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METABOLOMIC PROFILE OF PRETERM NEWBORN: ASSOCIATIONS WITH PRE- AND POSTNATAL GROWTH RESTRICTION

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A tutti i bimbi nati prima del tempo,
alle loro mamme e ai loro papà

"Il fiore che sboccia nelle avversità è
il più raro e prezioso di tutti"

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ABSTRACT

Background: Metabolomics is a science aimed to identify and interpret the metabolic profiles of a given biological system, namely the products of interaction between gene expression and environment. Among the "omic" sciences, it can be considered the closest one to phenotype.
The impact of growth and nutrition in the very early stages of life is a topic of great interest, considering not only the immediate effect on development, but also on lifelong health. Ensuring an adequate nutritional intake and growth is even more crucial in preterm babies. Intrauterine growth restriction (IUGR) and extrauterine growth restriction (EUGR) are two conditions in which the fetus and the newborn respectively, are not able to achieve their genetically determined potential size. Nowadays reliable biomarker to assess the suitability of nutritional status and growth in preterm infants are lacking.
Aim of the study: The aims of this study are to analyse and compare the metabolomic profile obtained by: 1) preterm infants diagnosed with IUGR during pregnancy versus preterm infants adequate for gestational age (AGA); 2) preterm infants who experienced EUGR and preterm infants who did not.
Material and Methods: This prospective observational study has been conducted at the third level Neonatal Intensive Care Unit (NICU) of Padova. Premature neonates born between 23 and 32 weeks post-menstrual age (PMA) have been enrolled. For each subject urine samples have been collected at three time-point: within 72 hours of life, at 21 +/- 3 days of life and at 36 +/-1 weeks PMA. The urine samples underwent untargeted metabolomic analysis using mass spectrometry combined with ultra-performance liquid chromatography. For each enrolled subject all relevant clinical data during NICU stay have been captured. IUGR was diagnosed through review of obstetrical history of the mother; EUGR was diagnosed when weight at 36 weeks PMA was $<10^{th}$ %ile of the predicted value. The data obtained were analysed using multivariate and univariate statistical analysis tools.
Results: 160 infants have been enrolled, with a median gestational age at birth of 195 days (interquantiles range: 185-207 days). Only urine samples collected within the first 48 hours of life were analysed (n=83). Among these 15 were collected from IUGR infants and were matched with 19 from AGA infants (controls). Untargeted metabolomic revealed evident clustering of the IUGR neonates versus the AGA ones. The metabolic derangements mainly involved were metabolism of tryptophan-serotonin and biosynthesis of steroid hormones. The comparison of urine samples collected from 9 infants with EUGR and 10 controls did not show any discriminant variables.

Conclusions: Neonates with IUGR showed a distinctive urinary metabolic profile at birth. Although data are still preliminary, metabolomics is proving to be a promising tool to explore biochemical pathways involved in impaired fetal and neonatal growth.

BACKGROUND

The first thousand days of life - between conception and a child's second birthday - are a delicate time, when the health foundations of a lifetime are laid. Appropriate nourishment during this crucial period of life has a profound impact not only on the early life growth and development but also on the lifelong health of the individual [1]. This concept should be expanded to include not only nutrition in the strict sense, as the whole environment to which the infant is exposed is extremely important. The emerging term "exposome" refers to the set of all non-genetic exposures that, together with the genome, determine the final phenotype during the course of a lifetime [2].

Anthropometry is a key component of nutritional status assessment and anthropometric data reflect general health status, dietary adequacy, and growth and development over time. Nutritional evaluation of a human being starts while he's in the womb through indirect echographic measurements, that are nonetheless unable to accurately estimate bodyweight and length. The first reliable anthropometrical assessment takes place at birth and provides useful "retrospective" information on intrauterine growth. From this point forward growth assessment is performed according to anthropometric charts, based on cross-sectional measurements taken in reference group of newborns and infants at different ages. [3]

Intrauterine growth restriction (IUGR) is a paradigmatic condition, in which a "hostile intrauterine environment" can hamper fetal development with a potential impact on long-term health [4]. Similarly, the baby born preterm, is exposed too early to an extrauterine environment, which is often not able to guarantee a growth comparable to the one occurring in utero in the last trimester of gestation, that is marked indeed by a peak of fetal growth. [3]

IUGR and preterm infants who fail to thrive during the first weeks of life and develop extrauterine growth restriction (EUGR), could therefore be subject to the same influence. Several longitudinal studies, mostly in subjects with a history of IUGR and, to a lesser extent, of EUGR, show that an impaired early growth can predispose to a number of major diseases later in life.

This study aims to explore which metabolomics perturbations occurs in a population of premature babies who experience pre and post-natal growth restriction.

Metabolomics

Untargeted metabolomics is evolving as a novel tool for studying the effects of the endogenous and exogenous environment. Taking an untargeted hypothesis-generated approach, metabolomics enables the simultaneous qualitative and quantitative analysis of thousands of different metabolites in a biological sample, enabling the identification of biomarkers and metabolic patterns characteristic of

a given condition. Among the "-omics" sciences, metabolomics is the one that comes closest to phenotyping. [5]

Two approaches can be applied in metabolomics: in targeted metabolomics, a fixed subset of certain metabolites is studied, and this method is generally used to verify a specific hypothesis; in untargeted profiling, all metabolites in a sample are measured (even with some detected metabolites not yet identified), and this approach is preferred for hypothesis-generating studies [6].

As a matter of fact, the study of metabolites of a biological system can be managed in different methods. Metabolite targeting aims to identify a specific metabolite. Metabolite profiling allows the identification and quantification of a particular set of metabolites belonging to a specific metabolic pathway. Metabolite fingerprinting tries to detect the metabolic characteristics that discriminate between groups of subjects, without any a priori hypothesis. It does not necessarily involve identifying each metabolite, but its goal is the discrimination of metabolite patterns associated with a given pathological condition. Since it is not guided by a researcher's hypotheses, it is open to new findings and unexpected or even unknown metabolites may turn out to be important in characterizing specific groups of subjects, so new pathophysiological hypotheses may be formulated.

Metabolomics studies are typically performed on biofluids, by analysing them with platforms such as nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS). 1H-NMR spectroscopy generates different signals for each metabolite in the NMR spectrum, thus favoring the identification of proton-containing metabolites. The sample, positioned in a NMR glass tube in a magnetic field, is excited with a radio frequency pulse; alternation between the lower and higher energy spin states of the electrons generates a resonance which is unique for every substance, depending on its chemical structure. Results are showed as peaks across a spectrum, and the area under each peak represents the relative concentration of that specific metabolite; by comparing the relative concentration to an appropriate reference signal, a precise quantification of the metabolite is provided. Advantages of 1H-NMR are minimal sample preparation, non-destruction of biofluids analyzed and execution speed [6]

MS is a powerful method for identifying and quantifying metabolites, and it generates a spectrum in which biocompounds are represented according to their masses (m/z). Liquid chromatography or gas chromatography, which improve mass separation, often precede MS. It is generally considered more sensitive than 1H-NMR, but MS-based platforms are more laborious and more destructive for the sample.55 Both techniques can be performed on very small samples (generally minimum 20 □L). Datasets deriving from NMR and MS analysis can be interpreted using appropriate multivariate statistical approaches (the "pattern recognition methods"). These approaches can be classified in unsupervised and supervised. The first ones, such as principal component analysis (PCA), represent

data by means of plots that the human eye can interpret as different cluster for distinctive subjects' categories (eg, healthy vs ill patients). The supervised methods, such as partial least squares - discriminant analysis (PLS-DA), use a training set of classified samples to create a mathematical model that is then used to test an independent dataset. This method enables us to predict which group a new sample belongs to according to its spectra characteristics.
From a clinical standpoint, metabolomics has the potential to contribute to the characterization of specific biomarkers of early diagnosis, prognosis or prediction of therapy response. For this reason, the metabolomics approach is considered promising with the view of the development of a more individualised medicine [5].

The pathophysiological and clinical aspects of IUGR and EUGR together with the existing evidence emerging from metabolomic studies on these conditions are outlined hereafter.

IUGR: definition and diagnosis

It is fundamentally important for obstetricians and neonatologists to recognize IUGR during a pregnancy because this condition can strongly influence the newborn's short- and long-term health. IUGR is estimated to affect between 5% and 10% of all pregnancies worldwide [4].
When dealing with IUGR, the first difficulty is posed by its definition. IUGR refers to a condition in which a fetus is unable to achieve its genetically-determined size. Although the term is often used interchangeably with the condition of Small for Gestational Age (SGA), IUGR is a prenatal finding of growth restriction, confirmed by a documented low fetal growth rate and/or by the presence of specific causes such as fetal infection, genetic abnormalities, an impaired placental blood flow, or toxicities. IUGR may be asymmetrical or symmetrical in nature, depending on the timing of the prenatal insult and diagnosis [7]. An international consensus effort has recently defined fetal growth restriction (FGR) as a fetus positive for at least one of the following parameters at <32 weeks: i) an abdominal circumference <3rd centile; ii) an estimated fetal weight <3rd centile; iii) no end-diastolic flow in the umbilical artery; iv) an abdominal circumference/estimated fetal weight ratio <10th centile combined with a pulsatility index (PI) >95th centile in the umbilical and/or uterine artery) (see reference for definition of FGR in a fetus at >32 weeks). Fetuses with congenital anomalies are excluded from this classification [8]. On the other hand, SGA refers to a postnatal condition when a baby's birth weight is below the 10th centile for gestational age. Both these definitions have their weaknesses. Considering birth weight alone can lead to an overestimation of cases of SGA, with the inclusion of newborns who are simply constitutionally small, without being at risk of adverse outcomes. On the other hand, considering only prenatal assessments can result in undiagnosed cases

due to cases going undiagnosed because a growth restriction or arrest occurring shortly before delivery, that may not be detected by ultrasound [9]. Ideally, our attention should shift from single growth parameters (head circumference, abdominal circumference, weight, etc.) to assessing a fetus's growth potential. In fact, individualized growth models have now been developed to predict third-trimester growth trajectories on the strength of second-trimester growth rate measurements [10]. It is noteworthy that 42% of 126 singletons judged to be SGA using conventional methods were actually found to be growing normally when assessed with individualized growth models [11].

