in 2 cases we found maternal infections (2/11 = 18.2%) and in both cases we had stage 3 and grade 2 at histology.

CONCLUSIONS

Our data suggest a correlation between HAC, Apgar score at 1 minute < 7 (54.5%), and IUGR (45.5%) although in both cases it was not possible to observe a correlation with the stage and the grade of HAC. We also found, in 27.3% of cases, oligoanhydramnios and, in 18.2% of cases, maternal infection. In conclusion, in spite of the low number of cases analyzed, we can hypothesize that HAC has a strong incidence on neonatal outcome, as suggested in previous literature [4].

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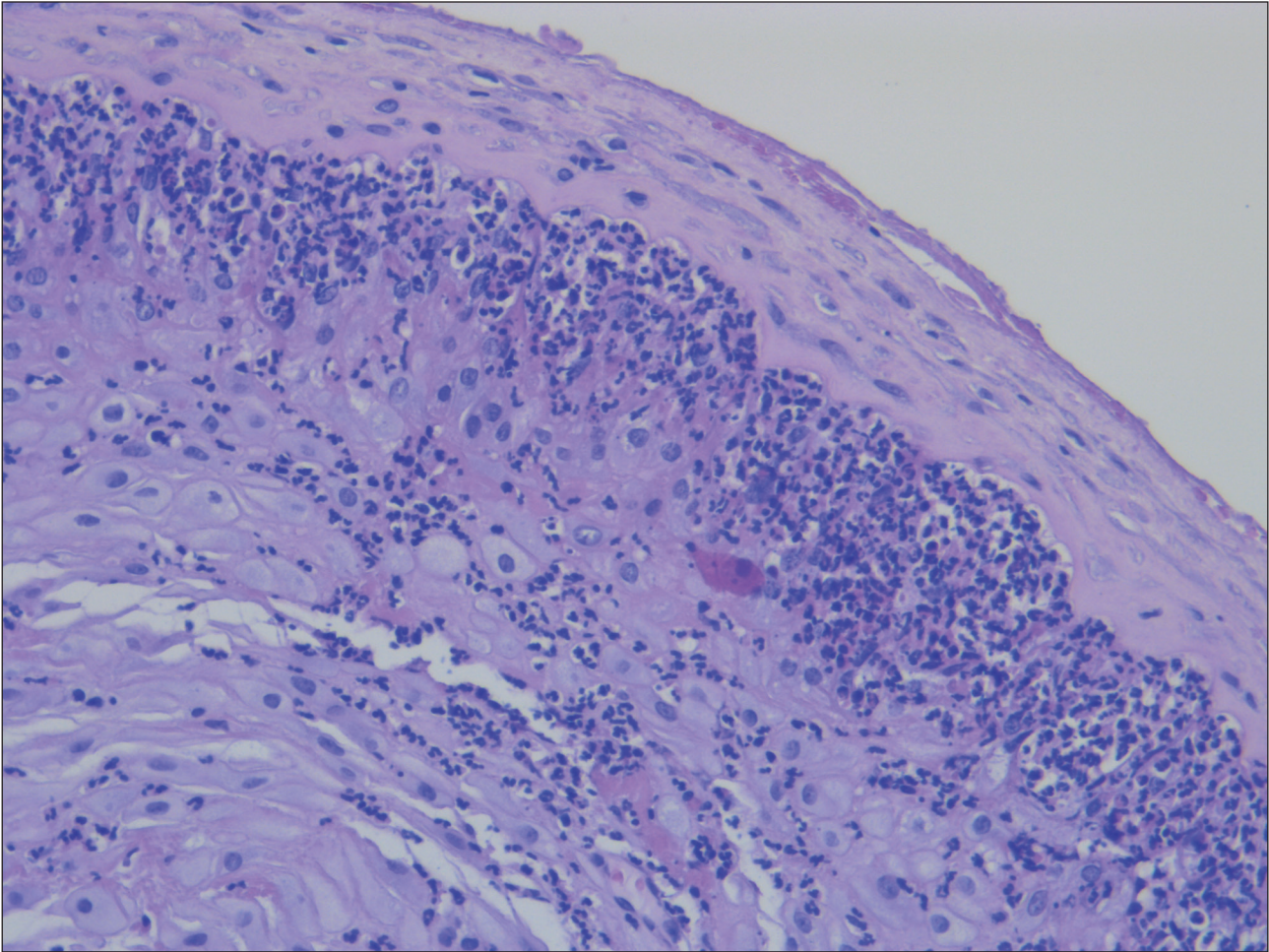


Figure 1 (LECT 47). Chorioamnionitis: stage 3 grade 2.

LECT 48

METABOLOMICS AND PLACENTA

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The placenta is a vital organ: it can be considered the connection between mother and foetus. It acts as a carrier (nutrients and oxygen, waste products and carbonic anhydride) as well as a barrier against infections. Pregnancy related metabolomics has examined different biofluids to have deeper insights into pathologies interesting this condition [1]. Amniotic fluid, urine and plasma were the most studied samples, while placenta tissue has been the subject of only few studies. The main technique employed was Mass Spectroscopy (MS), interfaced with Gas Chromatography (GC-MS) or Liquid

Chromatography (LC-MS). In 2008, Heazell et al. reported a study on placenta tissues, from 11 uncomplicated pregnancies, cultured at different oxygen concentrations: the aim of this study was to find metabolic changes in the culture medium and in placenta tissues lysates, possibly related to preeclampsia [2]. The same research group published an analogous study on samples from 6 uncomplicated pregnancies and from 6 women diagnosed with preeclampsia: the experimental treatment of samples was analogous, only the analytic platform changed, being used an LC-MS system [3]. The same technique was applied to the study of placentae from normal pregnancies (8 samples) and from women with suspected SGA (Small For Gestational Age) (9 samples) [4]. A different sample preparation was reported by the same group in 2012 [5]: placenta samples at different gestational ages (6 at 8 weeks and 6 at 10 weeks) and from uncomplicated term pregnancies (11 samples) were examined in comparison with preeclamptic term pregnancies (6 samples). The preanalytical sample preparation involved the homogenization of the placenta tissue

with a mixture of solvents, centrifugation and separation in two phases: hydrophilic and lipophilic. The obtained fractions were analysed on different platforms, the hydrophilic one by a GC-MS system, while the lipophilic was analysed on an UPLC-MS (Ultra Performance Liquid Chromatography-MS) instrument. The statistical analysis applied to the data matrixes obtained from the two platforms was a PCA (Principal Component Analysis) to find which metabolites change in a statistically significant way, i.e. with a p -value < 0.05 . In the hydrophilic fraction few metabolites were found significant in the comparison between different gestational ages (2-aminobutyric acid), or between uncomplicated and preeclamptic pregnancies (acetic acid, N-acetylglucosamine, a not identified C6-sugar, and succinic acid). The lipophilic fraction analysis resulted in a great number of statistically significant metabolites (≈ 150 for different gestational ages, and ≈ 70 for uncomplicated vs preeclamptic). In all these works the number of samples analysed may be considered low for a metabolomics study, always entailing a statistical analysis of the metabolite profiles. Higher numbers of placenta samples were reported by Chi et al. [6], who analysed 144 samples from normal pregnancies and 115 from women affected with different NTDs (Neural Tube Defects). The analytical method employed was analogous to that reported by Dunn et al. [5], but the following statistical analysis consisted of PLS-DA (Partial Least-Squares-Discriminant Analysis) and OPLS-DA (Orthogonal Partial Least-Squares-Discriminant Analysis). A recent report by Mumme et al. [6] examined 55 placentae from rats undergoing different diets, to find some correlation between obesity and placenta metabolite content. The samples were homogenized but no phase separation was performed: an aliquot of the tissue extract was analysed by GC-MS and another one by LC-MS [7]. The literature reports only three studies on NMR (Nuclear Magnetic Resonance) metabolomics analysis of placenta tissue. Tissot van Patot et al. reported the ^1H -NMR and ^{31}P -NMR study on 16 placenta samples from women delivering at sea level or at 3,100 m altitude, founding markers of oxidative stress in the first group [8]. The same group reported another similar work applying the same method to the study of placentae from first trimester (5 samples), second trimester (5 samples) and term (4 samples) pregnancies [9]. Whole placental biopsies were analysed by HR-MAS (High Resolution Magic Angle Spinning) NMR spectroscopy in a study on preeclampsia [10]. Our group recently examined 38 placenta samples from obese and normal weight

mothers to find metabolites significantly altered between the groups. An extraction method was developed to allow the separation of two phases, hydrophilic and lipophilic, both to be analysed through GC-MS.

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LECT 49

ARTIFICIAL PLACENTA

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Extremely premature infants, born ≤ 28 weeks of gestation, have the greatest morbidity and mortality because of respiratory distress syndrome (RDS) and bronchopulmonary dysplasia [1]. The gestational age limit for survival nowadays is on 23 weeks, which are the biological milestone of lung development, characterized by the early canalicular stage of lung morphogenesis. Thus, the 23-25 weeks are the so-called “grey zone” where vital organs have not developed just to sustain life [2]. Preterm newborns are at increased risk of early and late sequelae such as cerebral palsy and adulthood diseases (such as cardiovascular diseases). Today, the standard of neonatal assistance uses mechanical ventilation including: high flow nasal cannulae (HFNC); intermittent mandatory ventilation; continuous positive airway pressure (CPAP); high-frequency oscillator ventilation (HFOV); high-frequency jet ventilation. Steroids are used to accelerate fetal lung maturation. After delivery, surfactant therapy helps lung ventilation by increasing compliance. These techniques of mechanical ventilation permit to extend fetal survival from 32 weeks to the current limit of 25 weeks but limited improvement to survival and no changes to short- and long-term morbidity occur for infants born in the “grey zone” (23-25 weeks).

The functions of human placenta are gas exchange, adsorption of nutrients and elimination of waste. Furthermore, placenta is an endocrine organ secreting hormones, cytokines and growth factors that favor fetal growth and produces antioxidants to protect the fetus from free radicals [2]. The artificial placenta (AP) should retain the fetal circulation bypassing the developing lungs. In fact, the concept of AP and uterine environment gives an appealing option for extremely preterm infants by bringing them to a uterine-like environment [3]. The objective of AP should be to mimic the function of the native placenta, to decrease mortality of extremely preterm infants and to eliminate the early and late term survivor morbidity. The AP should retain: 1. fetal circulatory configuration and patency of major fetal shunts; 2. blood oxygenation and normal haemoglobin saturation without lung inflation; 3. fluid balance and electrolyte composition to ensure adequate hydration and elimination of surplus fluid; 4. kidney function to eliminate metabolic nitrogenous waste; 5. endocrine support. The history of AP spans 60 years. Extracorporeal life support (ECLS) is the term used to describe the preservation

of life by means of an external assistance device. Extracorporeal membrane oxygenation (ECMO) is the transfer of air or oxygen directly into blood across a gas permeable membrane. ECMO was adapted for use in an experimental AP and tested on preterm fetal lambs by Westin and Callaghan in 1958 and 1962, respectively [2]. However, extracorporeal systems have not achieved great success to extend survival. Partridge et al. [3], recently, reported the development of a system that incorporates a pumpless oxygenator circuit connected to the fetus of a lamb via an umbilical cord interface that is maintained within a closed ‘amniotic fluid’ circuit that closely reproduces the environment of the womb. The authors showed that fetal lambs, that are developmentally equivalent to the extreme premature human infant, can be physiologically supported in this extra-uterine device for up to 28 days. Lambs maintained stable hemodynamics, had normal blood gas and oxygenation parameters and maintained patency of the fetal circulation. With appropriate nutritional support, lambs on the system demonstrated normal somatic growth, lung maturation and brain growth and myelination. In conclusion, potential applications of AP could be: care of extreme premature infants of 23-25 weeks; treatments for delayed fetal growth related to placental insufficiency; rescue of a preterm infant with a threat of preterm delivery after fetal surgery; opportunity to give birth to newborns suffering from congenital malformations of the heart, of the diaphragm for an early correction; stem cell therapy or gene therapy. For the first time in the history of nature, a fetus has succeeded in making a path of its growth outside of the uterus of the mother, without apparent damage at the level of organs and the nervous system.

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LECT 50

LOW DOSE MEDICINE: A NEW PHARMACOLOGICAL PARADIGM. FROM PRINCIPLES TO RESEARCH

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INTRODUCTION

From the second half of the 1980s the development of the concepts expressed by Psycho-Neuro-Endocrine-Immunology (PNEI) has led to a change of perspective in the interpretation of the biological functions of the human organism, moving from an organicistic view to that of a cellular network. The PNEI approach highlights the importance of continuous cross-talk between cells, organs and systems both in physiological and pathological conditions, finally focusing on the role played by the signaling molecules and thus opening the way to a new therapeutic solution: the use of the same signaling molecules as medications to bring a sick organism back to its original physiological conditions [1-5]. In the following years, research in the field of Molecular Biology and Physiology has provided greater evidence of the fundamental role of signaling molecules such as hormones, neuropeptides, cytokines, and growth factors in all physiological and pathological processes by drawing new pharmacological scenarios [6-13]. However, the development of new drugs based on signaling molecules has been slowed down by the side effects that these molecules show when used at doses above the minimum pharmacologically active doses that are normally in use. In the early 1990s, in Italy, a new pharmacological and medical trend was developed: Low Dose Medicine. Applying innovative pharmaceutical techniques, it has been able to considerably reduce the degree of concentration of cytokine, hormone, and neuropeptide preparations; it has been possible to observe, firstly by baseline research on cells or animal models, and then in clinical trials, that the very low doses (sub-nanomolar) produce the same biological (and therefore therapeutic) effects without the side effects attributable at high doses. Inspired by the studies of Cooke and Bettelli et al. [14, 15], and based upon the studies of Méndez-Samperio et al. [13], the clinical use of low dose cytokines is based on the biological principle of physiological regulation through cross-regulation mechanisms. Taking into consideration that each disease is the expression of changed concentration of specific cytokines – with respect to the homeostatic physiological range – often due to over- or under-

expression of Th subsets, it is possible to up- and down-regulate the altered cytokine's concentration using the same or antagonistic cytokine according to positive and negative feedback mechanisms. In fact, as described by Cooke and Bettelli et al. [14, 15], Th subsets cross-regulate expansion and functions for each other via up- and down-regulation of specific cytokines, and all these mechanisms are led by sub-nanomolar cytokines concentrations.

LOW DOSE MEDICINE AND SCIENTIFIC RESEARCH

Ten years of scientific research in the field of Low Dose Medicine have demonstrated the validity of the conceptual approach and the effectiveness and safety of therapeutic intervention based on the oral administration of sub-nanomolar low doses of signaling molecules (**Tab. 1**) [16-29]. Today, we can state that scientific literature supports the therapeutic approach of Low Dose Medicine and that it is no longer a scientific theory, but it can be the basis for a new medical paradigm, particularly in the pediatric field.

All the works conducted so far show the ability of signaling molecules to modulate immune cell response in a highly selective manner; in particular, the immunostimulating and immunomodulating properties of the tested cytokines are clearly described. The ability to act in a refined way on the Th1/Th2 balance is crucial for the management of diseases characterized by the imbalance in the diametrically opposite cytokines concentration, such as allergic bronchial asthma (showing Th2 predominance), Crohn's disease, and psoriasis (Th1-predominant pathologies). One of the key points emerging from the analyzed scientific works is the efficacy of low dose treatment despite the fact that they have been utilized at concentrations lower than those generally considered pharmacologically active. The use of cytokines and other signaling molecules often dashes against the obstacle represented by the need of high doses, which carry these substances to final concentrations that induce a wide range of side effects alongside the desired pharmacological effects. The classic minimum active dose for these molecules is generally among the lowest pharmacologically active (10⁻⁵) and maximum physiological concentration (10⁻⁶) [8, 9]; Low Dose Pharmacology moves within the range of physiological concentrations of signaling molecules, thus, below the concentrations to which adverse effects appear but also achieving appreciable therapeutic results. The ligand-receptor binding properties are critical to explain how low

Table 1 (LECT 50). List of the major published works in the field of Low Dose Medicine (2009-2017) [16-29].

Year	Authors	Journal	Study type	Title	Molecules and tested drugs
2009	Gariboldi et al.	<i>Pulmonary Pharmacology & Therapeutics</i>	<i>In vivo</i> basic research	Low dose oral administration of cytokines for treatment of allergic asthma	IL-12, IFN- γ
2012	D'Amico et al.	<i>Journal of Cancer Therapy</i>	<i>Ex vivo</i> basic research	Low Dose of IL-12 stimulates T Cell response in cultures of PBMCs derived from Non-Small Cell Lung Cancer Patients	IL-12
2013	Cardani et al.	<i>Gastroenterology Research</i>	<i>In vivo</i> basic research	Oral Administration of Interleukin-10 and Anti-IL-1 Antibody Ameliorates Experimental Intestinal Inflammation	IL-10, Antibodies anti IL-1
2014	Radice et al.	<i>International Immunopharmacology</i>	<i>Ex vivo</i> basic research	Low-doses of sequential-kinetic-activated interferon-gamma enhance the <i>ex vivo</i> cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study	IFN- γ
2014	Roberti et al.	<i>Journal of Biological Regulatory & Homeostatic Agents</i>	Clinical trial	Immunomodulating treatment with low dose Interleukin-4, Interleukin-10 and Interleukin-11 in psoriasis vulgaris	IL-4, IL-10, IL-11
2015	Luchetti	<i>Minerva Medica Oftalmologica</i>	Clinical trial	Increasing of visual function in patients with retinal atrophy treated with drugs of Low Dose Medicine. Monocentric retrospective observational study	NT3, NT4, NGF, Retina suis Injeel, Solanum compositum, Ubichinon compositum
2015	Barygina et al.	<i>Journal of Dermatological Science</i>	<i>In vitro</i> basic research	Treatment with low-dose cytokines reduces oxidative-mediated injury in perilesional keratinocytes from vitiligo skin	IL-4, IL-10, b-FGF, β -endorphin
2015	Lotti et al.	<i>Journal of Biological Regulatory & Homeostatic Agents</i>	Clinical trial	Vitiligo: successful combination treatment based on oral low dose cytokines and different topical treatments	IL-4, IL-10, Antibodies anti IL-1, b-FGF
2015	Radice et al.	<i>Translational Oncology</i>	<i>Ex vivo</i> basic research	Enhancement of the Immunostimulatory Functions of <i>Ex Vivo</i> -Generated Dendritic Cells from Early-Stage Colon Cancer Patients by Consecutive Exposure to Low Doses of Sequential-Kinetic-Activated IL-4 and IL-12	IL-4, IL-12
2015	Lotti et al.	<i>Der Hautarzt</i>	Clinical trial	Successful combination treatment for psoriasis with phototherapy and low-dose cytokines: a spontaneous, retrospective observational clinical study	IL-4, IL-10, Antibodies anti IL-1
2016	Barygina et al.	<i>Journal of Dermatological Science</i>	<i>In vitro</i> basic research	Low dose cytokines reduce oxidative stress in primary lesional fibroblasts obtained from psoriatic patients.	IL-4, IL-10, b-FGF, β -endorphin
2016	Fiorito et al.	<i>Comparative Immunology, Microbiology and Infectious Diseases</i>	Clinical trial (veterinary)	Clinical improvement in feline herpesvirus 1 infected cats by oral low dose of interleukin-12 plus interferon-gamma	IL-12, IFN- γ
2016	Molinari et al.	<i>Cell Tissues Organs</i>	<i>In vitro</i> basic research	Stimulation of the Nonneuronal Cholinergic System by Highly Diluted Acetylcholine in Keratinocytes	Acetylcholine
2016	Genazzani et al.	<i>Bollettino di Ginecologia Endocrinologica Frontiers in Gynecological Endocrinology</i>	Observational pilot study	Pharmacological and Integrative Treatment of Stress-Induced Hypothalamic Amenorrhea	Beta-Estradiol
2017	Martin-Martin et al.	<i>Drug Design, Development and Therapy</i>	Clinical trial	An open randomized active-controlled clinical trial with low-dose SKA cytokines versus DMARDs evaluating low disease activity maintenance in patients with rheumatoid arthritis	Antibodies anti IL-1, IL-10, IL-4
2017	Galli et al.	<i>Italian Journal of Pediatrics</i>	Clinical trial	Long-term treatment with Low Dose Medicine in chronic childhood eczema. A double-blind two-stage randomized clinical trial	IL-12, IFN- γ

dose signaling molecules can be effective. The receptor's affinity for its specific ligand is critical to activate signal pathways downstream of the receptor itself; in conditions of saturation of the ligand, in fact, the receptor blockage and/or its down-regulation is generally induced. Low-dose molecules can, however, induce direct physiological receptor stimulation on immune cells (as described by Gariboldi et al.) [16] by modulating responses within the homeostatic range; Low Dose Medicine achieves one of the key points of the PNEI approach to the disease: restoring the physiological network of the signaling molecules. From a pharmacological point of view, the analyzed works highlight the importance of the activation of low-dose molecules through the drug delivery process called Sequential Kinetic Activation (SKA): low dose molecules not set up by this activation procedure are entirely ineffective, as always described by Gariboldi et al. SKA activation is crucial in overcoming the conceptual barrier represented by the minimal pharmacologically effective dose, being able to induce a release effect of low-molecule activity through interaction with the aqueous vehicle [16]. Eight years of scientific research on Low Dose Medicine has allowed scientists to provide scientifically relevant data that can demonstrate:

- i. the validity of the theoretical concepts underlying the Low Dose Medicine approach;
- ii. the centrality of the pharmaceutical process technology called SKA;
- iii. the effectiveness of the experimental and clinical use of low-doses of SKA activated signaling molecules;
- iv. the immunomodulatory and immunostimulating capacity of the test cytokines and the trophic activity of the growth factors;
- v. the safety of the tested preparations.

LOW DOSE MEDICINE AND PEDIATRICS

In general in all patients, and even more in children, the possibility of minimizing the side effects of the drug must become the north star of every therapeutic treatment: "*primum nihil nocere*" is the command of the fathers of medicine and never an affirmation was more true; but also evidence of effectiveness is needed. Low Dose Medicine is attracting a big interest both in Italy and in the whole world, and its acceptance by the medical community is mainly due to the fact that it shows to be the synthesis of these two needs: effectiveness and absence of side effects. The future of a person is partly prepared during his childhood and adolescence, and the same is for his "nosographic" future. It is well known, as

example, that antimicrobial resistance has become in the last years one of the most important and global problem, due mainly to the overuse and misuse of antibiotics [30]. Is there a chance to reduce the use of antibiotics in recurrent respiratory infections? Very possibly yes, by changing the paradigm of the therapeutic approach to this problem: give priority to therapy prevention. And in this, Low Dose Medicine can play a crucial role: the use of low cytokine concentrations helps to keep the "small" patient's body "reactive" by restoring and sustaining the physiological function of immune-competent organs without "stressing" them, as we know other types of drugs do. The basic and clinical research conducted to date is drawing very encouraging scenarios: the pediatric field, as mentioned above, will be fertile ground in many areas, from that – as has been said – of immunological disorders and recurrent infections, to that of endocrine (from alterations in puberty to thyroid diseases) and that of allergic fields. In particular, Galli et al. [28] studied the efficacy of a low dose SKA (IL-12 and IFN- γ 10 fg/ml) cytokine treatment in combination with a low-dose multicomponent medication with connective detox properties in a pediatric population with chronic Atopic Dermatitis. A randomized controlled double-blind two-stage experimental study has been conducted in order to evaluate a long-term treatment with Low Dose Medicine in a pediatric population (64 + 64 children) suffering from chronic Atopic Dermatitis. The clinical trial included children with low-medium Atopic Dermatitis (assessed according to the SCORAD – Scoring Atopic Dermatitis index, which should not exceed 40, with a minimum score of 6) with a recurrence rate of ≥ 4 /year and with skin lesions occurring for at least six months after the enrollment into the study. At the time of the enrollment, all children had to show an acute phase of the disease. Children with Atopic Dermatitis and IgE-mediated (*in vivo* and/or *in vitro* positive test) positive and non-IgE mediated (*in vivo* and/or *in vitro* negative-negative test) were included. As a primary outcome, the reduction in the severity of Atopic Dermatitis was evaluated through the SCORAD index with a 30% improvement in expected rate, while as secondary outcomes were taken into account: elongation of the "disease-free interval", tolerability and compliance of the treatment and management of adverse events, Skin Prick Test to the leading inhalants and food allergen, Skin Prick by Prick Test to the major food allergy, Patch Test to major food allergens, towards mites and nickel, total and specific IgEs for the

main inhalers and food allergens, characterization of lymphocyte subpopulations in cytofluorimetry by monoclonal antibody bacteria, cell and serum anti-inflammatory and pro-inflammatory cytokines IL-10, IL-13, IL-12, and IFN- γ . The results show that the low dose cytokine group has a decrease in SCORAD score between T0 and T8 of 54%, a decrease that continues in follow-up to 64%. In the same observation period, the treated group showed a significant reduction in the drugs for symptomatic control (antihistamines and topical corticosteroids). The study also showed a progressive improvement in the quality of life (itching and night-time disturbances) of subjects treated with low dose SKA cytokines throughout the survey period.

CONCLUSIONS

Perhaps more than a goal, we are at the beginning of a journey: we must always have a lay and non-ideological attitude, and we'll have to observe, measure, and reproduce. In other words: we have to continue and even increase commitments and investments in research; we should not let ourselves be too optimistic about positive results, or let negative ones disappoint us; we should always remember that Low Dose Medicine can enable us to achieve a use of drugs which matches the concept of the integrated vision of Pediatrics and, more generally, of Medicine.

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LECT 51

REASONABLE USE OF ANTIBIOTICS IN PEDIATRIC AGE: ALTERNATIVE THERAPIES OR CONCURRENT THERAPIES?

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Respiratory tract infections are some of the most common childhood illnesses and their management, both outpatient and home care, commits most of the pediatric working time, especially of national healthcare system's ones. Antibiotics are the most commonly used medications for the treatment of respiratory tract infections in Pediatrics, especially in preschoolers. In addition to the undisputed therapeutic value of antibiotics, the alarm on the abuse of these drugs and on the consequences of this cannot be ignored, as very well described in the 2013 *Consensus Conference* of the Italian Society for Prevention and Social Pediatrics (SIPPS) [1]. In the above mentioned *Consensus*, the ARNO Observatory 2011 data [2], referred to a pediatric population of 1,139,388 children, are reported: they show that the class of antibiotics is ranked first in the prevalence of use (48%), distributed among the age groups in this way: 42% in children under 1 year of age, 66% in children of 1 year of age, 65% in children 2 to 5 years of age, 41% in children of 6 to 11 years of age and 33% in children of 12 to 13 years of age. As emerged from the *Antimicrobial Resistance Surveillance in Europe 2011* of the European Centre for Disease Prevention and Control (ECDC) [3] report, Italy is ranked among the European countries with the highest levels

of antibiotic resistance for the following species: *St. pneumoniae*, *S. aureus*, *E. coli*, *E. faecalis*, *E. faecium*, *K. pneumoniae* and *P. aeruginosa*. But the problem is not limited to antibiotic resistance: the consequences of the quantitative-type microbiota homeostasis [4, 5] disruption induced by incongruous antibiotic therapies, are becoming a real health warning especially in pediatric age, and not just for the immunological aspects associated with the recurrence of respiratory tract infections. In fact, current knowledge on the microbiota and on the correlations between microbiota and gut, and between gut and other systems, make the central role played by the cross talk between microbiota, gut and other systems in the pathogenesis of several diseases easily understandable. In the last decade, in parallel with the works on the microbioma and its immunomodulatory role, a new pharmacological approach is underway: it involves the use of sub-nanomolar concentration of anti-inflammatory (IL-10, TGF-beta) and immunostimulating (IFN-gamma, IL-4, IL-2) cytokines in order to reestablish the Immune System homeostasis [6-15]. The first evidence is extremely encouraging and begins to outline new possible scenarios for the use of these low dose cytokines in pediatric age, even in overlapping with other immunomodulating therapies, both for their effectiveness and their safety, and the possibility to be used in long-term therapies.

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LECT 52

NEONATAL SEIZURES: AN ITALIAN SURVEY ON CURRENT TREATMENT AND MONITORING PRACTICES

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INTRODUCTION

In the last years the management of neonatal seizures has changed in several aspects. A widespread awareness of the inherent difficulties in recognizing neonatal seizures by clinical observation led to an increased use of continuous

EEG monitoring or amplitude-integrated EEG (aEEG) monitoring. There has been much debate about advantages and pitfalls of aEEG in neonatal seizures with results being mostly influenced by physician training, contemporary evaluation of the EEG raw trace and number of used channels. In the last decades there has been a higher precision in the etiological diagnosis thanks to neuroimaging and genetics with an increasing impact on specific therapeutic choices. New antiepileptic drugs have been available and increasingly used in neonates, but phenobarbital and phenytoin are still the preferred first option drugs for neonatal seizures. In the last years vitamins are increasingly used in refractory neonatal seizures in the suspicion of vitamin dependent epilepsy, but no clear guidelines are still available. Although there are concerns about the possible side effects on brain development of the old anticonvulsant drugs, they are still mostly used due to the limited evidence-based literature available and the currently approved national drug agency indications. Among the new antiepileptic drugs, levetiracetam appears as the most promising treatment in neonatal seizures with many observational data. There is an urgent need for more evidence-based data to guide neonatal seizure management and randomized controlled trials comparing old and new drugs are expected. Herein authors aim to study the choices in neonatal seizure treatment and neurophysiological monitoring in Italy among paediatric neurology specialists working for neonatal intensive care units around Italy.

MATERIALS AND METHODS

The Neonatal Neurology Working Group and the Paediatric Neurophysiology Working Group of the Italian Paediatric Neurology Society (SINP) proposed to study the current treatment and monitoring practices among Italian specialists managing neonatal seizures using a web-based questionnaire between March and June 2016.

RESULTS

24 specialists of neonatal epilepsy answered. Most participants indicate phenobarbital as first option, but percentage are different depending on seizure presumed etiology: 75% for acute symptomatic seizures (ASS), 63% for structural epilepsy (SE), 50% for presumed genetic epilepsy (GE). As second option phenytoin was indicated by 54% of participants for ASS, but 46% for SE, but only by 13% for GE, where a pyridoxine trial was preferred by many (29%). Taking together the data of the present survey indicate:

1. a clear sequence of choices for the ASS as follows: phenobarbital – phenytoin – midazolam – levetiracetam;
2. a higher concordance for the first choice (phenobarbital) in all three groups (ASS > SE > GE);
3. a higher discordance of choices for SE and GE compared to ASS;
4. a higher tendency to an early choice of vitamins (first, second, third option) in case of GE;
5. a general lower tendency to choose midazolam in case of SE and GE.

Use of continuous neurophysiological monitoring through conventional EEG was indicated by 58% of participants, whereas 1-3 channel aEEG/EEG by 38%.

CONCLUSIONS

This study represents the first survey among neonatal seizures specialists in Italy. It shows new interesting tendencies regarding the influence of etiology of seizures on the drug choices, more concordant in case of ASS and more variable in case of SE and GE. This may be due to difficulties in managing more heterogeneity, unpredictability and refractoriness of early onset epilepsy compared to ASS. Another original finding is the higher use of neurophysiologic monitoring in managing neonatal seizures. There is still poor evidence available to guide decisions on the most effective and safe anti-epileptic treatment strategies and no shared indication about appropriate monitoring for each clinical situation taking into consideration scientific advances, costs and resources. Current clinical practice surveys are useful instruments to monitor medical practice changes over time. Web-based questionnaires are an easy way to acquire information on the current trends on clinical practices, to understand needs, to better plan interventions or intervention effects.

LECT 53

A TAILORED CLINICAL APPROACH TO THE TREATMENT OF MONOSYMPTOMATIC NOCTURNAL ENURESIS

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INTRODUCTION

Nocturnal enuresis (NE) is an involuntary intermittent voiding while sleeping, up to 3 times per

week, in > 5-6 years old children without acquired or congenital central nervous system pathologies. It is a common problem that affects almost 10% of 7 year-old children. In clinical practice we tend to distinguish between Primary Enuresis, when the child has never acquired a nocturnal continence, and Secondary Enuresis if the child restarts to wet the bed after at least 6 months of dry nights. We also distinguish between Mono-Symptomatic Nocturnal Enuresis (MNE) when no daytime symptoms are associated and Non Mono-Symptomatic Nocturnal Enuresis (NMNE) when a daytime urinary dysfunction is associated. The etiopathogenesis is multifactorial and includes several causes such as a reduced bladder capacity (small size bladder respect to the age, detrusorial hyperactivity), increased production of urine during sleep (reduced production or response to ADH, reduced urine osmolarity), difficulty in awakening the child, presence of other family members affected. Therefore, treatment of MSE may comprise one or a combination of interventions including: behavioural approach (BA), pharmacological treatment (Desmopressin – DVAPP) nocturnal alarm (NA) [1]. Our goal is to give Pediatricians the rational basis for an initial reasoned and tailored approach to MNE using all validated tools.