Short- and long-term outcomes for IUGR neonates

For SGA infants, the mortality risk is 2 to 4 times higher than for infants born at term or preterm but not SGA [12]. For growth-restricted infants, the immediate consequences at delivery include hypothermia, hypoglycemia, hyperglycemia, persistent pulmonary hypertension, pulmonary hemorrhage, polycythemia, stillbirth, and intrapartum asphyxia [13]. Since IUGR is frequently associated with preterm birth, multisystem diseases of prematurity, such as anemia, respiratory failure, necrotizing enterocolitis (NEC), patent ductus arteriosus, and retinopathy may further complicate the clinical picture.

Intermediate and long-term outcomes of IUGR have been well documented, and include impaired physical growth, cognitive and motor development. Several studies also suggest that an impaired fetal growth can predispose to certain major diseases later in life, including metabolic syndrome, obesity, coronary heart disease, hypertension, dyslipidemia and type 2 diabetes, and chronic lung and kidney diseases [14-17]. The assumption is that the fetus makes a number of hemodynamic and metabolic adjustments to cope with the adverse uterine environment, which may lead to permanent changes in the function and structure of several organ systems. Postnatally, when the environmental conditions change, the growth-restricted newborn's response might be inadequate, further raising the risk of long-term consequences [18].

A few decades ago, Hales and Barker advanced the hypothesis of the so-called "thrifty phenotype", according to which insulin resistance and type 2 diabetes during adulthood might originate from a fetal adaptation to save glucose in response to intrauterine malnutrition [19]. Chronic diseases in adulthood may actually be the consequence of an "altered programming", when a stimulus or insult at a critical time in early life has permanent effects on structure, physiology, and metabolism. Previous studies have shown that particular body proportions at birth, rather than birth weight alone, can provide a stronger and more specific prediction of later disease [20]. IUGR fetuses have a disproportionately low fat mass compared to their lean mass [21], as well as an impaired skeletal muscle development [22]. When Barker studied various birth measurements, abdominal

circumference was the one best able to predict plasma concentrations of total and LDL cholesterol, and apolipoprotein B. A small abdominal circumference at birth reflects liver size and, since cholesterol metabolism is regulated by the liver, we can infer that an impaired liver growth in utero realigns cholesterol concentrations with a more atherogenic profile [23].

Prenatal exposure to undernutrition and hypoxia prompts the activation of the hypothalamo-pituitary-adrenal axis as an adaptive response to stress. Analyses on cord blood samples have revealed higher cortisol and lower ACTH levels in cases of IUGR [24]. Fetal growth restriction leads to a redistribution of the blood flow by reducing the supply to the kidneys and gastrointestinal tract and preserving the flow to the brain, myocardium and adrenal glands [16]. Low birth weight has also been associated with endothelial dysfunction [25] and an increased thickness of the aortic intima media has been reported in IUGR neonates through ultrasound [26].

An enhanced insulin sensitivity at birth, followed by an accelerated postnatal growth and the subsequent emergence of insulin resistance, is another feature of IUGR neonates [27]. Cord blood IGF-1 and insulin levels were found to be lower for IUGR newborns than for babies whose size was Adequate for Gestational Age (AGA) [28].

Interestingly, fetal macrosomia coincides with similar alterations in terms of fetal adipose tissue development, permanent changes in hormone function regulation, and a strong tendency for obesity and related metabolic disorders later in life [29].

Biomarkers of intrauterine growth restriction

Many efforts have been made to address the early detection or prediction of fetal growth restriction in the early stages of pregnancy with a view to implementing prophylactic strategies and stricter monitoring. β-human chorionic gonadotropin (β-HCG), pregnancy-associated plasma protein-A (PAPP-A), placental growth factor (PlGF) and soluble fms-like tyrosine kinase-1 (sFlt-1) are some of the biochemical analytes in the serum studied so far as potential predictors of placental dysfunction in early pregnancy. Unfortunately, when used alone, the value of these biomarkers in predicting IUGR is rather low. Combining these maternal serum markers with abnormal Doppler findings has been shown to increase their accuracy and sensitivity in predicting adverse outcomes -– especially for IUGR and pre-eclampsia - but their use has yet to take hold in clinical practice [30,31]. The search for biomarkers of IUGR has also focused on the newborn immediately after birth. Dessì et al. summarized some of the mediators found to be related to newborn anthropometry at birth [32] . Levels of leptin and adiponectin, two proteins secreted mostly by fat tissue, also correlate with birth weight [33]. In addition, adiponectin levels have been found to be significantly higher in AGA rather than in SGA neonates [34]. A recent study confirmed a positive association between leptin (but not

adiponectin) levels and anthropometric parameters in IUGR twins born from discordant dichorionic pregnancies [35]. One interesting finding concerns the higher concentrations of visfatin (a protein produced by visceral fat and associated with insulin resistance) in IUGR neonates rather than in those with a normal birth weight: this is probably due to a greater visceral adiposity or an altered fetal development of adiposity in cases of IUGR [36,37]. Florio et al. found increased urinary levels of S100B (an inflammatory protein expressed in cerebral and adipose tissue) in IUGR newborns [38]. Levels of S100B and neuron-specific enolase (another known brain injury biomarker) seem to relate to worse neurodevelopmental scores at 2 years of age, suggesting a possible link between these molecules and the neurological sequelae observed in IUGR [39]. Levels of ghrelin, a hormone with orectic properties, has been found in higher than normal levels in cord blood from SGA babies: this is likely to be an adaptive response to malnutrition in fetal life [40]. Epigenetic changes have also been demonstrated: IUGR neonates show significant DNA hypomethylation when compared with AGA newborn [41]. Heijmans et al. have shown a possible link between the Dutch Cohort and epigenetic markers. They have found that individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944–45 had, 6 decades later, reduced methylation of the IGF2 imprinted gene, leading to an overexpression of IGF2, compared with their unexposed, same-sex siblings [42]

Metabolomics as a tool to identify new markers of IUGR

The intrauterine metabolic and endocrinological adaptation mechanisms occurring in the fetus exposed to IUGR are not easy to investigate, for the time being at least. Our current knowledge comes largely from animal experimentations and studies on growth-restricted newborns at birth.

A number of researchers have applied the metabolomic approach in efforts to characterize the biochemical variations associated with IUGR [43] (Table1). Dessì et al. first compared the urinary metabolic profiles of IUGR and AGA preterm infants within 24 and 96 hours of birth. Among the discriminant metabolites, they found a significant increase in myoinositol, sarcosine, creatine and creatinine in the former [44]. Interestingly, an increase in plasma and urine myoinositol levels has been associated with adult glucose intolerance and insulin resistance [45]. A greater excretion of myoinositol - compared with AGA infants - was confirmed not only in IUGR neonates born preterm, but also in those born at term, and in newborn large for gestational age (LGA) [46,47]. This would suggest that a low carbohydrate tolerance is associated with both hypo- and hyper-nutrition in the uterus. The latest studies have found higher levels of several other metabolites in IUGR newborns, including creatinine, creatine, threonine, citrate, betaine, glycine, urea, glycerol, and uric acid. When Favretto et al. analyzed serum obtained from cord blood at birth, 22 metabolites were able to discriminate between IUGR and AGA newborns, with phenylalanine, tryptophan, and glutamate

emerging as the strongest predictors. The authors speculate that phenylalanine and glutamate accumulation in IUGR could be the result of an impaired placental metabolism or transport. Higher levels of tryptophan, a serotonin precursor, could be partially explained by an enhanced brain serotonin synthesis and activity, as seen in animal models of fetal growth restriction [48]. Notably, another study by the same group found a trend (not reaching statistical significance) towards phenylalanine upregulation, and valine, isoleucine, tryptophan, and proline downregulation in selective IUGR monochorionic twins with umbilical artery abnormalities on Doppler ultrasound, when compared with control AGA co-twins [49]. Similarly, Horgan et al. found upregulation of amino acids such as phenylalanine, tryptophan, and methionine in placental villous explants from SGA newborns (compared with AGA babies) that depended on O2 tension [50]. Sanz-Cortés et al. used NMR spectroscopy to analyze the metabolomic profile of umbilical cord blood plasma: they confirmed an increase in glutamine and creatine levels in IUGR, but found phenylalanine and tyrosine levels lower than normal in cases of IUGR. They attributed this reduction to an altered placental transport combined with a hyper-catabolic state in IUGR. The value of their study lies in the fact that different clinical subsets of IUGR neonates were compared, namely early- and late-onset IUGR and, among the latter, babies with or without signs of middle cerebral artery vasodilation.

The authors suggest that IUGR is a heterogeneous disease, with different metabolic profiles possibly underlying its different clinical presentations [51]. NMR spectroscopy was used also by Moltu and et al. colleagues to analyze urine samples of 48 premature infants to assess their metabolic status and the responses to different nutritional regimens. As secondary objective, the metabolites profile of AGA and SGA were compared, showing higher levels of glycine and threonine in the latter, although not significant at the adjusted significance level (p=0.027 and p=0,033, respectively). Glycine and threonine levels appeared to differ between SGA and AGA children in the first week of life, but not at later time points (weeks 3,5 and 7). The authors speculated that increased glycine levels may be caused by a reduced aminoacid oxidation or a reduced gluconeogenesis as a strategy to conserve aminoacids [52].

In a study by Liu et al., several metabolites (alanine, methionine, ornithine, serine, tyrosine, isovaleryl carnitine, and eicosenoyl carnitine), but not homocysteine, were found lower in peripheral blood sampled within 3-7 days of birth in IUGR babies with a birth weight below the 3rd %ile for AGA newborns. Interestingly, babies with a birth weight between the 3rd and 5th %iles showed an increase of the same amino acids, probably due to a compensatory mechanism [53].

Abd El-Wahed matched the metabolic profile obtained from cord blood of 40 SGA and 20 AGA neonates, identifying several metabolites at different concentration that clearly discriminated between

Study	Subjects	Sample	Method	Results
Dessì (2011) [53]	26 IUGR vs 30 AGA (preterm)	Urine • within 24 h • at 96 h	^{1}H-NMR	⇑ myoinositol, sarcosine, creatine, creatinine
Horgan (2010) [59]	9 SGA (BW<5th %ile) vs 8 AGA (term)	Placental villous explants	LC–MS	Difference in metabolites levels between the two groups, depending on O2 tension exposure
Favretto (2012) [57]	22 IUGR vs 22 AGA (GA: 32-41 wks)	Cord vein blood	LC-MS	⇑ phenylalanine, tryptophan and glutamate, methionine, proline, valine, isoleucine, dopamine, histidine, uric acid, caffeine, 5-methyl-2-undecenoic acid, oleic acid, 1-Hydroxyvitamin D3 3-D- glucopyranoside, L-thyronine, hexadecanedioic acid
Cosmi (2013) [58]	4 selective IUGR MCDA twins vs 4 AGA MCDA twins (GA: 28-36 wks)	Cord vein blood	LC-MS	⇑ phenylalanine, sphingosine, glycerophosphocholine ⇓ valine, tryptophan, isoleucine, proline, choline (not statistically significant)
Sanz-Cortés (2013) [60]	20 early IUGR (GA: 31.7 ± 2.2 wks) vs 23 matched AGA	Umbilical vein blood	^{1}H-NMR	⇑ VLDL, unsaturated lipids, acetone, glutamine, creatine ⇓ glucose, phenylalanine, tyrosine, choline
	56 late IUGR (mean GA: 38.3 ± 1.9 wks) vs 55 matched AGA	Umbilical vein blood	^{1}H-NMR	⇑ VLDL, unsaturated lipids ⇓ phenylalanine, glutamine tyrosine, choline, valine, leucine
Barberini (2014) [54]	11 IUGR (+ 12 LGA) vs 10 AGA (mean GA: 37 wks)	Urine • within 12 h	GC-MS	⇑ inositol ≠ urea, glycerol, glucose, citric acid, uric acid
Dessì (2014) [55]	12 IUGR +12 LGA vs 17 AGA (GA: 34-41 wks)	Urine • within 8 h	^{1}H-NMR	⇑ myoinositol, creatinine, creatine, citrate, betaine, glycine ⇓ urea, aromatic compounds and branched chain amino acids
Marincola (2015) [56]	8 IUGR vs 8 AGA (mean GA: 36.9 vs 37.5 wks)	Urine • within 8 h • at 4 days • at 7 days	^{1}H-NMR	⇑ myoinositol, citrate, glycine, ⇓ succinate, betaine, creatinine
Moltu (2014) [61]	16 SGA vs 32 AGA (mean GA: 29.9 vs 27.5 wks)	Urine • within 1 wk	^{1}H-NMR	⇑ glycine, threonine (not significant when adjusted for gestational age at birth)
Liu (2016) [62]	25 SGA (BW < 3rd %ile) vs 60 controls (mean GA: 36.8 vs 35.9 wks)	Blood spot • between 3 and 7 days	Targeted LC-MS	⇑ homocysteine ⇓ alanine, methionine, ornithine, serine, tyrosine
Abd El-Wahed (2017) [63]	40 SGA vs 20 AGA (mean GA: 34 ± 2.4 vs 35 ± 1.4 wks)	Umbilical cord blood spot	Targeted LC– MS	⇑ several acylcarnitines including C18–OH, C16–OH, alanine, arginine, aspartate, citrulline, glutamine, isoleucine, leucine, ornithine, phenylalanine, tyrosine, valine ⇓ histidine, methionine
Wang (2018) [64]	15 pairs of selective IUGR MCDA twins vs 24 pairs of uncomplicated MCDA twins (mean GA: 35 vs 36.5 wks)	Umbilical cord blood	GC-MS	⇑ methionine, phenylalanine, 4-hydroxyphenylacetic acid, 2-aminobutyric acid, decamethylcyclopentasiloxane, tyrosine, isoleucine, eicosapentaenoic acid ⇓ adrenic acid

Table 1. Post-natal metabolomic studies on fetal growth restriction
IUGR: intrauterine growth restriction, AGA: adequate for gestational age; ^{1}H-NMR: proton nuclear magnetic resonance spectroscopy; SGA: small for gestational age, LC-MS: Liquid Chromatography Mass Spectrometry; GA: gestational age; wks: weeks; MCDA: Monochorionic-Diamniotic; GC-MS: Gas Chromatography Mass Spectrometry; BW: birth weight

the two groups: among others, they found elevated levels of acylcarnitine (especially C18-OH and C16-OH), glutamine, leucine and valin Glutamine is known to be an important source of cellular energy during fetal life and it has a key role in fetal neurodevelopment. Increased glutamine levels could be explained by a hypercatabolic state associated with IUGR to compensate for the lack of other energy substrates, such as glucose. Acylcarnitines are also important for energy metabolism, as they are involved in fatty acid oxidation. Abnormal plasma acylcarnitine levels have been associated with type II diabetes [54]. Wang et al. recently compared umbilical cord and placental blood collected from 15 selective IUGR twins from monochorionic diamniotic pregnancies with 24 pairs of twins from uncomplicated pregnancies and 14 IUGR singletons. Untargeted metabolomic analyses revealed some metabolites capable of discriminating between the selective IUGR twins and the control twins. Methionine-cysteine, phenylalanine and tyrosine metabolism were among the metabolic pathways apparently most involved. The added value of this study lies in the comparison drawn within pairs of selective IUGR twins (i.e. between the larger and smaller twin), which led to the identification of those metabolites that correlated exclusively with birth weight discrepancy. Another intriguing finding was the higher level of environmental xenobiotics (identified as cyclic siloxanes) associated with selective IUGR [55]. The efforts to pool and interpret the metabolomic data generated so far on IUGR have proved to be very complicated and challenging. This is caused by several factors: the large numbers of metabolites generated, the non-homogeneous populations (preterm and term infants), the different analytical approaches (NMR or MS), the small sample sizes, the absence of a well-defined experimental design and the inconsistencies in the results obtained (not validated due to small sample size) from different studies.

EUGR: definition and pathogenesis

Extrauterine growth restriction is a common complication in very low birth weight (VLBW) preterm infants. Postnatal growth restriction is defined as body measurements (weight and/or length and/or head circumference) ≤ 10th percentile (z-score < -1.28) for post-menstrual age (PMA) at discharge [56]; in addition 36 weeks' PMA or 40 weeks' PMA (term-equivalent age) evaluation are often used to compare the incidence of EUGR between neonatal intensive care units [57]. Nonetheless, according to other authors it would more appropriate to consider EUGR as the reduction in weight z-score between birth and discharge >1 SD [58].

It should be noted that EUGR of a VLBW preterm newborn occurs in the same "window" in which the IUGR of the newborn at term is realized. Very preterm infants are exposed indeed to an extrauterine environment in a period normally characterized by a rapid intrauterine growth. A huge shift in energy expenditure is necessary to survive in this unexpected postnatal life.

The EUGR may be present both in AGA and in SGA infants at birth, with a significant further reduction in growth percentiles in the latter. Despite numerous efforts of improving the neonatal nutrition of VLBW infants during Neonatal Intensive Care Unit (NICU) stay, many of these do not receive an adequate nutritional supply for their increased demands, resulting in a growth restriction. The major factor is likely the development of significant protein and energy deficits during the first several weeks of life, which prove difficult to reverse. These deficits increase as gestational age decreases [59]. Furthermore, many of these infants develop chronic diseases in a critical period of life, when rapid growth is expected, with consequent high caloric requirements. Postnatal growth failure in VLBW infants is an almost universal phenomenon. Several factors have been found to be independently associated with EUGR including IUGR or SGA, male gender, need for assisted ventilation on the first day, prolonged need for respiratory support, length of hospital stay, and the development of neonatal morbidities such as bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), and late-onset sepsis.[60].

Data from the neonatal research network of the National Institute of Child and Human Development (NICHD) demonstrated that 16% of preterm infants with VLBW were SGA at birth; however, when they reached 36 weeks of corrected age, 89% of this same population of preterm infants had postnatal growth failure [61].

Similar prevalence was found by Stoll et al. who performed a large analysis on perinatal and neonatal data collected from 9757 infants of extremely low GA (22–28 weeks) and VLBW (401–1500 g) who were born between 2003 and 2007. Among the several parameters, a growth failure (weight <10th percentile for gender at PMA of 36 weeks) was found in 79% of infants. [62].

From the large analysis of the Spanish Neonatal Network database (21825 preterm infants < 32 weeks at birth) it has emerged that patent ductus arteriosus, bronchopulmonary dysplasia, necrotizing enterocolitis and late-onset sepsis had an independent effect on all growth parameters studied, which was greater in the case of necrotizing enterocolitis. In this study head growth was less affected than length and weight. Girls are at slightly higher risk of postnatal weight and head restriction after adjustment by morbidity [63].

In a study by Marques et al, among a total of 101 VLBW newborns (median gestational age: 29 weeks) 34.7% was diagnosed with EUGR. Fetal growth restriction, moderate-severe bronchopulmonary dysplasia, invasive mechanical ventilation, patent ductus arteriosus and its surgical treatment, retinopathy of prematurity, cystic periventricular leukomalacia, anemia requiring red blood cells transfusions, as well as duration of parenteral nutrition, day of start of enteral nutrition, day of achievement of full of enteral nutrition and a longer duration of hospitalization were identified as independent risk factors for EUGR [64].