MATERIALS AND METHODS

In our work for the management of MNE we use concepts that agree with the main studies in literature, such as the international guidelines issued by ICCS, NICE, *Cochrane* reviews and our personal experience gained over the years [2, 3]. A first evaluation of patients with suspected enuresis includes a careful investigation of the medical history to exclude the presence of Lower Urinary Tract Symptoms (LUTS), dysfunctional daytime wetting and related constipation, which suggest toward a Bladder-Bowel Dysfunction (BBD). A physical examination is aimed at excluding anatomical abnormalities (phimosis, labial adhesions) and spinal and neurological disorders. It is also necessary to carryout a urinalysis to exclude urinary tract infection and/or glycosuria. Although spontaneous remission is frequent, any MNE case should be evaluated and separated from non-monosynomatic forms, and the subsequent treatment should involve both the child and the family and consider possible pathophysiological mechanisms [4].

DISCUSSION

According to the most modern pathogenesis, enuresis can be the result of different mechanisms [5]:

1. uncontrolled night-time urine production;
2. ability to contain urine produced by the bladder during the night;
3. the detrusor response threshold to fill with a contraction.

There is no standard first approach to the treatment of nocturnal enuresis. Anglo-Saxon countries (UK, USA, Australia) use mainly the NA, while in most European centres Pediatricians recommend DVAPP as a first step.

According to the most recent guidelines, the treatment of MNE includes 3 possibilities, presented below.

- a. Behavioural approach (BA): it consists of instructions for proper hygiene in voiding, sodium intake reduction, reinforcement of motivation and a calendar of dry nights.
- b. Pharmacological treatment: the first choice drug is Desmopressin with a dose of 60-120 mg per day. Not all patients respond to standard doses, it may therefore be necessary to increase the dosage. Children with nocturnal polyuria respond better to treatment with DVAPP.
- c. NA: this device consists of a sensor that is activated with the emission of a sound whenever it detects the presence of wetness in the child's underwear.

However, each approach has advantages and disadvantages. From a review of main studies in literature and from our experience we can say that the response to these approaches differs depending on the age and sex of the patient. As it is important for pediatricians to have a simple tool to manage the first step approach to MNE, we propose a simple table for a first step practical approach in the treatment (**Tab. 1**). In younger children it can be useful as a first step to always use the BA, especially in females who seem to respond better to this therapy, and use the DVAPP as a second choice. In adolescents, where the problem is most emotionally felt and where the resolution of MNE is most urgent, we suggest starting immediately with NA.

CONCLUSIONS

Nocturnal enuresis is a common problem that can have a strong influence on children's self-esteem. All the approaches require a long-term effective therapy to be effective a long term therapy and a good compliance with the patient and his family. The progress of medical knowledge leads to more individual treatment of patients with NE. The characteristics of the patient and his family preferences must be taken into account, therefore a more tailored treatment can be prescribed.

Table 1 (LECT 53). First step approach in the treatment of Mono-Symptomatic Nocturnal Enuresis (MNE).

Gender	Age (years)	BA	DVAPP	NA
Male	6-8	++	+	-
	9-11	+	++	+
	12-16	-	++	+++
Female	5-8	+++	+	-
	9-11	+	++	++
	12-16	-	++	++

BA: Behavioural Approach; DVAPP: Desmopressin; NA: Nocturnal Alarm.

- not recommended
- + less suggested
- ++ moderately suggested
- +++ highly suggested

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LECT 54

UNFORGETTABLE CASE REPORTS

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INTRODUCTION

1. Ureterocele is the dilation of the distal ureteral portion, into the bladder or urethra. A prenatal ultrasonographic diagnosis can be made and its incidence is of about 1/4,000 neonates. Recurrent urinary tract infection (UTI) is the most frequent clinical presentation; ureterocele treatment is still debated and should avoid UTI and obstruction, preserving renal function and continence [1]. Duplication of collecting system is the most common congenital urinary tract anomaly (0,7% in the normal population) and predisposes to UTI and vesicoureteral reflux

(VUR). About 80% of ureteroceles occurs in patients with duplicated collecting system and ureterocele is bilateral in 10% of cases [2]. Treatment varies from endoscopic conservative approach to surgical reconstruction [1].

2. Prader-Willi Syndrome (PWS) is a genetic disorder whose prevalence is about 1/15,000-30,000. Neonatal presentation is often variable and diagnosis can be delayed, although hypotonia is very frequent. An early diagnosis is important for a correct management and to evaluate the possibility of familiar recurrence [3].

CASE REPORT 1

L. is a female neonate, born at 39 wks by vaginal delivery, Apgar 9'-10'. Prenatal diagnosis of dilated right ureter and ureterocele at 36 wks, dimension of renal pelvis (left 8.4 mm; right 7.8 mm). Renal ultrasound at 6 days of life evidenced bilateral duplex collecting system with a normal right upper one, without stasis, upper left pielocalicial dilatation with microcystic dysplasia, bilateral lower district pielocalicial ectasia and ureteral dilatation (10 mm of diameter) giving origin to two ureteroceles (2.5 and 2.7 cm). Prophylaxis with amoxicillin/clavulanic acid was started. At 6 wks, a UTI was treated with ceftazidime and gentamicin. At 10 wks, cystourethrography evidenced right VUR of 4th-5th grade and scintigraphy with MAG3 detected left kidney's dimension reduction, with residual excluded upper district, ureteral transit delay, reduced right secretory function and moderate upper and lower transit delay. Tubular Extraction Rate (TER) confirmed a prevalence of right kidney activity. At 6 months, an urosepsis, treated with meropenem, has been complicated by vaginal ureterocele prolapse (**Fig. 1**) (right ureteres diameters 3 and 11 mm, left 8 mm), so that a surgical approach has been decided. During the first intervention, a resection of necrotic prolapsed ureterocele (5 x 8 mm) has been performed; four wks later, bilateral resection of ureteroceles and bladder ureteral replantation have been done. At the follow-up, L. shows a preserved renal function (normal values of creatinine and cystatin C) and do not present UTI recurrence.

CASE REPORT 2

C. is a female neonate, born at 39 wks by cesarean section for podalic presentation, Apgar 9'-10', 2,335 g, small for gestational age (SGA). Since birth, C. manifested severe generalized hypotonia (mostly of the axial muscles), feeding allowed via nasogastric tube only, feeble cry, Moro reflex not completely elicitable, absent lower limb reflexes, reduced active movements and dysmorphic features, such as lower

limb and sacral hairs, large bregmatic fountain, microretrognathia, telecanthus, left epicanthus, thin eyelids, bell-shaped chest. Abdominal, cerebral, cardiac, sacral ultrasound and fundus oculi examination resulted normal. Metabolic screening was negative and all the blood exams were in range; cerebral-brainstem RM and electromyography were normal. At 11 days, a normal karyotype has been detected and molecular cytogenetic analysis with specific probe for Prader-Willi/Angelman region on chromosome 15 (FISH) resulted normal. Molecular investigation also excluded spinal muscular atrophy (SMA). At 20 days of life, C. presented amimic facies, absent spontaneous control of head and neck, non-use muscle hypotrophy, cingular and proximodistal hypostenia, absent reflexes and impossibility to cry. At 25 days, for the strong PWS suspicious, methylation test and PCR with specific primers for promotor exon 1 of SNRPM gene polymorphism from C. and her parents have been performed and confirmed the proposed diagnosis (detecting maternal uniparental disomy). C. has been discharged in a condition of generalized hypotonia; her parents have been educated about management, physical rehabilitation and nutrition of the baby at home.

CONCLUSIONS

1. Vaginal prolapse is a rare and severe complication of ureterocele; in our case report, it required surgical management, which resulted efficacious.
2. In the suspicious of PWS, the first exam to perform should be a methylation test of chromosome 15



Figure 1 (LECT 54). Vaginal ureterocele prolapse.

with southern blotting, whose negative result allows PWS exclusion. In case of positivity, more expensive tests can be required, such as FISH, able to detect the cases of PWS due to deletion on chromosome 15 (70%), and PCR to compare polymorphisms among the patient and his parents (detecting the cases due to maternal uniparental disomia or paternal deletion) [3].

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LECT 55

HIPS SONOGRAPHY IN THE THIRD MILLENIUM

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Developmental dysplasia of the hip is a cartilaginous capsule-ligamentous alteration of the coxo-femoral joint. It represents 2/3 of all the pediatric orthopedic pathologies, it is multifactorial and includes several anatomopathological and clinical pictures, starting from instability to the luxation.

Ultrasound reports were examined, according to the Graf method, of 7,027 infants: 3,523 males and 3,504 females of a mean age of 40 days at the time of the ultrasound examination. 501 patients enrolled for the study were born preterm and their mean gestational age was about 32.8 weeks. They were all born in province of Cagliari in the last 10 years, their geographical origin was mainly the South Sardinia. The Graf method is the mostly used, especially in Europe, for the screening of this pathology. The technique described by Graf, requires a lateral approach to the hip of the patients, with the probe put perpendicularly on the skin surface in respect of the great trochanter, in order to locate the correct articular section that requires the rectilinear visualization of the iliac wing, of the acetabular bone top with its inferior border, of the cartilaginous top that ends at its extremity with the

labrum and the metaphysarius femoral ossification front. On the obtained picture 3 lines are plotted: the basal line, parallel to the iliac wing; the bone top line that follows and overlaps the bone section of the acetabulum starting from the inferior border of the iliac bone; the cartilaginous top line that connects the labrum to the point in which the convexity of the bone edge becomes concavity. From the intersection of these 3 lines, the alpha and the beta angles are obtained, their measurement with the description of some joint features such as the conformation of the bone top, of the cartilaginous and the shape of the bone edge allows as to typify the hip by assigning a number and a letter. The Ia and Ib types represent normal hips, that are characterized by an alpha angle of at least 60° and differs for the beta angle that is lower than 55° in the Ia type and higher than 55° in the Ib type. The IIa type represents the immaturity of the hips in infants with an age of less than 3 months and it is divided in two subtypes, depending on whether the immaturity is physiological for the age (IIa+) or not physiological (IIa-). The type IIb, is a hip with a delay in the ossification where the age of the infants is above 3 months and maintains the same angle as the type IIa (alpha between 55° and 59° and beta > 55°). The type IIc, defined as critical hip, present an alpha angle between 49° and 49° while the beta angle is lower than 77°. The type IId is characterized by a hip that is about to decentralize where the cartilaginous acetabular coverage with a beta angle higher than 77°. The type III is a decentralized hip, subluxated. It is subdivided in IIIa if there is no structural alteration of the cartilaginous top and IIIb if there is any, furthermore the bone coverage is scarce, with an alpha angle less than 43°. Ultimately, the type IV is diagnosed when there is a clear dislocation in which it is impossible to measure the angles. The worsening of the ultrasound type reflects a progressive reduction of the acetabular coverage bone of the femoral head with a smaller and smaller alpha angle and a beta angle more and more wide that is instead the sign of the femoral push on the cartilaginous edge that can be reversed backward in the most serious cases. The results obtained in the study prove that 94.17% of the patients can be considered healthy and the remaining 5.83% affected by the pathology. In the latest group, greater than 70% of the hips were simply immature, the others displayed mild and moderate dysplasia and only the 0.06% was dislocated (seven dislocated hips in 10 years). A merely numerical confrontation between the incidence observed in this study and

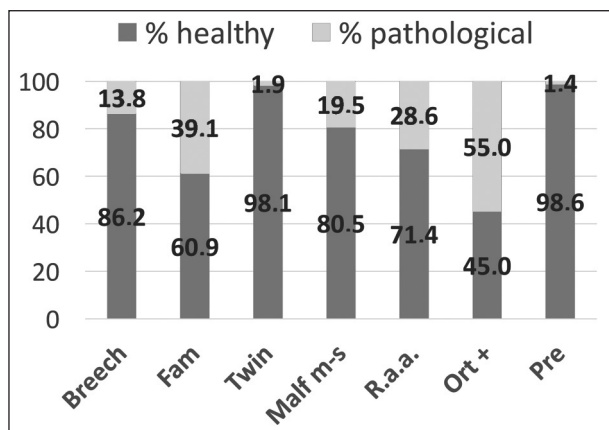


Figure 1 (LECT 55). Analysis of the risk factors in the studied population.

Fam: familiarity; R.a.a. reduced hip abduction; Ort +: positivity to the Ortolani maneuver; Pre: prematurity.

the data present in literature would not be a useful tool of comparison. Even with other studies using the same method, significant differences in the number of patients enrolled, in the enrolling modalities of patients and in the definition of the pathological hips were found. The female gender results to be more affected (F:M = 4.5:1) according to the literature. The twins and the premature birth are not related to the development of the hip dysplasia (Fig. 1). This study has confirmed in addition the already known reliability of the clinical signs such as the reduced abduction of the hips and overall the positivity to the Ortolani maneuver. Since more than 70% of the patients affected by this pathology do not present any risk factor, according to what was stated by our group in 2009, the universal ultrasound screening is necessary to reduce the number of late diagnoses. It would be useful a higher standardization in the diagnosis and in the management of this pathology with the aim of a better understanding of its real distribution.

LECT 56

NEONATAL PHARMACOLOGY: WHAT'S THE NEWS?

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INTRODUCTION

Different problems related to drug use in the neonatal population (lack of suitable formulations, presence of potentially toxic excipients, difficulty in detecting/signalling adverse drug reactions...)

still exist, but overall the scarcity of randomized controlled trials (RCTs) remains an important problem.

NEWS ON CLINICAL RESEARCH IN NEONATES

Despite some initiatives taken in the last years to increase clinical research in the paediatric population, in particular the introduction of the European Paediatric Regulation, the number of RCTs including newborns remains low. This was recently underlined by a resolution of European Parliament (2016/2902) that suggests to create a European network to favor paediatric clinical research. The WHO List of Essential Medicines for Children 2017, drawn up to promote clinical studies, comprises some specific medicines for neonatal care such as oral and iv caffeine and surfactant (suspension for intratracheal instillation). During the period January-July 2017, some approved Paediatric Investigation plans regarded new neonatal formulations (referred to ceftazidime/avibactam, dobutamine, fidaxomicin, iron preparations, melatonin and tapentadol) and RCTs specific for preterm (dopamine, dobutamine, tapentadol) or term neonates (anidulafungin, ceftazidime/avibactam, fidaxomicin, oseltamivir).

PARACETAMOL: NEW USES FOR AN OLD DRUG

Recently, paracetamol has been proposed intravenously as a supplement therapy to opioids for post-operative analgesia and suggested as a possible alternative to favor patent ductus arteriosus (PDA) closure in neonates. As regards its use in post-operative analgesia, i.v. paracetamol is still off-label for specific sub-populations (preterm newborns), but this formulation is increasingly used in these subjects in an attempt to reduce/avoid opioids [1]. In neonates, the benefits remain controversial, being the available pharmacokinetic data without any validated pharmacodynamics correlation. However, after non cardiac surgery this drug showed a very relevant opioid sparing effect [2].

In relation to PDA closure, paracetamol emerged as a possible alternative treatment to ibuprofen and indomethacin, but many aspects (efficacy in ELBW infants, the best route of administration, optimal dose, timing of the first dose) remain largely unexplored and information is lacking particularly as regards its long-lasting effects [3]. Some authors [4] compared the efficacy and safety of paracetamol, indomethacin and ibuprofen in 300 preterm neonates with a hemodynamically significant PDA: no difference has been observed between the three drugs as regards efficacy, while

paracetamol showed less side-effects on renal function, platelet count and GI bleeding.

CONCLUSIONS

In the last years, some initiatives contributed to increase clinical research in paediatric patients, but the assessment of the risk-benefit profile of medicines in neonates remains problematic. Therefore, RCTs are mandatory in this paediatric population not only to improve therapeutic approaches to specific pathologies, but also to explore new uses for old drugs such as paracetamol.