In a Brasilian research on 570 VLBW newborns (mean weight at birth: 1,113), 26 and 5% presented a weight and head circumference growth restriction at discharge, respectively. The authors considered a z-scores ≤ -2 for corrected gestational age for the diagnosis of growth restriction. Univariate analyses demonstrated that maternal hypertension, male gender, SGA at birth, respiratory distress syndrome, and length of hospital stay were significantly associated with weight z-score at hospital discharge. SGA at birth, length of hospital stay, respiratory distress syndrome and patent ductus arteriosus were the variables that showed a statistical significative association with EUGR in the final logistic regression analyses. It is worth noting that adding one day to the hospital length of stay of these neonates resulted in a 2% increase in the chance of having growth restriction at discharge. [65]
Shan et al. analyzed 2015 preterm infants with low birth weight born at four hospitals in Shanghai between 2003 and 2006 and found a frequency of 56.8% of EUGR (weight < 10 %ile at discharge). Higher incidence of EUGR was associated with lower birth weight. According to the logistic regression analysis male gender, gestational age at birth, birth weight, and length of hospital stay were factors related to EUGR. Interestingly this research also revealed an inverse association between EUGR incidence and the presence of NSTs (nutrition support team). NST is a multidisciplinary team comprised of physicians, nurses, dietitians, pharmacists, social workers, and medical technologists who provide nutritional management availability [66].
EUGR (< 10 %ile at discharge) incidence rates for weight, length, and head circumference were found in 57%, 49%, and 6%, respectively, in a population of 416 newborns born in 22 Japanese facilities in 2002. The authors assume that, although lower gestational age influences head circumference growth, nutrient supply to premature infants is somewhat able to spare brain from growth restriction. In addition, factors significantly associated with extrauterine weight growth restriction included whether or not the infant was SGA, if oxygen was administered at 36 weeks PMA, age in days when birthweight was regained and when enteral feeding was achieved [67].
Cooke and colleagues, in an attempt to compare EUGR in preterm infants discharged from level III tertiary care units and level I–II special care baby units in the former Northern Region of the United Kingdom found no differences in weight gain, weight at discharge, or degree of postnatal growth retardation between the two groups. This could be justified by the fact that higher risk infants, as soon as it is clinically indicated, were transferred from III to local I–II unit. Actually, also birth characteristics, CRIB score (a measure of illness in the first 24 hours of life) and length of hospital stay were not found to be significantly different between level III and level I-II units. It is noteworthy that significant variation in length of hospital stay, weight gain, weight at discharge and degree of postnatal growth retardation were recorded among level III units. Even greater variability was noted

in the same variables among the level I–II units, thus underscoring the need for shared nutritional and growth assessment protocols [68].

Clark et al. reviewed data from 24371 neonates born between 23 and 34 weeks PMA. The incidence of EUGR (growth value ≤10th percentile of intrauterine growth expectation at the time of discharge to home) was 28%, 34%, and 16% assessed by the weight, height, and head circumference, respectively. For each growth parameter, the incidence of EUGR increased with decreasing gestational age and birth weight. Factors independently associated with EUGR were male gender, need for assisted ventilation on day 1 of life, a history of necrotizing enterocolitis (NEC), need for respiratory support at 28 days of age, and exposure to steroids during hospitalization [57].

According to Radmacher et al, who conducted a 4-year retrospective chart review in 221 infants with birth weight ≤1,000 g and or ≤ 29 weeks gestational age, the incidence of EUGR (weight <10th percentile at time of discharge) was 59%. The added value of this study lies in having assessed also nutritional intakes during NICU stay. From this analysis, it emerged that mean energy and protein intake during hospitalization did not reach the recommended standard of 120 kcal/kg/day and 3.0 g/kg/day [69].

The variability in the incidence of EUGR in the studies presented so far is probably explained by the assessment of populations with different demographic characteristics, the cutoff used to define EUGR, as well as the use of different reference curves.

The haemodynamic or hormonal changes, as well as the metabolic pathways that are involved in EUGR pathogenesis have not been investigated thoroughly. Moreover, we lack reliable biomarker capable to assess the suitability of growth in preterm babies. In this connection some recent researches have highlighted some interesting results.

DNA methylation of Imprinting Centre 1 (IC1) was evaluated in 38 premature (gestational age at birth <34 weeks) newborns admitted to NICU at University Hospital of Pisa by Tozzi and colleagues. IC1 is an independent imprinting control region located in chromosome 11 at p15, a key region, involved in the control of growth and development. In particular, ICF1 regulates IGF2 gene expression, an important growth stimulator gene, and H19, a gene with unknown function. It is known how DNA methylation is a key component of epigenetic network and nutrients may modify the pattern of DNA methylation. For instance, alterations of the imprinting mechanism at this site could cause phenotypes of altered growth, as observed in Beckwith-Wiedemann Syndrome, in which IC1 hypermethylation determines overgrowth, and in Silver Russell Syndrome, in which IC1 hypomethylation causes growth restriction. A correlation between EUGR (defined as a reduction in weight z score between birth and discharge >1 SD) for weight and for head circumference and an increased IC1 methylation emerged from this investigation. In addition, a relationship between

reduced protein and lipid intake and IC1 hypermethylation was observed. The authors speculate that IC1 hypermethylation could be a reprogramming mechanism to promote a catch-up growth, by means of an increased Insulin-like growth factor 2 (IGF2) expression [56].

Li and colleagues recently compare microbiota from meconium and fecal samples at 28 days of life obtained from 12 EUGR and 11 AGA newborns. The genera *Aeromicrobium* and *Serratia* significantly decreased in the meconium samples of EUGR group, whereas genera *Parabacteroides, Ruminococcus, Blautia*, and *Aeromonas* were more prevalent. On postnatal day 28, the genera *Parabacteroides, Bacteroides, Eubacterium, Granulicatella* were significantly higher in EUGR, whereas genus *Salinivibrio was lower.* Overall, these findings showed that a distinct gut microbiota profile is present in preterm infants with EUGR. [70].

With regard to adipokines, a study comparing 43 term infants and 58 preterm infants (born before 34 weeks' gestation) found out higher levels of adiponectin in the latter. Further, body weight SD score changes were positively associated with adiponectin increases in preterm infants from birth to term-equivalent age. [71]

EUGR: is it preventable?

Growth failure of VLBW infants may result from a complex interaction of many factors, including inadequate nutrition, morbidities affecting nutrient requirements, endocrine abnormalities, central nervous system damage and administration of drugs that influence nutrient metabolism. The difficulties of feeding preterm infants, particularly VLBW infants, are well known. Most of these infants have weak sucking and swallowing mechanisms, decreased or absent intestinal motility, and immaturity of several metabolic pathways. They tend to have a high incidence of complications that interferes with nutrient absorption. In addition, the concern that feeding may contribute to the development of necrotizing enterocolitis often induces neonatologists to reduce or stop enteral feeding for variable periods of time. For these reasons, many very-low-birthweight infants are undernourished during the first weeks of life. Even if they receive nutrients by the parenteral route, the intake is often quantitatively and qualitatively below the nutritional requirement. Careful analysis of growth data from birth to discharge clearly suggests that the growth impairment in VLBW infants is mainly the result of nutritional deficit during the early weeks of life. After this time, healthy preterm infants on a full enteral feeding regimen with fortified human milk or one of the recent preterm formulas receive adequate nutrient supplies to support “intrauterine” rates of growth [3].

Recommendations by the American Academy of Pediatrics, the Canadian Paediatrics Society, and the European Society of Paediatric Gastroenterology, Hepatology, and Nutrition state that the nutrient supply for preterm born infants should ideally enable them to mimic intrauterine growth in the third

trimester of pregnancy, which is characterized by a huge increase in fetal body weight and brain volume [34].

Efforts during the past two decades to develop standardized feeding guidelines have begun to show some success in reducing the incidence of EUGR. Such guidelines provide intense nutritional support through a combination of early parenteral nutrition and early enteral nutrition, followed by a progressive reduction of parenteral nutrition, as enteral feeding volumes are steadily advanced to full enteral nutrition. Compared with historic controls, benefits of this approach have included an earlier regaining of birth weight, an earlier achievement of full enteral nutrition, reduction in the duration of parenteral nutrition, and improved anthropometrics at 36 weeks' PMA or discharge [60].

The implementation of shared and standardized nutrition protocols is encouraged in the NICU as well as the development of systems able to evaluate the amelioration in terms of EUGR incidence reduction. As an example, Darrow and colleagues recently introduced a new parenteral nutrition protocol designed to improve both energy and protein intake and consisted in initially targeting 3 g/kg/day of protein, and then increasing by 0.5 to a maximum of 4 g/kg/day by the third day of life. Lipids were infused on the first day of life at 1 g/kg/day, and then increased to 3 g/kg/day on the second day. Hypertriglyceridemia (TG > 200 mg/dL) would trigger a reduction to 2 g/kg/day for the next day, after which a return to 3 g/kg/day would be ordered if tolerance was demonstrated, The calcium and phosphorus content of the new standardized parenteral nutrition solutions nearly doubled. A significative reduction in z-score change from birth to 36 weeks PMA was obtained. Improvements in early weight gain and linear growth were seen as well. Quality improvement methodology may be utilized to design and make use of optimized, standardized parenteral nutrition, and this process can help improve extrauterine growth.[72].

Stevens et al. demonstrated that the implementation of the following practices was associated to better growth outcomes: earlier initiation of trophic feedings, earlier advancement of feedings and earlier attainment of full enteral feedings, greater use of breast milk and earlier breast milk fortification, and discontinuation of Total Parenteral Nutrition at a higher feeding volume. After the implementation of these practices, a 19% reduction (from 32.6% to 26.3%,) in postnatal growth restriction before hospital discharge, was achieved, as well as reductions in the difference in z-score between birth and discharge weights. [73].

Similarly Hu and colleagues compared parenteral and enteral nutrition practices between 87 EUGR and 41 non-EUGR VLBWs infants. From the comparison it emerged a higher enteral nutrition interruption rate, a lower average energy intake in the first day after parenteral nutrition termination and an overall lower energy and protein intake in the EUGR group [74]. The withholding of feeds was identified as a significant independent predictor of poorer growth at 36 weeks PMA, also in the

study of McKenzie, performed in 96 preterm infants. In contrast with other studies, the period of time to establish full enteral feeding was positively associated with weight-for-age z score at 36 weeks PMA [75].