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LECT 57

AIR NEONATAL TRANSPORTS IN SARDINIA: FROM STORK WINGS TO AIRPLAIN'S

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With the collaboration of all the Medical and Nursing Staff of the Neonatal Intensive Care Unit, Neonatal Pathology, and Neonatal Section, AOU and University of Cagliari, Cagliari, Italy

INTRODUCTION

The remarkable progress in Neonatology in the last decades increased the survival of preterms and critical newborns and decreased neonatal mortality and morbidity. Pathologic newborns and infants, who have undergone surgical and medical therapies in their first days of life, are today young healthy adults with a high quality of life.

On one hand, early prenatal diagnosis and the present network of pregnant and neonatal transport, on the other, in Highly Specialized Medical Centres, have been enabled to care for our critical babies in a short time, with an important decrease in mortality

and morbidity. Around the World, every state has a specific network of neonatal transport. In Italy, the Neonatal Transport Service (NTS, the Italian acronym is STEN) is the main transport network for pathological and critical newborns, extending over 90% of the Italian State, except for the Valle D'Aosta and Sardinia Regions. In Sardinia, NTS could only be a regional system of transport, but the insularity and geographical features determine the need for other neonatal transports to the Italian Peninsula.

Critical patients need immediate medical transfers to Highly Specialized Departments, such as Cardiac-Surgery, Neurosurgery or Neonatal-Surgery Centres in the Peninsula. At the moment, the collaboration between the Italian Military Air Force and the Medical Nursing Staff of the Neonatal Intensive Care Unit and the Neonatal Pathology is the best network of interregional transport for our sick babies.

Our knowledge about neonatal air transfer around the world includes the experience of other islands, such as the Isle of Man in the North of Europe [1].

AIR TRANSFER

Sardinia is a singular region: firstly, it's an island, with long distances of one thousand miles between Sardinia to Tertiary Centres in the Italian peninsula; secondly, the main problems are the weather and the time that a critical patient has. The main problems are that Sardinia doesn't have a regional NTS; it has a low density in population in the centre of the island, but a high density close to the major cities, Sassari and Cagliari, and there is an absence of Highly Specialized Departments, such as Cardiac Pediatric Surgery or Pediatric Neurosurgery.

The Italian Military Air Force represents the best transport system for critical Sardinian babies. On Italian Military Airplanes, parents can go on board with their babies, military and medical staff.

We evaluated the epidemiology of critical and pathological newborn transport in our singular island



Figure 1 (LECT 57). Elmas Airport, 2014. A neonatal transport.

and emphasized the leading role of the Italian Military Air Force (**Fig. 1**), namely the 31st Wing “Special Transport” based in Ciampino (Rome).

OUR DATA

Critical and sick newborns in the first thirty days of life are admitted from other regional and city hospitals in the NICU and the Neonatal Pathology of Azienda Ospedaliero-Universitaria in Cagliari. Inter-regional transfers were carried-out over a five-year observational period (from the first of January 2012 to the first of September 2017) have been examined and data has been extracted from the SISAR Regional System, the paper registers of the NICU and the Neonatal Pathology, and clinical cases of every patient transferred. Destinations and clinical diagnosis were evaluated.

The total number of interregional air transports over a five-year observational period, from the first of January 2012 to the first of September 2017, is one hundred.

The number of neonatal interregional transfers carried out in the period from the first of January 2015 to the first of September 2017 is forty-nine: 24 (49%) from NICU, and 25 (51%) from Neonatal Pathology.

The destinations were: thirty-four (70%) to the Pediatric Hospital Bambino Gesù in Rome; four (8%) to the Pediatric Meyer Hospital, Florence; two (4%) to the Pediatric Cardiology Department

Pasquinucci, Massa Carrara; seven (14%) to the Hospital IRCCS Policlinico San Donato Milanese, and two (4%) to the Gaslini Pediatric Hospital, Genoa.

Diseases or problems that required immediate air transport were: thirty-four for abdominal and thoracic diseases and surgery, nine for congenital heart diseases; six for neurologic and metabolic diseases (**Fig. 2**).

SARDINIA: A UNIQUE ISLAND, A UNIQUE EXPERIENCE

Insularity, a high density in population near the coast and a low density in the centre of Sardinia make the singular experience of interregional neonatal transfer to the peninsula clear. A low total density in population and a low level of births don't justify Highly Specialized Department, such as pediatric and neonatal neuro-surgery or cardiac pediatric surgery, but the high percentage of air transports of critical and life-threatening patients could be justified. The Italian Military Air Force offers the best collaboration twenty-four hours a day, seven days a week, in adverse weather conditions. In the same way, the medical and nursing staff are readily available for neonatal interregional transport, even outside their shifts if it's necessary.

WHAT CAN WE DO FOR OUR NEWBORNS?

Birth is the best event in a family and in a society, it's the parents' best dream. The diagnosis of an

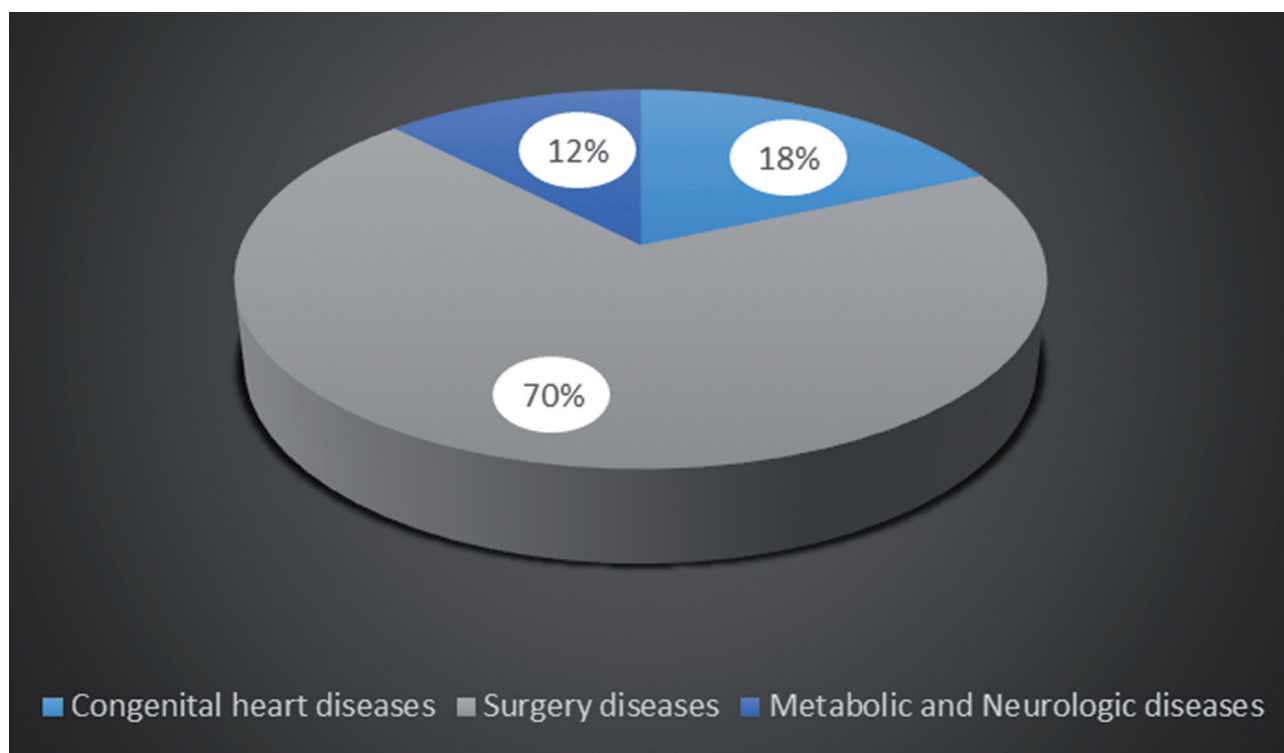


Figure 2 (LECT 57). Diseases or problems that required immediate air transport.

important pathology, such as the diagnosis of congenital heart disease in a life-threatening patient, is a shock for the family.

These conditions request immediate air transfers: happy feelings change in to disperation. In the last decades, the Italian Military Air Force has supported Sardinian babies' health, to give our babies the best choice in surgical and medical therapies. In every transfer, the Military, the medical and nursing staff can see their own children in the eyes of these sick babies. Finally the stork's wings are the image of birth just like the air wings of the Italian Military Air Force are the image of a healthy life for the newborn and their family.

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LECT 58

MORE THAN 50 YEARS OF HOSPITAL FLIGHTS FOR THE ITALIAN AIR FORCE

Italian Air Force, 31st Wing, Captain D. Sgambati

The 31st Wing “Special Transport” based in Ciampino (Rome) is the Italian Air Force unit in charge of the air transport to satisfy the mobility requirements of the high State, Government and Military VVIP Offices, as well as emergency medical transport for humanitarian reasons of patients who are severely traumatized or in imminent danger of life, organs and medical teams for lifesaving transplants (Fig. 1). The vision of the Wing is to always represent a

reality of excellence and professionalism in order to accomplish the assigned mission with the highest level of readiness, accuracy, safety, effectiveness, discretion and participatory spirit, guaranteeing worldwide airlift capability for State and emergency transport to assist Italian citizens and anyone else is required to be rescued, even in territories where the Italian Armed Forces are deployed to take part in international military operations. The “twilight” of the sanitary service was between 1962 and 1965 with the arrival of the new DC6B aircraft, but the real hospital flight started with the introduction in 1974 of two airplane DC9-30. With these new airplanes the 31st Wing started to perform the hospital flight service 24 hours, 365 days. The back boarding stair was used to load passengers and stretchers on the airplane. They were used to accomplish several medical tasks all over Europe during the period 1974-1985. The big change in the hospital flight service took place in 1985 when the first Dassault Falcon 50 (SN151) was assigned to the 31st Wing and landed at Ciampino airport; it was Serial Number 151, the unforgotten “I-2020” for the 31st Wing personnel. Shortly afterwards three other Falcon 50 arrived at the base. Two of these legacy Falcons are still in service. Future use of the vector would be for hospital and State flights. An airplane that at the time, for technical features



Figure 1 (LECT 58). The logo of the 31° Stormo (the 31st Wing).

and versatility, was able to renovate the activity of the Wing, thanks to performances, flexibility and high overall safety standards and reliability. Still today, with the legacy Falcon 50, along with the Falcon 900EX-EASy (introduced in 2006), the 31st Wing crews, on behalf of the Italian Government, provide the high readiness emergency air transport service 24 hours a day, 365 days per year. It has been 32 years of intense partnership, celebrating the thirtieth anniversary at Ciampino airport in April 2015. During this period the 31st Wing crews have flown an astonishing total of 140,000 flight hours, an average of 5,000 per year for special emergency hospital and State flights, touching every continent and flying the Falcons around the world with high readiness global reach. Only in the last five years of emergency medical air service, with its Dassault airplanes, that can be quickly converted from utility to medical evacuation transporters, the Wing has flown an average of 1,200 flight hours per year, transporting more than 350 patients and 600 medical staff members, equipment and organs (Fig. 2). Very often emergency hospital flights involve children or infants. IAM3168, a F900 EASy flight, is to date the longest hospital flight in favor of a newborn patient ever made in the history of the aero-medical transport. 26-hours of flight to transport a one month

old infant, the son of a couple of Italian citizens, from China to Italy. Departed from Ciampino on Friday, July 22, after boarding in Genova the Gaslini hospital special medical team, the F900EX EASy landed at the Shanghai airport, after a 13 hour flight. The ambulance with the little patient and his parents was already waiting for them, for the immediate return to the homeland and hospitalization of the child in Genova. The mission was accomplished in less than 70 hours from the task, thanks to the efficacy of the alerting system and the reliability and readiness of the support chain. These are considerable numbers, that can be achieved through the 31st Wing airmen and women’s preparation, skillfulness and passion, combined with the excellent technical support of the maintenance departments, both military and the Dassault Falcon Service. Since the arrival of the first Falcon 50 at Ciampino, in fact, a field support team, the Dassault Falcon Service Roma, was established in the military airport. A small dedicated field team of highly skilled French technicians works side by side with the military to maintain the Falcon fleet to the highest level of efficiency and continuity required to support the readiness and the reactivity of the mission assigned to the Italian Air Force 31st Wing. The efficiency level has always been higher than 85%. This has created a valuable mix

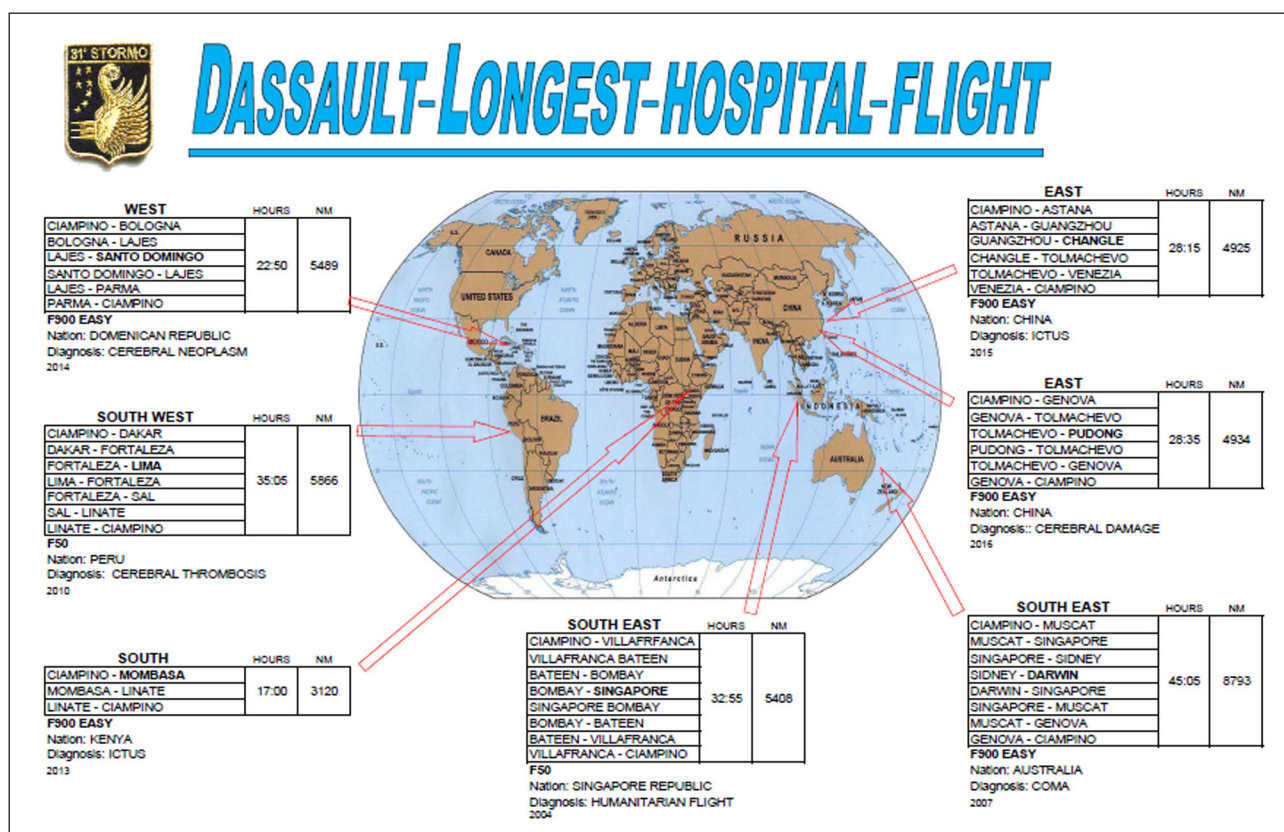


Figure 2 (LECT 58). 31st Wing Falcon longest hospital flights: “Readiness for Global Reach”.

of knowledge and experience and, most of all, a full sharing of the 31st Wing mission and vision, with total effort and commitment for its support. The chain of command to activate the Air Force squadron is practically a connection line among hospitals, Prefectures and the Air Force Chief of Staff mission tasking room. The process starts with the request for airlift support from the hospital manager to the headquarters of a Prefect that, after a detailed evaluation, will forward the request to the Air Force Headquarter in order to task the crew, who is standing by, ready to respond and take off within 2 hours. For emergencies abroad it is the local Embassy or Consulate who receives the request from the hospital and afterward forwards it to the Italian Air Force. The 31st Wing constantly works to set an example of highly specialized international synergy, and to maintain a level of excellence and success for “Special Needs”.