In a study conducted by Genoni, 100 infants with a gestational age of ≤34 weeks were enrolled, 50 before and 50 after the introduction of a nutritional intervention including: total parenteral nutrition with a high level of amino acids (3 g/kg/day) from the first hours of life; lipids from the first 24 hours of life at 1.5–2.5 g/kg/day increasing to 3.5 g/kg/day and minimal enteral feeding at 10–20 mL/kg/day from the first one to two days of life. They observed that prevalence of EUGR at discharge was significantly lower after the introduction of this regimen for weight (34% vs. 66%), head circumference (22% vs. 42%) and length (20% vs. 48%). In the new regimen group, weight velocity was significantly higher and maximum weight loss and negative changes in the Z-scores from birth to discharge for weight were lower than in the pre-intervention controls [76].

Similarly, two French cohorts of 66 and 78 premature children were compared before and after the introduction of nutritional protocol providing higher intake of protein, lipids and glucides. A reduction of moderate EUGR (changes in weight z-score between 1 and 2 SD) rate from 86.4 % to 39.7 % and of severe EUGR (changes in weight z-score between 1 and 2 SD) from 21.2 % to 5.1 %, were observed. [77].

Izquierdo et al. assessed growth parameters in 142 VLBW infants ≤32 weeks of gestation, after implementation of nutrition policies including higher nutrient and protein supply from day 1, the start of lipid infusion within the first 24 hours, and the availability of ready-to-use standard parenteral solutions in the unit. Weight at 14 days of life, but not at day 28 of life or discharge, was significantly higher after implementation of the new protocol [78].

Theile and colleagues have retrospectively evaluated growth in 88 ELBW, diagnosed with bronchopulmonary dysplasia. It is known how these infants expend considerable amounts of energy to maintain their metabolic rate, synthesize new tissue and support the increased respiratory work, physical activity and thermoregulation. It is therefore even more difficult to meet the nutritional requirements in lieu of fluid balance concerns and other co-morbid conditions in this subpopulation of preterm infants. Among BPD subjects, those whose growth was under fetal rate ($\leqslant 14\ g\ kg^{-1}$ per day) received more systemic pressor agents, doses of surfactant, diuretic medication and had more days of supplemental oxygen and of mechanical ventilation and more severe BPD compared with the cohort with greater growth ($\geqslant 16.1\ g\ kg^{-1}$ per day). There was less EUGR by weight and head circumference for those ELBW infants with BPD receiving higher amounts of protein, aggressive early total parenteral nutrition and caloric-dense milk [79]

In view of this it seems that an “aggressive” nutrition could prevent a catabolic state in the first days after birth and later assure an appropriate growth. This can be obtained by initiating total parenteral nutrition on the first day of life with a protein intake (>2 g amino acids/kg/d) that prevents catabolism and ideally allows a sufficient protein deposition, as long as the protein intake is tolerated, and with the use of i.v. lipid (0.5–1.0 g lipid/kg/d) from the first day of life. Glucose and lipid intakes advance as rapidly as tolerated, and “minimal enteral feeding” is started in the first 1–2 d of life in order to stimulate the gut, with advancement to “nutritive” feedings, taking into account clinical tolerance and risk factors [3].

Long term consequences for EUGR infants

In humans, the importance of nutrition in the early period of life is now well known, and the term “programming” has been proposed to emphasize that early nutrition should be considered not simply in terms of providing immediate nutritional needs but also for the potentially life-long biological effects. Studies of monozygotic twins have demonstrated that among environmental factors, nutrition is the most important in determining diverse phenotypes, independent of genotype, including susceptibility or chronic disease in adulthood. Early growth failure may “program” various adverse long-term effects, including cardiovascular disease and type II diabetes [3]

The impact of growth restriction and nutritional problems at such early age can influence the future quality of life, as it can affect brain growth and, consequently, development, and contribute to the onset of chronic adult diseases. [59]

Of the 322 Japanese VLBW infants studied by Takayanagi et al. 47.5% showed EUGR (*z*-score for weight or length of <−2.0 at term or at a corresponding age if the subjects were discharged before). At growth assessment of approximately six years of age the *z*-scores for the height and BMIs of the infants who experienced EUGR were significantly lower in comparison with those without EUGR, regardless of whether they were SGA at birth. In addition 11.8% presents short stature, 11.8% were thin and 1.9% were obese. Among the 38 VLBW infants with short stature, nine were diagnosed with growth hormone deficiency. The univariate analysis revealed that a history of SGA and EUGR during NICU admission was a risk factor for subsequent short stature and thinness. A logistic regression analysis revealed that the *z*-score for body length at term was a significant factor for short stature, with an odds ratio of 0.53, while the *z*-scores for body weight at term (OR 0.44) and male gender (OR 3.10) were significant factors for thinness. The prevalence of growth hormone deficiency in VLBW infants was between 1.8% and 2.8%, which seemed to be markedly higher than the rate in the general Japanese population. This suggested that being born with a VLBW may be a significant risk factor

for the subsequent occurrence of growth hormone deficiency. However, the pathophysiological reasons for this association are not clear [80].

Past studies have shown a positive correlation between physical growth and neurodevelopmental outcomes in preterm newborns. In-hospital growth correlates positively with neurologic outcome [35,36], and energy and protein intakes in the first week of life are each independently associated with better Mental Development Index (MDI). Malnutrition during rapid brain growth results in fewer neurons, which might lead to future behavioral problems, and memory and learning difficulties [37,38]. A comprehensive review by Chan et al. confirms that supplementing nutrition after birth with macronutrients or multinutritional interventions can increase the likelihood of survival without neurodevelopmental impairment in VLBW infants [39].

Within the EPIPAGE (Étude Épidémiologique sur les Petits Âges Gestationnels -Epidemiological Study on Small Gestational Ages) cohort follow-up project, medical examination (n = 1305), cognitive evaluation with the Kauffman Assessment Battery for Children (n = 1130) and behavioral difficulties assessment were performed at 5 years of age. Risk of cerebral palsy was greater for neonates who were AGA at birth but presented catch-down-growth (defined as loss ≥1 SD between birth and six months weight z-scores) compared with AGA neonates whose growth follows the curve (OR 2.26 [95% CI 1.37-3.72]). Irrespective of post-natal growth, all children who were SGA at birth showed increased risk of cognitive deficiency and inattention-hyperactivity [81].

A Spanish research has investigated metabolic changes in a group of 38 prepuberty children (mean age about 8 years) with a history of EUGR (weight < 3rd %ile at 36 weeks and at the time of discharge from the NICU) compared with a control group of 123 born at term children with similar age and gender. To note that EUGR group birth weight was above 10th %ile. At time of evaluation the EUGR group had height and body mass index values significantly lower than in the control group and higher systolic and diastolic blood pressure. The EUGR group had higher glucose levels and lower high density lipoprotein cholesterol (HDLc) than the control group, although within normal range and without clinical relevance[82].

In the same cohort circulating adipokine levels were also measured in order to evaluate any changes in adipose tissue and adipocyte function. Prepubertal children with a history of EUGR exhibit lower plasma adiponectin concentration and higher resistin concentration compared with healthy children, even after adjustment for gestational age, weight, and size at birth, while leptin was non significantly different between the two groups. Similarly, children with a history of IUGR have been reported to present lower adiponectin concentrations. Hypoadiponectinemia has been associated with the metabolic syndrome, hypertension, dyslipidemia, and an increased risk for CVD in prepubertal children with a history of IUGR. [83].

Interestingly, in a study performed by Saito and colleagues increase in adiponectin levels from birth to term-equivalent age was significantly higher in the AGA than in the SGA group, and was positively correlated with the weight gain rate (g/kg/day) [84]

On the other side resistin, a cytokine specifically secreted by adipocytes and macrophages, has a proinflammatory action. Increased resistin concentration is strongly correlated with an increased risk for CVD later in life [83].

It is noteworthy that, adipose tissue development is probably altered already from the first weeks of life. Indeed, infants who reach term-corrected age have a lower lean mass and a higher percentage of body fat than term infants before and after hospital discharge [85]. An Italian research has assessed fat mass in 195 infants with birth weight ≤1500 g by an air displacement plethysmography system. At term, both the group of SGA infants and the group of AGA who experienced growth restriction showed a significantly lower fat mass than the AGA group not presenting EUGR. In the first three months, change in fat mass was comparable between the group of AGA with a history of EUGR and the group of SGA and significantly higher than that of the group of AGA without EUGR. Nevertheless, the mean fat mass value presented at term by all the infants enrolled in the study, regardless of categorization, was much higher than that found in full term neonates at birth. Mean fat mass values attained at three and five months by all infants, regardless of group categorization, were comparable to those of full term breastfed infants [86].

A retrospective investigation included 68 VLBWs infants, comprising 45 appropriate-for-gestational-age (AGA) and 23 SGA infants who underwent lumbar spine dual-energy X-ray absorptiometry (DSA) at term-equivalent age. Bone mineral density (BMD) and bone mineral content (BMC) did not differ between the two groups. In contrast, infants with EUGR (weight below the 10th percentile of the reference values at term age on DXA measurement) showed significantly lower values than those without. Multiple regression analyses showed GA, weight and head circumference at birth, and weight percentile at term correlated with term BMD [87]

EUGR in premature neonates is also likely to have significant implications on ongoing nephrogenesis and consequently on renal function in adult life. [88].

In a study performed by Bacchetta et al., renal function at a mean age of 7,6 years was assessed in a cohort of 50 ex premature children (<30 weeks gestation). Among the included children, 23 had intrauterine and 16 extrauterine growth retardation. When comparing both of these groups to 11 children with appropriate growth, significantly lower glomerular filtration rates (measured by inulin clearance), emerged, although still within the normal range. Although affected by some bias, the EUGR group had indeed lower GA, lower BW and suffered more often from bronchopulmonary dysplasia than controls, this is the first study suggesting that adequate postnatal growth of the preterm

neonate is very important in determining later renal functional capacity. Future research in this area is essential in order to ascertain ways in which to achieve optimal renal development in the NICU setting. Potentially, renal size can be measured postnatally in living preterm neonates to compare renal growth in neonates that have grown appropriately to those that have been growth-restricted ex utero. Furthermore, longitudinal studies examining both renal function and size in EUGR preterm individuals in adulthood will be necessary in order to determine whether these observations persist into adulthood [89].

If on one and all these evidence support the position of enhance nutritional intakes with the aim of optimize infants growth, on the other hand many studies, mostly conducted on term born infants, suggest that greater catch up in weight could also be detrimental and lead to long term consequences. Some evidence suggest that this might happen also in subjects with a history of prematurity.

For instance, in a study by Toftlund, a cohort of 281 VLBW infants was followed-up at 6 y of age, by means of height, weight, and body mass index measurement, a dual-energy X-ray absorptiometry scan and blood sampling. The results of the analysis show how early rapid growth (crossing of weight percentiles with >1 SD in either direction from 34 weeks of postmenstrual age and until 2 months of corrected age) was seen in 53% of the children and was significantly correlated with several metabolic outcomes at 6 y of age [90].

Kerkhof et al. determined and compared determinants of cardiovascular disease and type 2 diabetes mellitus in 162 young adults (18-24 years) born preterm (gestational age <36 weeks) and 217 young adults born term. They found out that gain in weight for length in the period from preterm birth up to term age, and in the first 3 months after term age, was positively associated with body fat percentage and waist circumference at 21 yr. Gain in weight for length in the first 3 months after term age was also positively associated with total cholesterol and low-density lipoprotein cholesterol levels in early adulthood. Subjects with the highest gain in weight from birth to term age had significantly higher body fat percentage, waist circumference, acute insulin response, and disposition index in early adulthood than the subgroups with moderate and low gain in weight. Rapid catch-up in weight during the first 3 months after term age resulted in a higher fat percentage, waist circumference, and serum triglycerides level than slower catch-up in weight.[91].

On the contrary, Embleton et al. did not find any association between infant weight gain (before 1 year of age) and metabolic outcome at a median age of 11.5 years in a cohort of ex-preterm children. However, there were strong associations between more rapid childhood weight gain (after 1 year of age) and subsequent body composition (higher fat mass %, fat mass index and waist circumference) and metabolic markers (higher fasting insulin, blood pressure and lower insulin sensitivity) [92].

Interestingly, according to the research conducted by Belfort and colleagues in 945 subjects with a history of prematurity, more rapid linear growth from term to 4 months was associated with lower odds of Intelligence Quotient <85 at age 8 years (OR, 0.82; 95% CI, 0.70-0.96), but higher odds of overweight/obesity (OR, 1.27; 95% CI, 1.05-1.53). BMI gain over the entire 18 months after term was associated with later risk of overweight/obesity, with less evidence of a benefit for IQ.

This study suggests that faster linear growth soon after term was associated with better cognition, but also with a greater risk of overweight/obesity [93].

In addition, Singhal et al. reported that low nutrient conditions following birth have a positive effect on adult insulin receptivity. Actually, fasting proinsulin concentration was greater in subjects aged 13-16 years born preterm (n=216) and fed with a nutrient-enriched diet than in those fed with the lower-nutrient diet. Proinsulin concentration was associated with greater weight gain the first 2 weeks of life [94].

In the same group flow-mediated endothelium-dependent dilation of brachial artery was lower in in adolescents with the highest rate of weight gain in the first 2 weeks after birth. These findings are consistent with an adverse effects of accelerated neonatal growth on long-term cardiovascular health [95].

In particular in IUGR newborn's, weight gain during infancy and early childhood was found related to their body composition and cardiovascular risk in adolescence [96]. In a study by Mortaz et al., children born with SGA (whatever their gestational age) revealed a lower cholesterol absorption efficiency at 8-12 years of age, and cholesterol synthesis was predicted to be higher in those children whose weight centile had increased most during their follow-up [97]. In other words, a greater catch-up in weight was strongly correlated with an increased cholesterol synthesis. In a group of term-born infants judged to be SGA, using a nutrient-enriched formula (containing 28% more protein) raised their blood pressure at the age of 6-8 years [98].

Judging from these reports, it seems that both overnutrition and undernutrition at a crucial time of life are associated with adult diseases. It is mandatory to promote adequate growth after birth, but we still do not know how to judge the quality of an infant's growth - especially in the case of newborns who have suffered from IUGR. We also lack reliable biomarkers that can tell us whether the nutritional status and growth rate of a preterm baby is appropriate.

Noteworthy, it is well known that prematurity is per se a strong risk factor for later development of metabolic syndrome diseases [99,100] and is related to higher mortality from infancy into mid-adulthood [101]. The role of post-natal growth in this issue has nonetheless not been completely clarified.

Metabolomics as a tool to identify markers of adequate nutrition and growth in newborns

NMR-based metabolomics was used to compare the metabolic urinary profiles obtained from term infants receiving different nutritional regimen: exclusively breast-fed, fed with standard formula or with an enriched infant formula. A common age-dependent modification of the urine metabolome was observed from birth to 4 months of life, mainly characterized by similar temporal trends of choline, betaine, myoinositol, taurine, and citrate. Significant differences in the metabolic profiles were identified according to the type of diet (human versus formula milk), while no significant difference was observed between the two formulas [102]. Dessì et al. used a gas-chromatography mass spectrometry (GC-MS) method on urine and showed that metabolomic profile of IUGR, AGA and LGA newborns, after the shift from placenta to milk supply, was more affected by diet than by their anthropometric parameters. After three days of formula milk nutrition, urine had higher levels of glucose, galactose, glycine and myo-inositol, while up-regulated aconitic acid, aminomalonic acid and adipic acid were found in breast milk fed neonates. Noteworthy, at 7 days newborns fed with formula milk shared the same representative metabolites (pseudouridine, myo-inositol and glycine) with IUGR and LGA neonates at birth, which raises questions about the quality of formula milk, especially when compared with breast milk. On the other hand, breastfed neonates shared up-regulated pyroglutamic acid, citric acid, and homoserine, with AGA at birth [103]

To the best of our knowledge no studies have investigated the relationship between EUGR and metabolomic perturbation in preterm newborn.

STUDY DESIGN and METHOD

Our setting

Almost 350-400 newborns per year are admitted to the Neonatal Intensive Care Unit (NICU) of the Women and Children Health Department of Padova. Among these patients, about 110 are VLBW Infants (birth weight <1500 g) and about 50 are Extremely Low Birth Weight Infants (birth weight < 1000 g). Around 15-20% are diagnosed as intrauterine growth restricted.

Aims of the project

The main aims are to analyse if the metabolomic profiling of urine is able to discriminate between:

- IUGR and AGA premature babies at birth
- EUGR and non-EUGR premature babies at birth, 21 days of life and at 36 weeks postmenstrual age.
-

Design of study

This is a prospective observational study.

Population:

Inclusion criteria

Each infant must meet all the following criteria to be enrolled in the study:

- admission to the NICU
- age between 23 and 32 weeks
- written informed consent for participation of a legally acceptable representative.

Exclusion criteria

- infants who had a major congenital abnormality or a chromosomal abnormality,
- infants with known or suspected congenital metabolic disease,
- infants with hemodynamic instability not allowing early enteral feeding
- refusal of consent.

Collection of data

Clinical data evaluation:

Primary outcomes evaluation:

- Intrauterine growth restriction was diagnosed by expert obstetricians based on prenatal ultrasonographic anthropometric and doppler parameter

- Extrauterine growth restriction was defined as a weight below 10th %ile at 36 weeks postmenstrual age

Other clinical data evaluation (potential confounders)

For each enrolled subjects the following data were collected:

- Demographic data (gestational age, birth weight, birth weight %ile, sex, ethnicity)
- Pregnancy and delivery (premature rupture of membranes (pPROM), multiple pregnancy, type of delivery, antenatal steroids administration),
- Birth (Apgar score at 5 minutes),
- Respiratory support (need for surfactant, days of mechanical ventilation, BPD diagnosis, need for post-natal steroids),
- Hemodynamically significant patent ductus arteriosus (need for medications and/or surgery)
- Intraventricular hemorrhage (grade)
- Sepsis (documented or suspected)
- Necrotizing enterocolitis (documented or suspected)
- Retinopathy of the prematurity (need for laser therapy)
- Cholestasis
- Growth parameters during NICU stay (maximum weight loss, time to regain birth weight, mean extrauterine growth*)

  Mean extrauterine growth (from birth to 36 weeks PMA or earlier, if the baby was transferred to other facilities) was calculated according to the formula proposed by Patel et al. [104]:

  [1000 x ln(36 weeks PMA Weight*/ Birth Weight)]/252**–Birth PMA (days)

  *or PMA weeks at time of transfer to other facilities

  **or days of life at time of transfer to other facilities
- Maximun azotemia and maximum creatinine during the first month of life
- Osteopenia defined as zBTT < 2 SD at bone ultrasound examination performed at birth and at 36 weeks PMA
- Nutrition: mean energy intake in the 1st week of life and mean energy intake in the 1st month of life, days of parenteral nutrition, time to full enteral feeding, type of feeding: defined as proportion of human milk versus formula:
  - > 80% of human milk,
  - 80% < human milk > 50%
  - < 50% of human milk
- Drugs: spironolactone-hydrochlorothiazide, furosemide, fentanyl, ibuprofen, paracetamol
- Death during NICU stay

Standard feeding regimen of preterm newborn: after birth all enrolled preterm infant received either human milk or preterm formula within three days. Standardized intravenous parenteral nutrition (glucose, aminoacids, lipids minerals and electrolytes) was provided. Parenteral nutrition and volume and number of the feeds were decided by the attending physician following our nutrition protocol.

Sample collection:

At least 2 ml of urine collected within 48 hours of life (T0), at 21 +/- 3 days (T21) of life and at 36 weeks postmenstrual age (T36). Urine samples were collected using a non-invasive way (a cotton swab) and subsequently transferred into a provette.