LECT 59

NEONATAL TRANSPORT IN ITALY AND BEYOND

S. Rugolotto

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Neonatal Transport Service (NTS) is a complex pivotal element within a regionally based perinatal care program. It needs a specific transport team (a neonatologist and a neonatal nurse), dedicated devices such as a transport incubator, a neonatal ventilator, a multi-parameter monitor, infusion pumps, the possibility to use NO, and other ancillary devices; last but not least the possibility to switch among transport systems like ambulance (the most common), airplane, helicopter, and boat. As a matter of fact it is a sign of a highly organized health system, of a good perinatal network, and it has also been associated with improved neonatal outcomes, and enhanced neonatal survival [1]. These are the reasons why the Italian National Ministry of Health has always recommended the institution of specific NTSs for critical ill newborns in the Italian Regions [2, 3]. However, the development of regionally based NTSs in Italy occurred slowly. This happened because after the 80s, and mainly after the 90s, the Italian Health system became, gradually, a more and more regionally based health system, with a high number of Newborn Intensive Care Units (NICUs). For this reason, despite the recommendations of the National Ministry of Health, many variations in

practice existed throughout the country. Mainly, not all of the Italian regions began NTSs at the same time, and did it in the same way. The Italian Society of Neonatology recently provided an excellent survey of the NTSs available in Italy up to 2015 [4]. According to this survey, in Italy 44 NTSs are available; 11 regions have a total coverage of their territory, 3 regions have a partial coverage, and in 5 regions a NTS is missing. Over a year, a total of 6,300 neonatal transports are provided, however not all of the critical ill transported newborns are counted because some of them are transported by 118 emergency service or other systems. The average transfer time is 112 minutes, 8% only of these transports are about preterm infants (< 28 weeks of gestational age). The most common indication for neonatal transport is the respiratory problem (76%); other common indications are cardiac disease (9.5%), surgical disease (9.5%), and congenital disorders (4.8%). In 8 regions only is present a regional coordinating center for NTS: Basilicata, Campania, Veneto, Trentino Alto Adige, Friuli Venezia Giulia, Lazio, Liguria, Molise. Most of the NTSs in Italy rely on hospital-based transport teams (a neonatologist and neonatal nurse), and use local emergency medical services vehicles (ambulance, helicopter and more rarely boat and aeroplane), within the national emergency system (118).

In Italy, the last data (2017) on the NTS reports an extension to over 90% of the Italian State, except for the Valle D’Aosta and Sardinia Regions.

The main differences rely on the size of populations served and on the model of the NTS. Let’s review the three main models available. The first model is a hub and spoke model and it is the most common. In this model a referral NICU provides NTS for a small group of birth centers (spoke centers) and transports those newborns who will be admitted to its unit. In case the neonate of a spoke center cannot be admitted to the referral NICU, the spoke birth center has to look for another close referral NICU, which will transport the neonate to its unit. The personnel of this NTS is the same of the NICU, thus their activity is mainly within the NICU while the neonatal transport activity is just a small part. The Italian regions, which adopted this model, have therefore many NTSs available, which do not provide many neonatal transports per year. The second model is the less common. In this model a NTS (not NICU based) is available for a large group of birth centers. It provides a NTS 24 h/day, 7 days per week, with a neonatologist and a nurse. In this model the area is pretty large, and the transport time and transport distance may be very

long. It is a dedicated NTS where the neonatologists and neonatal nurses do neonatal transports only. This NTS provide a big number of neonatal transports per year. The third model is still a hub and spoke system but with a few centers of NTS NICU based available. An example of this model is the one available in the Veneto Region. In this region two NTSs only are available, based on Padua and Verona NICUs. They have their own specific group of spoke birth centers and they take care of all the requests from their birth centers. When a birth center needs to activate the NTS, this service will accept the request and will transport the newborn where a bed is available, either in its own NICU or in others. In Veneto region a specific regional budget is dedicated to the service and the personnel receive extra payment for the NTS. After about a couple of decades of NTS activity and the actual recent drop in birth rate, the time has come in Italy to review NTSs, NICU network and birth centers.

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LECT 60

A HEART OF... JERSEY

A. Brandi

Cuore di Maglia Association, Cagliari, Italy

“Cuore di Maglia” association was born in 2008 in Alessandria, and it is now active in 60 hospitals and in 6 life-help centres. Our branch in Cagliari was founded in November 2012. Our little works warm the babies up while embracing them; they try to make their parents smile; they are part of the Care Protocol. We hand make clothes for premature babies hospitalised in Neonatal Intensive Care Units. The clothes we realise are conceived and tested with the support of doctors and nurses of the unit. Moreover, we only use merino wool for winter clothes and cotton baby for summer ones, without any kind of acrylic material. Our main items are: *sacco nanna* to feel snuggled up and cuddled; little hats (**Fig. 1**) and shoes (**Fig. 2**) in every possible colour; doudous to hold



Figure 1 (LECT 60). Hats realised by “Cuore di Maglia”.

close to smell mummy's scent (Fig. 3 and Fig. 4); mini twist for the kangaroo; little sweaters for the discharge from hospital; little blankets warm



Figure 2 (LECT 60). Shoes realised by “Cuore di Maglia”.



Figure 3 (LECT 60). Doudous to hold close to smell mummy's scent.



Figure 4 (LECT 60). Doudous to hold close to smell mummy's scent.

and full of colour. With *Destinazione Paradiso*, “Cuore di Maglia” gives warmth and love to the little ones who cannot make it.

LECT 61

THE ITALIAN REFORM CONCERNING CIVIL AND CRIMINAL LIABILITIES IN THE HEALTH FIELD

F. Tregnaghi

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In Italy, the recently enacted Law no. 24 of 8 March 2017, commonly known under the names of the proposers Gelli and Bianco, contains provisions concerning the safe treatment and safety of persons receiving treatment, including the responsibilities of health professionals. It was published in Official Gazette no. 64 of 17 March 2017, and came into force on 1 April 2017. This law represents an organic reform of the liabilities of all those who operate in the health field. It substitutes previous laws dealing with single aspects, which in the reality of the courts had little impact on a jurisprudence consolidated in often quite strict positions. The objectives of the reform are many:

1. The most evident and publicized is the goal of “severing ties” with the jurisprudence that had developed under the previous legislation. To that end, it deals with civil liability by establishing that the single health professional is to be liable for so-called extracontractual, or Aquilana (from the name of the magistrate who introduced it in ancient Rome), liability, should the accusations be verified, with the advantages provided for in the overall Italian regulations concerning liability, both in terms of the statute of limitations – shorter terms – and of the extent of damages payable – more limited. Contrary to the previous situation, the major “contractual” liability now lies only with the health structure, since it is a party to the sort of contract between the patient and the structure, with direct rights and duties to the patients.
2. The “new” civil liability, modified in substance, is also applicable through a special procedure, which has raised certain perplexities in the minds of its first interpreters. It states that the patient (or his/her heirs in the case of death) who intends to sue for damages cannot immediately apply to a court, but must necessarily first try

to reach an out-of-court settlement in front of a professional mediator or begin the procedure of a preventive technical verification leading to a settlement, during which one specialist, appointed by a judge, must evaluate the fact or omission subject of the complaint, and attempt to reach an agreement. The presence of the opinion of an authoritative third party, who would have a voice in future litigation for damages, may be particularly useful in “demolishing” from the very beginning demands for exceptional compensation or baseless complaints.

Also introduced is the patient’s faculty of beginning litigation directly with the insurance company of the health professional or structure, which is obliged to respond in accordance with the minimum coverage set by law, and which only later will have the faculty, if provided for in the terms of the policy, to take legal action against the insured health professional or structure for the amounts disbursed.

Insurance coverage is made obligatory for health structures:

3. as concerns criminal liability, the new law introduces a new offence in the criminal code (Article 590.6) which defines “culpable responsibility for death or personal lesions in the health care field”. This distinguishes it from manslaughter or culpable lesions previously applicable.
4. Procedures that regulate and encode the “validation” of good practices and guidelines that should help health professionals, but even more so the judges who must settle litigation, in establishing eventual liabilities should a treatment lead to an inauspicious conclusion. The new law thus introduces “open” directions for sanctions, since at least a part of the effective field refers to “norms” not of legislative, and not even statutory, origin (still to be adopted by ministerial decree), but which are substantially private, although hopefully of great scientific value.

Time and jurisprudence will tell if the basic objectives of the law (to lighten and in any case clarify the ambit of health care liabilities) are reached and to what extent.

LECT 62

A MEDICO-LEGAL VIEW AT THE MEDICAL LIABILITY CONTEXT : LIGHTS AND SHADOWS OF THE ITALIAN “NEW LAW NUMBER 24/2017 GELLI-BIANCO”

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At the beginning of the 2017, the Italian Parliament enacted a law concerning the Patient Security and the medical liability system, the so-called Bianco-Gelli law (no. 24/2017). The main declared goal of this legislative act was to improve the creation of a patient-centered health system and, at the same time, to reduce all the medical activities put in place by physicians to reduce indemnity claims. The main innovative issues were those which have been long considered by all the scholars as the more detrimental ones for the medical activity, namely the penal punishment for a physician misconduct, the reversal of the duty to prove in the civil code context, the absence of an obligation for the Hospitals and all the other health facilities to hold the health workers harmless by subscribing an insurance against damages to patients. One, and for some reasons the only, positive aspect of the law is the creation of a systematic body of rules dealing with the complex world of the medical liability system. But several criticisms have been raised by judges, lawyers, medico-legal expert and by citizens’ organizations, postulating that the rules may be from one side unconstititional – by infringing the right to health stated by article 32 of the Italian Constitution – and from the other not suitable to prevent the ‘tsunami-like’ effect of medical malpractice suits. Although not fully implemented in the everyday courtroom life – being necessary the creation of the National Guide Lines System (SLGN) – and being the effects of the new rules waited in a five years period, the first comments on the single articles seem to underline a substantial futility of the rule there stated. Looking at the article 6 – in which a penal liability subsystem for the health workers has been depicted – the limitation of the negligence only to the lack of skill may be taken into account from the Judge only in those cases in which a guideline among those included in the SLGN. Being the medical conditions in which the physicians may refer to a coded guideline residual in the clinical scenario and being generally all the patients affected by one or more comorbidities – while the guideline is created for a single disease – the practical effect of this subsystem in the wider context of the medical malpractice world is a priori limited. In the lack of coded guidelines, the Judge has to base his/her decision on the prior rule,

vanishing the innovative effect of the law. One praiseworthy change in the general rule of medical liability in the civil code context is the clear-cut statement of the contractual nature of the liability for the Health Facilities while for all the health workers employed in the facilities a non-contractual relationship with patients has to be considered. As well known, the two different liability profiles differ by the duty to prove (being born by the hospital in the case of contractual relationship and by the patient otherwise). This rule seems to put an end to a vexed quaestio dealing with the possibility for health employees to be charged with a contractual liability, based on the so-called ‘social contact’. As a personal opinion, we believe that one of the more problematic aspect of the new law is the ‘inner’ duty for the Hospital General Director to inform the Corte dei Conti Prosecutor everytime a compensation is paid for a medical liability. The loss of revenue and the insurance company’s reimbursement are going to become the new frontier in the medical liability arena. The articles 9 and 12 addresses the unclear issue of the right/duty for the Hospital and for the Insurance Company to ask the physician to return the undue payment of the compensation, being the conduct of the physician/s characterized by a wrongful behaviour which may be evaluated as wide fault (*‘culpa lata’*). So not to be obligated to pay instead of the physician the loss of revenue, the hospital management may be forced to report all the compensation – even those in which the physician conduct may not be considered as a wide fault – paid to the patients. This general rule may be, in a near future, responsible of a huge number of reimbursement sentences by the Corte dei Conti. If so, the new law will only create a delayed in time request of compensation. As the Prince of Salina states in the *Gattopardo*, “we have to change everything to let all the things unmodified”.

LECT 63

WHAT LEGAL MEDICAL RESPONSIBILITY FOR THE NEONATOLOGIST?

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In recent years, neonatology reached unthinkable goals as to the survival rate and quality of life of extremely preterm infants. These results sparked a series of medical and legal issued concerning

the survival of infants with extremely low EG (< 23 weeks) that many national and international protocols failed to resolve. However, the medical and legal responsibility of the neonatologist cannot be limited to these extreme situations. There are many procedures involving neonatologists, often with obstetricians, for civil and criminal liability in neonatal care. Even in this case the neonatologist almost always has no objective responsibility. However, over the last decade, the neonatologist’s responsibility has undergone substantial changes in neonatal care: early discharge, spasmodic exclusive breastfeeding, lack of controls shortly after discharge lead to the problem of returning to hospital neonates with more or less severe pathologies. Hence the involvement of the neonatologist in objective responsibilities that do not find an adequate response in ministerial or corporate guidelines adapted to the change of events. In this roundtable my goal will be to emerge these issued/problems stimulating our colleagues to do research aimed to resolve this kind of problems.

LECT 64

A NEW LAW ON PROFESSIONAL LIABILITY: BETWEEN UNSOLVED TANGLES AND ONGOING COMPLIANCE TO NEW RULES. THE ROLE OF SCIENTIFIC SOCIETIES

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In the Official Gazette no. 64 of 17 March was published the law of 8 March 2017, n. 24 laying down “*Provisions on the safety of care and the assisted person as well as on the professional liability of practitioners in health professions*”, known as “Gelli Law” from the rapporteur to the Chamber of Deputies. The law, which has been waited for many years, intervenes in a complex matter with the aim of addressing and containing two serious health problems:

- the amount of legal medical disputes (with the consequent substantial increase in the cost of insurance for professionals and healthcare facilities);
- the phenomenon of defensive medicine (responsible for the inappropriate use of resources for public health).

With the new provision, the civil and criminal liability of healthcare providers changes; it

becomes mandatory for every public and private healthcare provider to have an insurance coverage and the compulsoriness of insurance for all health professionals is reaffirmed.

The main changes made by the Law on Responsibility are presented below.

- Criminal liability – The law introduces art. 590-sexies of the criminal code, whose second paragraph excludes the liability for manslaughter or for personal injury caused by the health professional's inexperience, provided that the professional has complied with good clinical-welfare practices and the recommendations contained in the guidelines appropriate to the concrete case.
- Civil liability – The new law establishes that the liability of a medical doctor who has no direct/contractual relationships with the patient becomes "extra-contractual", which means that the responsibility is centered around the provider's fault, which must be proven by the injured party with a limitation period for liability action set to 5 years (from the moment at which the damage comes up).

However, the responsibility remains "contractual" for the healthcare structure, and for the professional provider who has a direct/contractual relationship with the injured patient. For a full execution of the law, however, a few months are still required, because of the various references to the decrees that need to be implemented to make various measures operational, such as the mandatory nature of insurance policies, the definition of minimum insurance policy requirements for the public or private health and sanitary structures and health professionals (by identifying risk classes to which differentiated ceilings apply), the establishment of a National Observatory on Good Health Practices and the setting-up of a list of the scientific societies, scientific technical associations and public and private entities that are called for the development of the guidelines. Therefore, a challenging period is in front of us, both for interpretation and application, that regards the scientific level (formulation of the guidelines in compliance to the law) as well as the jurisprudential level, with an evolution of the concepts of professional guilt in health care. As far as insurance is concerned, the law establishes that insurance companies must extend the insurance coverage to events occurring within ten years prior to the conclusion of the insurance contract, provided that they are reported to the insurance company during the policy term. In the event of a permanent termination of professional

activity for any cause, an overdue coverage period must be foreseen for compensation claims received in the ten years after the cessation date, referring to the facts that occurred during the period in which the coverage was operative. This overdue coverage period is extended to heirs and is not subjected to a termination clause. The Italian Society of Pediatrics (SIP) has for many years been involved in identifying tailor-made policies for this specialty, basing the demands to the insurance market on the concrete experience of risk management on behalf not of a individual professional provider but rather of over 6,000 medical doctors. The SIP collective policy already has characteristics that are in line with the requirements of the Gelli Law, having anticipated some aspects, such as those that regard retroactivity and ultraactivity in case of a cessation of professional activity. It will in any case be a concern of the SIP to follow carefully the legal developments resulting from the application of the legislation, adapting the policy features to the changes that may occur.