All samples were stored in a freezer at -20°C until analysis.

Analysis of data

Descriptive analysis of clinical data

Descriptive analysis of the sample is reported as median and interquartile range (I-III quartile) for continuous variable and as absolute number and relative percentage for categorical variable.

The population has been stratified for the presence or not of IUGR and EUGR. The distribution of variables has been assessed initially for IUGR, then for EUGR by excluding IUGR from the second analysis. The existence of statistical significant difference between the two groups has been assessed by use of Kruskal-Wallis test for continuous variables a and chi-square for categorical variables. For categorical variables with a frequency lower than 5, p-value of chi-square test has been approximated using Monte-Carlo simulation. Significant level was set at $p < 0.05$.

Analysis have been conducted by means of software R [105] e RMS library [106]

Metabolomic analysis

The analysis was performed at the Mass Spectrometry and Metabolomics Laboratory of the University of Padua's and Women's and Children's Health Department (Città della Speranza Foundation, Pediatric Research Institute). Metabolic profiling was done in positive and negative ionization modes on an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, U.K.) coupled to a Quadrupole Time-of-Flight (QTof) Synapt G2 HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.). The UPLC-MS conditions were optimized in terms of the peak shape, reproducibility and retention times of the analytes. Chromatography was performed using an Acquity HSS T3 (1.7 µm, 2.1 x 100 mm) and an Acquity HILIC (1.7 µm, 2.1x 100 mm) column (Waters Corporation, Milford, U.S.A.), at 50°C and 40°C, respectively. For mass accuracy, a LockSpray interface was used with a 20 µg/L leucine enkephalin. Data were collected in continuous mode in a scanning range of 20-1200 m/z, with lockmass scans collected every 10 s, averaging over

3 scans for mass correction purposes. In the analytical sequences, quality controls (QC) were run with a different dilution factor.

Data were pre-processed using Progenesis software (Waters Corporation, Milford, U.S.A.). The ion intensities for each peak detected were normalized on the basis of the calibration models obtained for the QCs, then Probabilistic Quotient normalization was applied to remove dilution effects on the sample concentrations. The data tables generated were submitted to statistical analysis.

Statistical data analysis

Both univariate and multivariate statistical data analyses were run to characterize the two groups of newborn. First, an exploratory principal component analysis (PCA) was performed to identify outliers, trends and clusters of observations. Then a univariate analysis, based on the t-test with a false discovery rate (q-value<0.15), was used to compare the means of each variable in the two groups investigated. Projection to latent structures regression-discriminant analysis (PLS-DA) was applied to distinguish infants with IUGR and those without, based on their metabolomic profiles. The PLS-DA models were post-transformed to improve the model's interpretation. Stability selection was used to select the subset of relevant variables (100 subsamples were extracted by Monte-Carlo sampling with a prior probability of 0.70) [107]. N-fold cross-validations with different values of N (5,6,7) and permutation tests on the class response (1000 random permutations) were conducted to assess the robustness of the model and avoid over-fitting. The results of the multivariate data analysis were merged with those of the univariate data analysis to obtain a set of relevant variables, which was submitted to the annotation step. Receiver operating characteristic (ROC) curve analysis was used to describe the discriminatory power of the annotated variables.

Metabolite annotation

The main available metabolomic databases (Human Metabolome DataBase and METLIN) and our in-house database were searched to identify the relevant metabolites characterizing each group. In addition, urine samples with a higher ion intensity were injected in MS/MS mode to obtain more information on the ions' structure by studying the relative fragments. If the commercial standards were already available at our laboratory, the mass-to-charge ratio (m/z), retention time (Rt), and ion fragments in the urine were compared directly with the corresponding standard. These procedures enabled us to classify the selected features with different levels of confidence, as established by the Chemical Analysis Working Group of the Metabolomics Standards Initiative [108].

Variables annotated were investigated using over-representation pathway analysis. In this last step of the data analysis, we tested whether compounds involved in a particular pathway were enriched compared with random hits. We considered 80 pathways for Homo sapiens. The data analysis was

implemented with the R 3.1.2 platform (R Foundation for Statistical Computing), the PCA was performed with SIMCA 14, and the pathway analysis with MetaboAnalyst 3.0.

Ethical approval for the study was obtained from the local ethics committee (protocol number: 4374/AO/17) and informed consent was collected from parents.

RESULTS

Recruitment started in September 2016 and lasted until January 2019. All subjects were recruited in the NICU of Women's and Children's Health Department of University-Azienda Ospedaliera of Padova.

Clinical data descriptive analysis

160 infants were enrolled, among these 40 were diagnosed as IUGR. Among the AGA infants, 101 were followed-up until 36 weeks PMA and 51 developed EUGR. The main clinical feature of the two cohorts of subjects are presented in table 2 and table 3.

	Tot. (n=160) Median (IQ range) or Prevalence (absolute number)	AGA (n=120) Median (IQ range) or Prevalence (absolute number)	IUGR (n=40) Median (IQ range) or Prevalence (absolute number)	P level
Gestational age (days)	195.0 (185.0-207.0)	192.0 (170.0-203.2)	211.0 (196,0-216.2)	<0,001
Sex (males)	52% (83)	52% (62)	52% (21)	1
Ethnicity				0.13
Caucasian	90% (143)	87% (101)	98% (39)	
African	7% (11)	9% (11)	0% (0)	
Asiatic or Hispanic	3% (5)	3% (4)	2% (1)	
pPROM	21% (34)	26% (31)	8% (3)	0.02
Multiple pregnancy	32% (51)	32% (39)	30% (12)	0.845
Mode of delivery (CS)	89% (143)	87% (104)	98% (39)	0.143
Prenatal steroids				0.833
no	10% (15)	11% (12)	8% (3)	
< 7, >1 days before delivery	31% (47)	30% (34)	33% (13)	
< 1 days before delivery	27% (42)	29% (33)	23% (9)	
> 1 days before delivery	32% (49)	31% (45)	36% (14)	
Apgar score at 5 minutes	8 (7-8)	7 (7-8)	8 (7-8)	0.031
Surfactant	72% (116)	75% (90)	65% (26)	0.154
Surfactant (doses)	1.0 (1.0-2.0)	1.0 (0.7-2.0)	1.0 (0.0-2.0)	0.781
Mechanical ventilation (days)	1.0 (0.0-8.0)	2.0 (0.0-8.5)	1.0 (0.0-6.0)	0.025
BPD	35% (53)	34% (38)	41% (15)	0,548
BPD severity	1.0 (1.0-2.0)	1.0 (0.7-2.0)	1.0 (1.0-3.0)	0.352
Postnatal steroids	13% (18)	15% (16)	5% (2)	0,165
Postnatal steroids (n° courses)				0.412
0	88% (129)	86% (93)	95% (36)	
1	5% (8)	6% (7)	3% (1)	
2	6% (9)	7% (8)	3% (1)	
PDA				0.531
no	48% (77)	47% (56)	52% (21)	
Only medications	46% (73)	46% (55)	45% (18)	
Medications + Surgery	6% (10)	8% (9)	2% (1)	

	Tot. (n=160) Median (IQ range) or Prevalence (absolute number)	AGA (n=120) Median (IQ range) or Prevalence (absolute number)	IUGR (n=40) Median (IQ range) or Prevalence (absolute number)	P level
IVH (grade)	0 (0-0)	0 (0-0)	0 (0-0)	0.43
EOS				0.24
no	74% (117)	76% (90)	69% (27)	
suspected	23% (37)	21% (25)	31% (12)	
proven	3% (4)	3% (4)	0% (0)	
LOS				0.063
no	62% (99)	59% (71)	70% (28)	
suspected	12% (20)	11% (13)	18% (7)	
proven	26% (41)	30% (36)	12% (5)	
NEC				1
no	90% (142)	91% (107)	90% (35)	
suspected	4% (7)	4% (5)	5% (2)	
proven	5% (8)	5% (6)	5% (2)	
Laser therapy for ROP	18% (21)	21% (19)	8% (2)	0.164
Cholestasis	6% (8)	3% (3)	14% (5)	0.031
Maximum weight loss (%)	13.30 (9.78-16.61)	15.00 (11.99 -17.23)	11.11 (9.68-13.55)	0.31
Time to regain BW (days)	14 (12-17)	15 (12-19)	11 (9-13)	<0.001
Mean EUG (g/kg/day)*	10,66 (7,89-13,47)	10.26 (7.08-12.32)	12.63 (10.38-15.99)	0.003
Mean EUG (g/kg/day)**	12.18 (9.92-13.38)	11,92 (9,89-13,23)	13,08 (11,56-14,46)	0.003
Maximum azotaemia*	9.55 (7.10-13.47)	6.45 (5.22-8.15)	11.40 (8.30-14.80)	<0.001
Maximum creatinine*	77.5 (68.0-88.0)	76.0 (70.0-87.0)	78.0 (60.2-90.0)	0.943
Osteopenia at birth	10%(11)	11%(8)	9%(3)	1
Osteopenia at 36 weeks PMA	34%(25)	37%19()	29%(6)	0.59
Death	5% (8)	4% (5)	8% (3)	0,687

Table 2: Clinical data of the enrolled patients and comparison between IUGR and AGA premature newborn

IQ: interquartile; pPROM: premature rupture of membranes; CS: caesarean section; BPD: bronchopulmonary dysplasia; PDA: patent ductus arteriosus; IVH: intraventricular haemorrhage; EOS: early onset sepsis; LOS: late onset sepsis; NEC: necrotising enterocolitis; ROP: retinopathy of prematurity; EUG: extrauterine growth; BW: birth weight

*calculated from birth to 28 days of life

** calculated from birth to 36 weeks PMA

From table 2, it can be noted that there were no significant difference between IUGR and AGA preterm babies except that for higher gestational age, Apgar score at 5 minutes, cholestasis prevalence, mean extra-uterine growth rate during first month of life and from birth to 36 weeks PMA and for lower prevalence of pPROM, time to regain birth weight and maximum azotaemia level in the first month of life in IUGR group of subjects.