LECT 65

THE ROLE OF SCIENTIFIC SOCIETIES WITHIN THE FRAMEWORK OF THE NEW ITALIAN LEGISLATION ON PROFESSIONAL LIABILITY

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INTRODUCTION

In 2004, the so-called Sirchia Decree attempted to establish the requisites that scientific societies were to possess for their accreditation and their performance in name and for account of the institutions, of their specific functions in promoting research and the diffusion of medical and scientific knowledge. Following the rejection of the decree by the Italian Supreme Court some years ago, there still remained the need for regulations of the institutional and organizational aspects of scientific societies. Up to the present, the exact number of scientific societies is unknown: we have a "jungle" of their acronyms, some with few members and/or scanty or no scientific activities, which lead to the suspicion that their transparency is also scarce. The main point of reference in this "jungle" is the FISM (Federazione Italiana delle Società Medico-Scientifiche), a federation that includes some 180 societies with a total membership of about 120,000. Created in 1984, on the initiative of some 30

scientific societies, FISM has become a stable point of reference for the institutions in its over thirty years of productive and communicative activities. Since to become an affiliate of FISM scientific societies must possess the requisites of national representativeness both geographically and within their specific fields, their financial standing must be transparent and they must be recognized on the EU or international level, FISM can be considered a high and qualified representative. Up to now, we can say that scientific societies have had no juridical standing in absence of specific legislation concerning the sector and, to fill this gap, Law no. 24 of 2017 has been passed. Within the context of an overall revision of safety in treatments, this law, the so-called Gelli Law, dated 17 March 2017, establishes the criteria for accreditation of scientific societies, and sets the functions and roles they must perform and play, above all in the production of guidelines which, once drawn up with stringent and transparent methodologies, must also be the basic point of reference for the entire scientific community, also from the juridical standpoint. We are well aware that for many years guidelines have played an essential role in the medical and legal fields, but now, for the first time, they have assumed a role different from the one they originally had; they go from the application in clinical practice of scientific evidence emerging from standardized studies (evidence-based medicine), to their use in health risk management for the obvious purpose of limiting the phenomenon of defensive medicine. The “involvement” of scientific societies within Law 24 is found mainly in Articles 3 and 5. Article 3 provides that scientific societies and technical-scientific associations in the health sector shall act as consultants in drawing up policies for, and upon the request, of the national observatory of good safety practices in health as refer to the management and prevention of health risks. Article 5 sets the requisites that scientific societies must possess to be included in the list created and regulated with a decree of the Ministry of Health to be issued within 90 days from the date the law comes into force. It also attributes to them the task of drawing up the guidelines that must necessarily be followed, except in specific concrete cases, by all those belonging to the medical professions; the latter must also apply good clinical and caregiving practices. Proper conduct, which is to say respect for the recommendations of the guidelines, is to be taken into account in criminal proceedings (Article 6) with the exclusion of liability for incompetence,

as well as civil suits (Article 7), in determining damages. As it is easy to imagine, this formulation and imposition have led to much complaint within the scientific and forensic worlds. Here we need not go into detail on the dispute since it is beyond our purpose, but we can briefly summarize it with a question: is it opportune, faced with a certain proper, sacrosanct guarantee from the viewpoint of forensic medicine, to accept an excessive rigidity in our daily work with the evident prejudice to all autonomy? In any case, willingly or unwillingly, scientific societies will have to cooperate with the institutions in coming to terms with the conditions set in the implementation decrees about to be issued. The predominant function will undoubtedly be to draw up guidelines together with the technical and scientific associations working in the medical field and with yet-to-be defined public and private authorities and institutions as required by paragraph 1 of Article 5.

WHAT KIND OF GUIDELINES ARE TO BE DRAWN UP?

Taking for granted that the drawing up of guidelines is normally dependent, or at least strongly conditioned, by the reaching of the goal set by the same subject who is drawing them up, or by others (in our case the legislator) and, given that there can be guidelines for diagnostics, therapeutics, economics, organization, and risk management, we may say that with different objectives and different fields of implementation there will be guidelines with different characteristics, even in the same nosological field. A guideline with a strong clinical characterization will be based on a strict methodology, and the stricter the methodology, the less autonomy the physician will have, while in the field of health management the guideline will be based on economic meticulousness, perhaps with ceilings on expenses which, lacking a certain flexibility, will invariably limit the autonomy of health professionals. The drawing up of guidelines pursuant to Article 5 of Law 24 will have to take into account the objectives previously set, and in fact declared by the legislator as follows:

1. reduction of the number of adverse events through the management of health risks, of which we shall speak in greater detail in the following paragraph;
2. the limiting of defensive medicine, which this time involves extended guarantees in court cases involving health professionals, who may then be able to count on greater peace of mind while on the job;

3. aiding magistrates and their technical consultants in the delicate task of reaching a judgement.

It is evident that the same guidelines cannot be functional to the reaching of three predetermined objectives at the same time, just as the drawing up (or choice) of guidelines with a strong procedural basis and rigid protocols may be excellent in ascertaining responsibilities, but certainly not in increasing the guarantees offered to health professionals. To that end, more flexible guidelines may be useful by including for example reference to good practices, which in the text approved by the Chamber of Deputies, were placed on the same level as the recommendations of the guidelines, while in the final text approved by the Senate, they were placed at a lower level: Lacking the aforementioned recommendations, health professionals shall apply good clinical and caregiving practices. Thus, the duty of scientific societies and technical-scientific associations will be that of assisting the National Observatory in deciding on policies leading to the identification of proper measures for prevention, health-risk management and the training of health professionals. This is an activity in which the scientific societies have been working for many years now. But the law introduces important new features with respect to the different scenarios in which to prepare measures for clinical risk management. Indeed, if in recent years risk management has mostly, if not exclusively, involved health professionals working in hospitals, the law states that from now on all health professionals are to participate in clinical risk management, including the 46,000 general practitioners, the 7,700 family paediatricians, and all the personnel who in different roles work on the basis of agreements with the National Health Service.

This necessity arises from the overall provisions of the law, which introduces important innovations as concerns professional liability and insurance coverage:

1. the contractual obligation will apply to public and private social health structures, which will be liable for any damages caused by health professionals operating within or for account of such structures pursuant to Articles 1228 and 1218 of the Italian Civil Code (Article 7, paragraphs 1 and 2 of the Gelli Law). Such professionals may be employees, those working as freelance professionals in public structures, those using telemedicine, even when patient-chosen. They will have extra-contractual liability pursuant to Article 2043 of the aforementioned

code, as described in paragraph 3 of Article 7 of the Gelli Law;

2. public or private social health structures are obliged (Article 10, paragraph 1) to have insurance coverage or equivalent coverage (self-insurance or risk retention) for damage caused by personnel working within them in any form whatsoever.

To sum up, by contract, health structures respond for all services provided by the health professionals working for their account, while health practitioners who work in the structures by contract no longer respond, as they did with the consolidated jurisprudence up to now, but pursuant to Article 2043 of the Civil Code. Furthermore, the structures must have proper insurance coverage for damages caused by health professionals providing such services, and therefore provide them (even those working under the terms of an agreement) with direct coverage, save recourse against them for malice or gross negligence. Considering that a structure with contractual liability and the obligation to hold harmless the property of its service providers, it must also necessarily govern the risk factors and, considering that this is relatively simple in the case of its employees, since it is already customary, this does not appear to be the case for those providing services on the basis of an agreement. So how can we allow a Local Health Agency (ASL) to manage the clinical risk of general practitioners' surgeries and Family Pediatricians, in the light of their capillary distribution and relative autonomy in management? As mentioned above, the answer lies in paragraph 3 of Article 1 of the law:

All personnel of public and private social health structures that hold conventions with the National Health Service, including the freelance professionals who provide services on the basis of an agreement with them, are obliged to participate in activities for the prevention of risk. One ought to ask oneself why in all these years the care provided by health professionals within the National Health Service was kept outside of the activities of risk management, since the specific National Collective Agreements (ACN) define surgeries with a convention as "health facilities of the National Health Service" and "destined to provide a public service". This is thus a new challenge for scientific societies if they are called in by the National Observatory to assist in the drawing up of guidelines for clinical risk management, both in hospitals and regional structures, for the purpose of revising the processes of treatment which finally call for the "integration"

of hospitals and regional structures. Also in this sense, the Gelli Law may represent an “opportunity” for making treatment safe in all environments.

LECT 66

THE NEW REALITY OF MEDICAL PROFESSIONAL RESPONSIBILITY IN ITALY

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In Italy and in most Western Countries, the services of the Emergency Department, usually called ER (i.e. Emergency Room), have been steadily increasing in the last few decades about 8% per year. Despite the decline in birth rate in Italy, this phenomenon affected also the Pediatric Emergency Care Services in our country (*Pronto Soccorso Pediatrico*). This phenomenon and the cultural, social and, why not, legal issues that characterized this era have led to the development of new organizational models. The Triage activity was established to distinguish real urgencies from the less demanding cases: i.e. the patients involved in the ER have a priority code to access to the medical examination. Also in our reality we have witnessed a steady increase in the number of users. In our ER, which is currently dealing exclusively with Internal problems since surgical and orthopedic-traumatological urgencies still relate to General ER, the true emergency (i.e. Red and Yellow Codes) represent less than 10% of the total. Most patients are then classified as a Green Code (diffusive urgency) or White Code (no emergency). The data is shown in **Fig. 1**. In Italy the attribution of this code is relevant to nursing staff, which

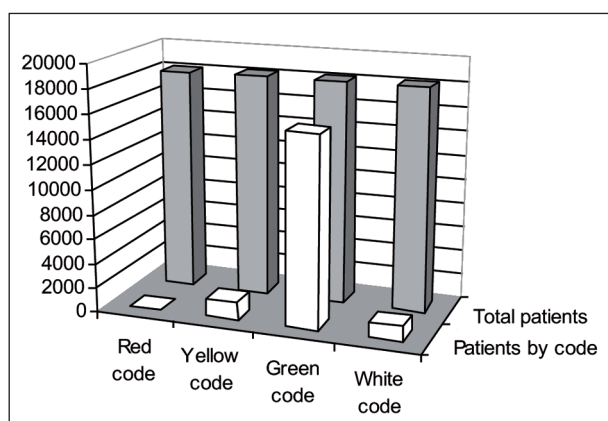


Figure 1 (LECT 66). Pediatric emergency room, “G. Brotzu” Hospital: access/year (2016) by triage code.

“operates under the supervision of a physician in charge, responsible for the activity, and according to predefined protocols, recognized and approved by the service manager” (DPR 27/03/1992). This “supervision” has often been the subject of discussions because this term should mean a “direct control” by the physician, that cannot exist because the nurse actually operates in autonomy with full responsibility for his/her work. There are, therefore, contradictions that have not been followed by an adequate case law. Furthermore the “See and Treat” procedure was introduced to improve patient care in ER. This method is for patients with low priority codes (i.e. white or green) and is still established by the nursing staff that follows protocols shared and approved by the service manager. In the children, the use of “See and Treat” is typical in case of fever or pain. From a legal point of view, the “See and Treat” was accompanied by many controversies. However, this procedure, if it complies with the established protocols, does not determine the offense of abusive practice of the medical profession. Increasing access to Pediatric ER may result in overcrowding of Pediatric Department, in which, on the other hand, there is a drastic reduction in receptivity in terms of Ordinary Hospitalization. Pediatric ER is therefore an important filter to avoid improper hospitalization, however, this only occurs if other appropriate services are present, like Radiology, Analytical Laboratories, and Short-Stay Observation (*Osservazione Breve Intensiva*). Often in Emergency Services, telephone counseling requests arrive. Even in this case, there are some possible legal medical responsibilities, which, in some respects, are similar to those of the operator 118/112 that must determine the apparent degree of criticality and establish time and mode of intervention. In the case of the child, an absolutely prudent attitude is suggested as the collected data is always mediated by a third person (preferably advising a physician or coming to ER). Other issues are being made to protect privacy and informed consent. All procedures which can be considered invasive, such as blood tests or X-rays, must be authorized by the parents: as well as the child’s entry in a computer archive must be authorized. A particular problem arises with the children of separated parents, as if they both have the parental authority it would be advisable to obtain the consent of both. The child’s consent must also be sought, but there are no age limits as well. After 14 years of age the opinion of the child capable of understanding and will is still binding. Of course

these problems do not arise in cases where the imminent danger of life can be seen.

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LECT 67

MEDICAL PROFESSIONAL ACCOUNTABILITY: THE NEW REALITY IN ITALY. THE OPINION OF THE PEDIATRIC SURGEON

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To be sued for malpractice is absolutely devastating for a surgeon: anxiety, seclusion, distrust of anyone, guilt, loss of self-esteem, bitterness against the company in which you work, uneasiness towards coworkers and patients, seen as enemies and not like actors in the process of diagnosis and treatment. I will try to analyze our Country psychosocial contest, with the aim to give an explanation for the increasing number of malpractice claims who afflict our health system. In the meanwhile, I will try to analyze the health context taking into account the considerable organizational and managerial changes, which had deeply marked the relationship between doctor and patient, "historical" foundation of our profession. I will analyze our behaviour evolution, more and more defensive and in good part responsible for the distance between the patient's expectations and the doctor. The health care is one of the most complex working activity: a) the multidisciplinary work produces a complex condition bound to the different "training and educations" of the actors involved in the process; b) the high and growing level of technological sophistication in diagnosis and therapy implies an increasing number of professionalism aligned to the correct use of this technology and centre equipped from an organizational point of view, without considering that the most sophisticated technologies are not available everywhere; c) the fragmentation of the medical knowledge, the specialized language that comes from it, coupled with a very high number of employees at all levels, presents a serious

communication problem, transforming more and more our hospitals in Tower of Babel, meanwhile providing high expertise in the treatment of various pathologies. Another reason for this increasing difficulty is the deep change in the patient-doctor relationship. The Hippocratic approach, which has anyway conditioned the behaviour of so many doctors generations, has insuperable application difficulties in the era we live, I would justly say. From a paternalistic imprint interaction model doctor-patient, we have now reached a situation in which the informed consent and the shared construction of the diagnostic-therapeutic pathway are of vital importance, according to the guidelines validated by the scientific community. The stressful work rhythms of our hospitals do not free us from the moral obligation to dedicate to our patients all the time that will be necessary to make the diagnosis and care path appropriate to their needs and to give a high-quality professional performance. The diagnosis and therapeutic pathway should be guided by these fundamental principles: total quality management, evidence based medicine, personalization of the care. In times of economic tightness, the State, in addition to spending management measures, uses, even more, an occult rationing of health care spending through discouragement methods, represented by a various type of taxes, but above all by the notorious waiting list. The latter, in particular, certainly not determined by the professionals, generate violent protests by the citizen, amplified by politics and the media, which contribute to discrediting the medical figure and to fall back on the professional the fault of the only partial satisfaction of the demand for health service. These generate additional clinical risk due to the doctor's high exposure, considered responsible for the disruption. Historically, the doctor has played the role of mediator, between the need for health by the citizen and the supply of health care. At present, the media, coming directly to the user with messages that are often devoid of scientific, obscure, or worse, market-oriented, have largely expropriated the doctor's role. Equally, they have done bureaucratic figures, the expression of politics, which have replaced the role of mediation of the medical figure. The lack of a traditional figure of mediator between health needs and healthcare supply, replaced by the information find on the internet, sensitive to the market logic, has created a disaffection towards public health, the explosion of health spending and paradoxical phenomena in the modern era of denial of the choices of scientists,

like the Di Bella or Stamina cases or the current anti-vax campaigns proposed by political parties or committees of various kinds. The retrieval of a proper relationship between doctor and patient and the recovery of a physician's figure in a modern society is not only beneficial to the well-being of the citizen and the health professionals but is strategic to the balance of the national economic system.

LECT 68

A NEW APPROACH IS NEEDED FOR EFFECTIVE PREVENTION AND MANAGEMENT OF NEONATAL SKIN INJURIES

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INTRODUCTION

Neonatal pressure injuries are still an open issue as there is a lack of high-quality studies about their prevention and management. Neonatal Intensive Care Unit (NICU) patients have many risk factors, such as limited or fixed postures, cognitive or mobility deficits, altered tissue perfusion, altered nutritional status, skin immaturity, temperature instability, skin moisture, friction and shear. Moreover, all these risk factors are negatively influenced by a low gestational age and use of medical devices, especially if more of them are needed or placed in the same area for a long period, like for example using invasive and non-invasive ventilation.

MATERIALS AND METHODS

Constitution of a team composed of NICU nurses, periodic literature review, development of procedures and protocols for the prevention and treatment of skin injuries; nursing staff training through structured courses, supervision and periodic control of the correct application of protocols and procedures; data collection and their periodical elaboration.

RESULTS

Thanks to literature revision and our data collection, we are able to say that neonatal pressure injuries appear different from the adult ones, as they seem to be mainly associated with use of devices instead of immobility. Therefore, areas of pressure injuries in neonatal population and their treatments differ from adult population. For example, the time of wound

closure appears to be different and all the advanced medications available on the market for the adult population are not appropriate for newborns immature skin. It is clear how NICU wound care has to consider also other kinds of skin injuries as moisture-associated skin damage (MASD), which occupies a preponderant place in this field. In addition, cases of medical-adhesive-related skin injuries (MARS) and traumatic wounds (skin tears, burns e abrasions) have been observed.

CONCLUSIONS

Initially our working team was created to structure and uniform NICU wound care in order to decrease the incidence of pressure injuries, but during our study we had to match the needs and peculiarity of new-born skin compared to poor literature available. Moreover, it is important to extend our researches to other kind of skin injuries too. For these reasons, we concluded that neonatal healthcare professionals should consider neonatal pressure injuries as a new approach and not to exclude other kind of skin injuries, in order to be truly effective and significantly decrease the incidence.

LECT 69

NURSING PRIORITIES TO NEWBORNS WITH DIGESTIVE OSTOMIES

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INTRODUCTION

In the neonatal period of life several pathological conditions, mostly congenital, typically require procedures that result in ostomy whose complications are associated with higher morbidity and mortality rate. Ostomies can be created at any level of the digestive tract, as needed. Quality of the nursing care to newborns with ostomies might significantly affect outcomes and survival rate.

OBJECTIVE

Pre- and post-operative aspects and priorities of the nursing care to neonates with a digestive ostomy.

METHODS

The clinical records of all the infants who required assistance at our NICU in the 6-year period from

2010 to 2016 were reviewed to identify those who underwent a digestive ostomy. Data considered for the study included gestational age, diagnosis, need of parenteral nutrition, central venous catheter and mechanical ventilation.

RESULTS

Study population. In the 6-year period studied, total 2,630 newborns received assistance at our NICU. Fourteen (0.53%) of them underwent a digestive ostomy; these were 9 (64%) at term and 5 (36%) preterm infants (M = 9: 64%). The ostomies were 1 jejunostomy, 6 ileostomies and 7 colostomies. Among the 5 preterm infants, the reasons of ostomy were necrotizing enterocolitis in 3, jejunal atresia in one and anal atresia in another one, while among those at term, meconium ileus was the cause in 3, congenital anorectal malformations in 4, Hirschsprung disease in one and intestinal duplication in another one. All neonates with digestive ostomy received parenteral nutrition, all but two central venous catheter; half of them needed mechanical ventilation. The period at NICU ranged from 2.5 weeks to 3 months. Overall survival rate was 93% because 1 of the 14 infants with digestive ostomy unfortunately deceased. The main aspects of the nursing care. The assistance to neonates with digestive ostomy consisted in both pre- and post-surgery nursing care aimed to gain satisfactory vital signs' records during the period spent at the NICU, either before or after surgery. In the post-surgery period at the NICU, nurses were aimed to the strict observance of the treatment protocols and to the accurate monitoring of vital signs, the hydration status and bowel functioning. Assistance to neonates with digestive ostomy required specific and complex interventions by highly professional and experienced nurses, who were capable of managing the multiple aspects of the nursing care to neonates with digestive ostomy, including the proper use of the required devices, monitoring the respiratory function and providing adequate nutrition, rest and sleep. Another important aspect was pain evaluation and treatment, when necessary. Care of the ostomy reduced the occurrence of skin injuries and infections. Post-surgery change or removal of the stool pouch was performed using clean and aseptic materials; the nurse gently shifted it from a higher to a lower level, and took care of ensuring moist skin. Skin was cleaned by warm water only, avoiding the use of any detergent or soap, and dried by the gentle application of soft paper press. These procedures were able to reduced skin complications as peristomal injuries or infections. Ostomy devices

were applied having care of selecting the right ostomy adhesive to guarantee perfect adhesion to the skin, therefore protecting peristomal skin from the material passing through the ostomy. Usually the need of changing the ostomy adhesive should be no more than once a day and it can be done every 3-4 days. Finally yet importantly, nursing care involved also the psychophysical assessment of each neonate. As part of the nurse role was also parents' educational training for the home care of the ostomy bag. Nurses played an important role in educating parents to the use of a teaspoon until the baby was capable of suckling directly from the breast, and in ensuring, as early as possible, the transition toward breast feedings according to the guidelines recommended by WHO/UNICEF.

CONCLUSIONS

The assistance of newborns with digestive ostomy is a complex and multidisciplinary team task that requires the intervention of several professional figures of which that of the nurse is essential during all the different and articulate phases, from the pre-surgery to the post-surgery and follow-up. During the pre-surgery, the skilled nurse begins to take care of monitoring the life signs of the infant, which includes all that is necessary, i.e. respiratory machines when needed, enteral or parenteral nutrition and by promoting breast milk feeding. As part of the nursing care is awareness of the potential serious complications, that can occur both at short- and long-term. During the first month, is essential that the nurse immediately recognizes peristomal skin complications such as skin irritation, ischemia and necrosis, retraction or detachment, edema, hemorrhage, infection, abscess or fistula. Later complications include parastomal herniation, stomal prolapse, stenosis or stomal granuloma. The nurse has to take care of all the many details that allow preventing the occurrence of serious complications, often reached by simply applying the recommended therapeutic protocols. Techniques to favor sufficient rest and sleep hours to all the newborns at the NICU should be routinely applied. Parents' education and autonomy is another important aspect of nursing care to the neonate with digestive ostomy. In conclusion, nursing care of newborns with digestive ostomy is a professional task that requires skills and expertise to ensure the desired and complication-free outcomes. Nursing care regards not only highly qualified assistance to the neonate but it must include the parents' education, training and psychological support to guarantee proper home care of the neonate with digestive ostomy.

LECT 70**INTEGRATING MICROBIOMICS AND METABOLOMICS DATA IN BEHAVIORAL COMPLEX TRAITS: A ROLE IN AGGRESSION?**M. Manchia^{1,2}, V. Fanos³¹Section of Psychiatry, Department of Medical Sciences and Public Health, University of Cagliari, Cagliari, Italy²Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada³Neonatal Intensive Care Unit, Neonatal Pathology and Neonatal Section, AOU and University of Cagliari, Cagliari, Italy**INTRODUCTION**

Aggression is a widespread complex behavior affecting a significant proportion of the general population, particularly individuals in their adolescence and early/middle adulthood. Despite the elevated economic and societal costs associated with aggression, the implementation of preventive strategies has led only to a marginal reduction of the impact of this disruptive behavioral trait. This might be explained by the scarce knowledge of the biological underpinnings of aggression, which in turn has impacted on both accuracy and precision of predictive models. One possible solution to this quandary might be offered by “omics” data, particularly microbiomics and metabolomics, which can clarify the biological basis of aggression and increase the ability of prognostic models to detect individuals at risk of developing this disruptive behavior. In addition, the discriminative properties of prognostic models might be further refined by the integration of microbiomic and metabolomic information. Here, we performed a qualitative review of the available evidence on the role of microbiomic and metabolomics data, and their integration in aggression.

MATERIALS AND METHODS

We performed a systematic search (performed in September 2017) for peer-reviewed articles in English using Medline and Embase. The terms used for the electronic database search were (“metabolomics” OR “metabolomics”) AND (“microbiomics” OR “microbiome”) AND (“aggression” OR “violence”). Backward and forward citation tracking was selectively performed to identify articles eligible for inclusion in our qualitative review.

RESULTS

We identified one article through our search [1]. In addition, other two articles were found and

included through backward and forward citation tracking [2, 3]. The first paper is a selective review of finding on the biological underpinnings of aggression with a focus on genetic, epigenetic, metabolomic and microbiomic determinants of risk. This study highlights how perturbations of gut microbiota, detected through metabolomics, have been detected in individuals affected by autism spectrum disorders who often manifest externalizing (aggressive) behavior of severe entity. The second study focus on the role of microbiome/metabolome targeted therapies, which might inhibit detrimental microbes/metabolites as well as promote beneficial microbes/metabolites. In addition, Shaffer et al. highlight the importance of microbiome/metabolome for personalized medicine, particularly if the treatment of an individual is tailored based on the composition or metabolic capabilities of their microbiome [2]. Although this approach has not been yet implemented in aggression, the potential for preventive strategies is substantial. Indeed, as shown by the systematic review by Mussap et al. [3], metabolites derived from the gut microbiota can modulate the behavioral phenotype of the autistic children, greatly influencing host metabolic pathways and the immune system, ultimately shaping the individual susceptibility to aggression.

CONCLUSIONS

Our qualitative review highlights the emerging role of microbiomics and metabolomics in targeting, and potentially treating, individuals at higher risk for aggression. Although most of the available evidence is indirect, i.e. derived from studies on autism spectrum disorders individuals, who are at higher risk for aggression, studies assessing microbiomic and metabolomic changes in accurately phenotyped aggressive individuals are needed.

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LECT 71**AGGRESSIVENESS FROM FETUS TO ADULT: THE TWINS CASE**

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Aggressiveness and violence are major issue challenging modern societies, so much that some says that they are “endemic”. Even though aggressive behavior is evolutionary preserved as a defense mechanism and a way to survive among species, when it comes to humans and it is out of control due to several reasons (psychiatric disorders and substance abuse among others) it could be extremely dangerous for both the victim and the agent. Several investigators are today focusing their attention on the possible neurobiological substrates that causes aggressive behavior and bursts of violence in humans and some fascinating hypotheses have been made, thanks also to the development of the new “omics” sciences such as metabolomics. Metabolomics is a technology that allows obtaining a screenshot of the metabolic state of an individual through the analysis of biological fluids [1]. Furthermore, scientists according to the DOHaD theory state that there is a fetal programming of health and diseases. This means that if some negative events happen during the critical time window in pregnancy it may affect the neonates’ health status up to their adult life, even from the behavioral point of view. For instance, it is well known that children born from mothers that took antidepressants (SSRI in particular) during pregnancy could develop several malformations and the most affected organ in this case is the heart. They could also present a dysregulation of the HPA axis damaging their adaptiveness to stress and making them more prone to develop anxiety and depression. Speaking about tweens aggressiveness according to recent literature there is a strong genetic component, thus it seems that heritability in aggressive behavior is high. Nevertheless in a recent work analyzing pre-scholar twin pairs born from obese mothers, since there could be a deficit in the vitamin D synthesis and high levels of glucose during pregnancy that may affect the brain development they could be more aggressive than their peers born from lean mothers. Then the uterus environment seems to exert a role as well. Even the post-natal environment could play a role in the development of aggressive behavior [2]. In fact, according to Dickson at al. twins that grow up in a family with high parental coercion and hostility tends to be more aggressive since very young age and one twins aggressiveness at 6 years

of age is predictive of the aggressiveness of the other twins at 7 [3]. Analyzing the neurobiological substrates of this behavior from an imaging point of view in adolescent twins, it has been found that there are volumetric and thickness disturbances in the cortico-limbic striatal circuits, in particular there is a striatal enlargement that has been proposed as potential biomarker [4]. The causes of these differences in neuronal networking are still under investigation, but some hypotheses have been proposed. Lipids, cholesterol in particular and mitochondria seems to be the main actors involved in these dysfunctions. According to some authors, even if the matter is still controversial, it seems that people that displays aggressive behavior towards others and themselves, show cholesterol alterations in their blood. Even if peripheral cholesterol is not directly linked to brain cholesterol, the alterations of its levels in neurons may lead to demyelination, cellular membrane modifications and compromised lipid rafts formation. These events may ultimately lead to neuronal death and reduced synaptic efficacy. Not only that, but also since the synaptic function is directly linked to mitochondrial activity, there could be also a mitochondrial damage that worsen the functionality of neuronal network. These neural circuits modifications may results in poor impulse control and aggressiveness (**Fig. 1**). Several questions remain open: are these modifications reversible? Given all this different influences since genetics even if seem to be the major contributor, at least in twins, is only one variable involved can clinicians and parents prevent aggressive behavior in children? Since there is a high probability that aggressive children will become aggressive adults and some neuronal modifications are seen both in adolescents and in adults, further investigations are needed to clarify the underlying mechanism to finally solve the mystery of aggressiveness in humans.

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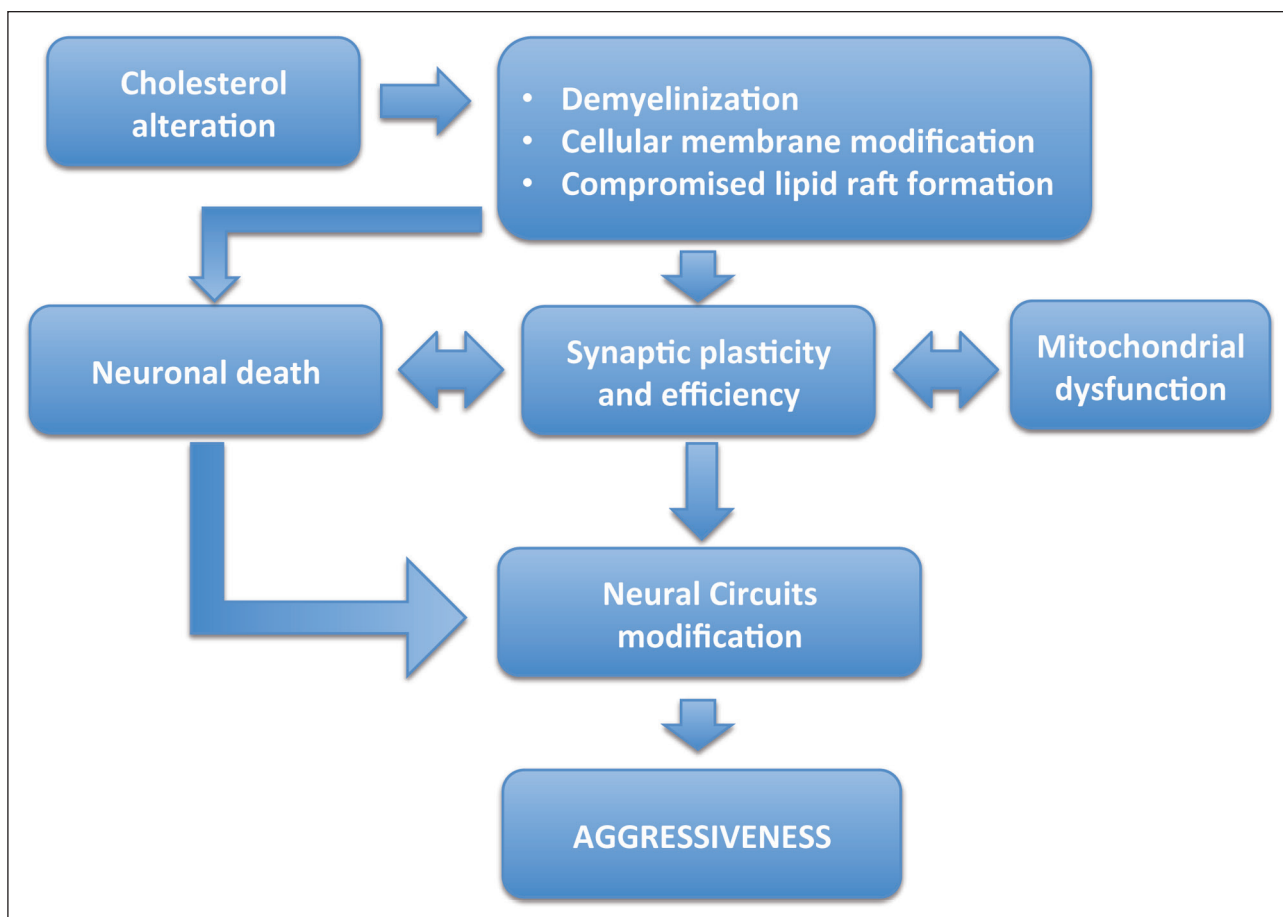


Figure 1 (LECT 71). Potential biological mechanism of aggressive behavior.

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LECT 72

DOHaD, NUTRITION AND BASIC RESEARCH

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The “DOHaD” (Developmental Origin of Health and Disease) theory describes how *in utero* exposure to environmental factors may have long-term effects on the structural and functional development of the fetus. Extensive retrospective studies, such as those on the Dutch famine of 1944, have reported correlations between maternal diet or nutritional status and the risk of pregnancy pathologies or to develop adverse conditions in the future adult. Indeed, macro- and micronutrients taken with the maternal diet can regulate the stability and expression

of fetal/placental DNA and phenotype adaptations through epigenetic modifications, reversible mechanisms that occur without changes in the DNA sequence (DNA methylation, histone acetylation, microRNA) [1]. Recently, a large prospective longitudinal cohort study in humans (MANOE study) reported that maternal intake of methyl donors, especially during the periconceptual period, can affect the epigenoma of the offspring in genes related to obesity and diabetes. However, many observations on this issue are born from basic research studies performed on the placenta: placental epigenetic modifications are one of the main mechanisms through which nutritional and environmental factors affect fetal growth. Epigenetic regulation of placental phenotype and function has been extensively studied in the mouse. For example, “imprinted” placental genes (IGF2, H19) act as “nutritional sensors” by varying their methylation status based on environmental conditions. In our lab, we have recently reported lower functionality in the placenta of overweight/obese women with high gestational weight gain, with an important role in fetal sex [2]. Those placentas also exhibit alterations

in mitochondrial content suggesting a bioenergetic placental imbalance resulting from an altered nutritional intake. Methylation of mitochondrial DNA may also be involved in these mechanisms [3]. Future research will allow to fully understand the underlying mechanisms of pregnancy pathologies in relation to maternal-fetal nutrition.

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LECT 73

ENVIRONMENTAL FACTORS AND EPIGENETIC MECHANISMS

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Epigenetic mechanisms (DNA methylation, histone tail modification and chromatin remodeling, ncRNA interventions) contribute to gene expression regulation. Several environmental exposures, in particular to endocrine disruptors, are able to interact with genetic susceptibility factors, by interfering with our epigenome, increasing the occurrence of complex diseases such as obesity, behavioural disorders and other pathological conditions. The first stages of development of an organism represent a particularly sensitive period, as well as childhood and adolescence. The entire differentiation process that characterizes intrauterine development is the result of a complex series of cell-specific epigenetic events, including “reprogramming” of specific epigenetic patterns of DNA methylation in each individual cell. There are therefore a number of crucial windows in the early stages of embryonic development during which the epigenetic mechanisms responsible for differentiation are particularly sensitive to influences by environmental factors. Some of the environmental exposures that are likely to have a greater impact on epigenetic mechanisms will be considered. The most studied are exposures to heavy metals, particularly arsenic, to endocrine disruptors, including obesogens,

diet and some lifestyle, including alcohol, some pharmacological treatments, and stress. Complex diseases with an epigenetic etiology can be prevented through primary prevention actions, moreover it has been seen a great potentiality in therapeutical interventions.

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LECT 74

PERINATAL PROGRAMMING AND BRAIN

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The Developmental Origins of Health and Diseases theory that states that the health state of a human being is determined during pregnancy meaning that “what happens in pregnancy does not stay in pregnancy” but it will affect the entire life course. This is particularly true when we focus our attention at the brain, according to recent literature. Furthermore, this organ complete its development in the first years of life, thus investigators are studying any possible epigenetic factor that could modify, alter or disrupt this process. First of all, maternal factors such as diet, lifestyles and drugs assumption are of primary importance [1]. According to recent studies, other than in case of malnourishment that can result in low weight at birth or several deficiencies of nutrients that are fundamental for an appropriate brain development; even children born from obese mothers can display alterations in brain maturation (high levels of glucose seem to be “toxic” and possible vitamin D deficit) that could lead to behavioral disturbances. For what concerns lifestyle, leaving aside substances abuse, smoking during lactation and gestation is associated with serious brain malformations with several regions to be hypotrophic. Even drinking is related to brain abnormalities (Fetal Alcohol Syndrome, FAS): it literally kills the neurons giving rise to phenomena such as apoptosis and autophagy among others, at least from evidence coming from animal studies.

Children born from heavy drinker mothers show severe cognitive deficit with lower IQ compared to their peers, behavioral problems and psychiatric disorders. Another factor that may lead to brain abnormalities is maternal stress. Prolonged stress or anxiety disorders alter brain structures and their physiology during pregnancy. Moreover, they impair the functioning of the Hypothalamus, Pituitary and Adrenal Gland (HPA) making this newborn more susceptible to stress, leading to maladaptation and eventual mental disorders. On the other hand, antidepressant or anxiolytic must be administered with special precaution, even if the studies results are sometimes controversial, due to the fact that they may impair fetal growth (for instance: selective serotonin reuptake inhibitors are linked to cardiac malformations) and brain structures. Brain growth during pregnancy is a six-step procedure: neurogenesis, migration, apoptosis, synaptogenesis, pruning and myelination. Recently, another class of drugs, which is very demanding to administer to mother, fetus and newborn, has been related to possible interactions with developing brain, that is anesthetics. Even if the evidences come from animal studies, since there is no study so far on human fetal anesthetic exposure, this topic has gained more and more attention over the past few years. The most affected process seems to be the apoptosis: it is increased up to 20 times in treated animals and it lead to behavioral and cognitive impairment [2]. The findings of studies on neonates and infants are controversial. Some authors state that there is an anesthetic neurotoxicity that leads to lower cognitive abilities, even in case of short time administration, others negate this association. Further studies are needed, since there is no study concerning long time or repeated administration. Perinatal programming of the brain is a matter of fact, but there are several question that still need to be answered especially those concerning drugs, their pharmacodynamics and their mechanisms of actions on the developing brain and the individual responses of neonates that are characterized by high inter-individual variability. Maybe thanks to new technologies such as metabolomics, in the future clinicians could be able to individually predict the effect of a specific drug and prevent any abnormalities related to it and prescribe the best treatment for these frail little patients [3].

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LECT 75

ANESTHESIA ON THE DEVELOPING BRAIN OF THE FETUS AND INFANT: THE DARK SIDE OF THE COIN

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The first surgical procedure under general anesthesia was performed the 16th of October in 1846. That day, in the surgical theater of the Massachusetts General Hospital, Edward Gilbert Abbott underwent the removal of a neck tumor performed by Surgeon John Collins Warren while rendered completely unconscious by dimethyl ether inhalation administered by Dr. Thomas G. Morton [1]. It is quite easy to understand how the introduction of such innovation revolutionizes surgery since then. Total absence of conscience and motility and with later improvements various degrees of analgesia and stunning of autonomic responses to surgical tissue destruction, made possible operations before unimaginable. During the 170 years since then, dozens of different molecules able to produce a state of general anesthesia have been developed. Various ethers and hydrocarbons both simple and halogenated have those properties. We remember vapors like dimethyl ether, chloroform, cyclopropane, alothane and the more recent alogenated ethers (sevoflurane, desflurane) [2]. Inhalational agents include gases such as nitrous oxide and the noble gas xenon [3, 4]. Injectable anesthetic drugs came later on: potent GABAergic agents like barbiturates (sodium pethatol, metoexital) [5], injectable fast acting benzodiazepines (mostly midazolam) [6], propofol are the most common [7]. Ketamine and more recently adrenergic α_2 agonists clonidine and the more selective dexmedetomidine use different mechanisms [8, 9]. The development and diffusion of relatively safe and handy mechanical ventilation techniques during the XX century, made preferable pharmacological regimens characterized by various degrees of ventilator depression and the abandon of unstable and flammable chemicals. Moreover,

modern anesthesia practice warrants the association of multiple drugs, which are synergic in determining the best operative conditions minimizing adverse reactions associated with single molecules. Not all of them are used to determine loss of consciousness, for example myorelaxants are usually administered to abolish muscular tone or opioid agonists are administered in order to blunt autonomic responses to pain stimuli. More than 300 million patients undergo major surgical procedures worldwide every year [11]. In the US, an estimation of the number of surgeries in children reaches six millions in the USA, nearly three million of them are under 36 months of age and 1.5 million are infants younger than 12 months with 2-3 million of these anesthetics in children and there approximately 1,500 fetal interventions and surgeries are performed [12]. Given the huge number of procedures a legitimate rise in the attention toward safety of these techniques has arisen. Acute complications are extensively studied since the dawn of the discipline. Predictable adverse reactions of the commonly administered drugs, particularly respiratory depression, whereas intubation or ventilation by external means proves impossible, or cardiovascular depression or arrhythmias are well known. Even knowledge of much more rare, though potentially catastrophic, such as malignant hypothermia, is part of the culture of every anesthetist, and is explained in biomolecular details [13]. Though less known as early as 1945, Levy described behavioral alterations lasting up to six months in healthy children submitted to general anesthesia for brief procedures such as appendectomy, tonsillectomy or adenoidectomy, with an increase of terror, dependency and disbehavior [14]. The effect was more severe in children under two years of age. In a prospective multicenter published by Campbell and colleagues in 1988, Campbell found a 47% overall incidence of new behavioral problems, including anxiety, nightmares, and attention seeking, in 551 operated children, with a relatively more higher risk in those younger than two years. Alterations were not associated to a particular drug regimen, by then mostly cyclopropane e barbiturates [15]. More recently a population-based retrospective birth-cohort study of 5,357 children by Wilder and associates found that children who received two or more general anesthetics before the age of four years were at increased risk for learning disability as adolescents with an increased risk with cumulative exposure duration longer than two hours. The cohort exposed to anesthesia before

4 years of age had cognitive scores that were two standard deviations below predicted, possibly preventing them from development of full cognitive potential [16]. In a large (228,961 individuals) population study, Sun and colleagues found that children who underwent general anesthesia before three years of age required more Medicaid services for learning disability than did children not having these procedures [17]. Procedures in premature infants have also been associated with an excess of cognitive and behavioral disabilities later in life. For example, surgically treated premature infants with patent ductus arteriosus or necrotizing enterocolitis had worse neurologic outcomes than did premature infants who were treated medically [18, 19]. Biologic causative mechanisms implied are multiple and extremely complex. Events such as transient severe hypoxia, commonly observed during pediatric anesthetic procedures, with no immediate clinical significance could determine long term impairing, as seen in animal studies [20, 21]. Mitochondrial mediated apoptosis seems to have a role. So does intracellular disruption with lysosomal hyper activation. GABAergic and glutamatergic transmission are implied in central nervous system apoptosis modulation. Those pathways are brutally disrupted by halogenated vapors, barbiturates and propofol (GABAergic) and ketamine, nitrous oxide and xenon (glutamatergic, through NMDA inhibition). By now it is not possible to make definitive statements regarding which general anesthetic regimen is safer for long term cognitive outcomes in children or fetuses [22-24]. Some authors suggest a preventive role of drug regimen that implies dexmedetomidine sedative action through adrenergic pathways, sparing the glutamatergic and GABAergic ways [25]. It is nevertheless mandatory to contemplate the matter, for which there is now enough evidence and limit as much as possible surgical procedures in children, especially in the first two or three years of life and whenever it is possible, associate or prefer loco-regional techniques.

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LECT 76

EXPOSOMICS: ENVIRONMENT, EPIGENETIC FETAL PROGRAMMING AND PREVENTION OF CHRONIC PATHOLOGIES

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The epidemiologic scenario has radically changed, in the last decades, all over the world. The dramatic reduction of acute, infectious illnesses has been replaced by an equally remarkable increase of chronic, degenerative, inflammatory, neoplastic pathologies: endocrine-metabolic diseases (the “pandemics” of obesity and diabetes) [1], neurodevelopmental disorders [2], with particular reference to autism spectrum disorders [3]; neurodegenerative diseases [4]; immune-mediated inflammatory diseases (allergies, autoimmune diseases) [5]; reproductive anomalies [6]; cancer, especially in children [7]. The growing burden of chronic diseases is associated with the particularly alarming evidence of the progressive anticipation of age at onset of many of them. Such a worrying

epidemiological transition [8], that is rapidly spanning from the North to the South of our planet, was totally unexpected and difficult to interpret according to traditional epidemiological and etiological models. Traditional medical genetics, the usual considerations about modifiable behaviors (smoking, unhealthy diet and sedentary lifestyle) and the most accredited pathogenic models concerning specific diseases are totally inadequate to explicate the entire picture. In this scenario, a new explanatory model is needed in which the complex “molecular dialog” between the “information” coming from a rapidly changing environment and the one inscribed in the human genome (and its effects on the human phenotype) are better defined. For this purpose, it is useful to remind that the embryo-fetal ontogenesis represents by far the most sensitive period to the “information” coming from the environment and in particular to maternal stress, nutritional errors, pollutants, since differentiating cells are extremely plastic: fetal programming refers to the ability and necessity of embryo-fetal cells to define their epigenetic setting as a response to the information coming from the mother and, through her, from the external world, in a predictive and adaptive way. This knowledge led to the formulation of a basic pathogenetic model (now validated by hundreds of epidemiological and experimental studies) able to provide a reference framework to the ongoing epidemiological transition: the theory of the embryo-fetal origins of chronic diseases in adults (DOHaD) explains how, in the very first phase of life (in particular during the embryo-fetal ontogenesis), the information coming from the environment can induce modifications in the epigenetic package of the organism that may have long-term effects, both on the directly involved organism and on the next generations. In the light of what has been said so far, DOHaD has important implications for our societies and for the health policies and even for the evolutionary future of our species [9]. Genomic imprinting is an epigenetic process independent of the classical Mendelian inheritance, by which epigenetic marks are established (“imprinted”) in the germline (sperm or egg cells) of the parents and are maintained through mitotic cell divisions in the somatic cells of an organism, causing these genes to be expressed in a parent-of-origin-specific manner. Genomic imprinting is predicted to involve only about 1% (~200 genes) of the expressed genome with only about 90 imprinted genes that are well characterized at present, with many being tissue-specific (27 of them are imprinted only

in the placenta) [10]. The fact that these genes are imprinted in the first stages of development suggests that these epigenetic marks play a key role in fetal programming and can have extensive effects on health [11], as evidenced in a study performed on the survivors of the Dutch famine (Second World War, winter 1944-45) that had undergone a severe caloric restriction *in utero* in whom has been observed, 6 decades later, the hypomethylation of the IGF2 imprinted gene [12]. The fetal-maternal exposure to several epigenetic stressors can contribute to explain the ongoing increment of preterm births, that determine an increase of the risk of endocrine-metabolic and neurodevelopmental dysfunctions and renal, cardiovascular and neoplastic pathologies in infancy and adult life. In this context, the placenta can be considered the “pregnancy flight recorder”. Actually, an increasing number of studies is highlighting the main role of the placenta in the “epigenetic reprogramming” of the fetus, demonstrating the fundamental role of this organ in the control of the fetal development and the interaction among the fetus, the uterine microenvironment and the external environment. As a result of many recent studies, it is becoming increasingly evident that imprinting is differently distributed to individual tissues, with the placenta being a main target. Yet many questions remain unresolved about the biologic significance of placenta imprinting. In particular [13]: are changes in imprinted gene expression a direct cause of pregnancy complications, or do they represent an adaptive compensatory mechanism (particularly those playing a crucial role in the control of the fetal development)? Only a deep and combined research – histopathological epigenetic and metabolomic – will be able to address these questions.

From what has been said so far, it seems obvious that the best way to deal with the ongoing epidemiological transition can only be primary prevention, intended as the set of strategies targeting the reduction of the exposure to risk factors during the embryo-fetal life and in infancy. Since epigenetics studies the inheritable modifications of the expression and programming of the genes that do not modify the sequence of the DNA, and metabolomics studies the functional metabolic patterns starting from the genetic expression to the protein synthesis [14], the potentiality of an integrated use of these two approaches appears evident to improve our knowledge of the relationship between environmental pollution, embryo-fetal development and the possible outcomes, and to define an exposure

biomarker panel necessary to the development of an health-environment advanced surveillance system.

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