In table 3 data on neonates born AGA are reported. Infants who experienced EUGR were smaller at birth, received more surfactant doses, experienced more BPD, patent ductus arteriosus, intraventricular haemorrhage and late onset sepsis than non-EUGR infants. They took longer time to reach full enteral feeding and spent more days on parenteral nutrition. In addition they received lower

total energy in the first week and in the first month of life but higher amount of post-natal steroids, spironolactone-hydrochlorothiazide and fentanyl during NICU stay.

	Tot. (n=101) Median (IQ range) or Prevalence (absolute number)	Non-EUGR (n=50) Median (IQ range) or Prevalence (absolute number)	EUGR (n=51) Median (IQ range) or Prevalence (absolute number)	p level
Birth weight (g)	1085.0 (798.7-1253.7)	1139.5 (1003.7-1250.)	840.0 (692.5-1162.5)	<0.001
Birth weight percentile	54.00 (40.00-73.00)	68.50 (53.00-78.75)	41.00 (30.00-55.00)	<0.001
Gestational age (days)	192.0 (179.0-203.2)	194.0 (188.2-203.0)	185.0 (173.5-204.5)	0.078
Sex (males)	51% (52)	54% (27)	49% (25)	0.686
Ethnicity				0.354
Caucasian	90% (91)	86% (43)	94% (48)	
African	6% (6)	8% (4)	4% (2)	
Asiatic or Hispanic	4% (4)	6% (3)	2% (1)	
pPROM	25% (25)	26% (13)	24% (12)	0.827
Multiple pregnancy	30% (30)	34% (17)	25% (13)	0.371
Mode of delivery (CS)	87% (88)	90% (45)	84% (43)	0.654
Prenatal steroids				0.019
no	7% (7)	2% (1)	12% (6)	
< 7, >1 days before delivery	36% (34)	47% (22)	25% (12)	
< 1 days before delivery	28% (27)	32% (15)	25% (12)	
> 1 days before delivery	28% (27)	19% (9)	38% (18)	
Apgar score at 5 minutes	7 (7-8)	7 (7-8)	7 (7-8)	0.537
Surfactant	75% (76)	68% (34)	82%/ (42)	0.104
Surfactant (doses)	1.0 (0.7-2.0)	1.0 (0.0-1.0)	1.0 (1.0-2.0)	0.015
Mechanical ventilation (days)	2.0 (0.0-8.5)	1.0 (0.0-3.0)	8.0 (1.0-28)	0.001
BPD	38% (38)	24% (12)	51% (26)	0.011
BPD severity	1.0 (1.0-3.0)	1.0 (1.0-1.0)	2.0 (1.0-3.0)	0.005
Postnatal steroids	13% (18)	15% (16)	5% (2)	<0.001
Postnatal steroids (n° courses)				<0.001
0	85% (85)	98% (49)	72% (36)	
1	7% (7)	2% (1)	12% (6)	
2	8% (8)	0% (0)	16% (8)	
PDA				0.002
no	43% (43)	58% (29)	27% (14)	
Only medications	49% (49)	40% (20)	57% (28)	
Medications + Surgery	9% (9)	2% (1)	16% (8)	
IVH (grade)	0 (0-0)	0 (0-0)	0 (0-0.5)	0.018
EOS				0.521
no	72% (72)	76% (38)	68% (34)	
suspected	24% (24)	22% (11)	26% (13)	
proven	4% (4)	2% (1)	6% (3)	
LOS				0.023
no	54% (55)	68% (34)	41% (21)	
suspected	13% (13)	10% (5)	16% (8)	
proven	33% (33)	22% (11)	43% (22)	

	Tot. (n=101) Median (IQ range) or Prevalence (absolute number)	Non-EUGR (n=50) Median (IQ range) or Prevalence (absolute number)	EUGR (n=51) Median (IQ range) or Prevalence (absolute number)	p level
NEC				0.866
no	90% (89)	88% (44)	92% (45)	
suspected	4% (4)	4% (2)	4% (2)	
proven	6% (6)	8% (4)	4% (2)	
Laser therapy for ROP	22% (19)	12% (5)	30% (14)	0.068
Cholestasis	3% (3)	4% (2)	2% (1)	0.611
Maximum weight loss (%)	15.00 (11.76- 17.24)	15.50 (12.68-17.32)	13.83 (9.80-16.99)	0.682
Time to regain BW (days)	15 (12-19)	15 (13-18)	14 (12-19)	0.302
Mean EUG (g/kg/day)*	10.26 (7.08-12.32)	8.05 (4.49- 12.01)	10.57 (8.70-12.44)	0.07
Maximum azotaemia*	11.40 (8.30-14.80)	11.05 (8.65-14.05)	11.95 (8.05-15.25)	0.68
Maximum creatinine*	76 (70-87)	77 (71-84)	74 (69-87)	0.816
Osteopenia at birth	12%(8)	17%(6)	7%(2)	0.27
Osteopenia at 36 weeks PMA	37%(19)	41%(11)	32%(8)	0.549
Mean energy intake 1st week (kcal/kg/day)	77.9 (69.8-91.7)	84.4 (76,2-95.1)	69.85 (64.0-77.8)	<0.001
Mean energy intake* (kcal/kg/day)	107.2 (96.1-120.3)	111.6 (103.4-127.4)	102.7 (90.5-109.3)	0.001
Days of PN	16.0 (11.0-25.2)	15.0 (10.0-22.0)	21.0 (14.5-32.0)	0.005
Time to FEF achievement (days)	17.5 (13.0-27.0)	16.0 (12.0-24.0)	24.0 (17.0-33.5)	<0.001
Type of FEF				0.747
HM >80%	39% (39)	38% (19)	39% (20)	
HM > 50%, <80%	17% (17)	14% (7)	20% (10)	
HM < 50%	45% (45)	48% (24)	41% (21)	
Spironolactone-hydrochlorothiazide administ.	31% (24)	12%(5)	51% (19)	0.001
Furosemide administ.	37% (29)	27%(11)	48% (18)	0.062
Fentanyl administr.	34% (26)	20%(8)	49% (18)	0.007
Days on fentanyl	0 (0-2)	0 (0-0)	0 (0-9)	0.002
Ibuprofen	69% (38)	70%(14)	69%(24)	1
Paracetamol	60% (33)	5%(11)	63% (22)	0.781
Death	1% (1)	2% (1)	0% (0)	0,488

Table 3: Clinical data of the patients followed-up till 36 weeks PMA and comparison between EUGR and non-EUGR premature newborn

IQ: interquartile; pPROM: premature rupture of membranes; CS: caesarean section; BPD: bronchopulmonary dysplasia; PDA: patent ductus arteriosus; IVH: intraventricular haemorrhage; EOS: early onset sepsis; LOS: late onset sepsis; NEC: necrotising enterocolitis; ROP: retinopathy of prematurity; EUG: extrauterine growth; BW: birth weight; PN: parenteral nutrition; FEF: full enteral feeding.

*calculated from birth to 28 days of life

** calculated from birth to 36 weeks PMA

Metabolomic analysis

In order to minimize the confounding effects of all therapies and procedures performed in these patients during the first days of life, only urine samples collected within the first 48 hours of life were considered.

Among enrolled neonates, urine samples were adequately collected within the second day of life. in 83. Fifteen of 83 presented with a diagnosis of IUGR and were matched with 19 AGA infants. The two groups were comparable as regards gestational age, sex, pPROM history, multiple pregnancy, type of delivery, time of administration of betamethasone during pregnancy, Apgar score at 5 minutes, surfactant administration, presence of patent ductus arteriosus or intraventricular haemorrhage and early sepsis, as shown in table 4.

	ANOVA test	Kruskal-Wallis test	Fisher's exact test
Birth weight (g)	<0.001	<0.001	
%ile of birth weight	<0.001	<0.001	
Gestational age (days)	0.353	0.296	
Sex			0.075
pPROM			0.113
Multiple pregnancy			0.555
Mode of delivery			0.492
Prenatal steroids			0.262
Apgar score at 5 minutes	0.465	0.423	
Surfactant			0.080
PDA			1
EOS			1
IVH			0.187
NEC			1

Table 4: Comparison of clinical data between the group of 15 infants presenting with IUGR and 19 not presenting IUGR, selected for metabolomic analysis.

Among 120 neonates, who were born adequate for gestational age, 101 were followed up till 36 weeks PMA and in 34 urine samples were adequately collected at all the three time-points. Nine developed EUGR and were matched with 10 controls who did not present EUGR. The two groups were comparable as regards gestational age, sex, pPROM history, multiple pregnancy, type of delivery, time of administration of betamethasone during pregnancy, Apgar score at 5 minutes, surfactant administration, presence of patent ductus arteriosus or intraventricular haemorrhage, early and late onset sepsis and BPD, as shown in table 5.

	ANOVA test	Kruskal-Wallis test	Fisher's exact test
Birh weight	0.459	0.653	
%ile of birth weight	0.08	0.19	
Gestational age (days)	0.913	0.713	

Sex			1
pPROM			0.582
Multiple pregnancy			0.474
Mode of delivery			1
Prenatal steroids			0.417
Apgar score at 5 minutes	0.463	0.492	
Surfactant			1
BPD			1
Postnatal steroids			0.58
PDA			1
EOS			1
LOS			0.65
NEC			1

Table 5: Comparison of clinical data between the group of 9 infants presenting with EUGR and 10 not presenting EUGR, selected for metabolomic analysis.

Metabolomic investigations through positive ionization mode gave rise to one data set that included 1080 RT_mass variables.

Based on Hotelling's T2 test and the DModX test, PCA did not reveal any outliers in either group (significance level alfa=0.05). We obtained a PLS-DA with 2 latent variables for the POS data set, R2=0.67 (p-value<0.01), Q2-fold=0.34 (p-value <0.01). Figure 1 shows the score scatter plots and the clear clustering of samples belonging to the two different groups of subjects.

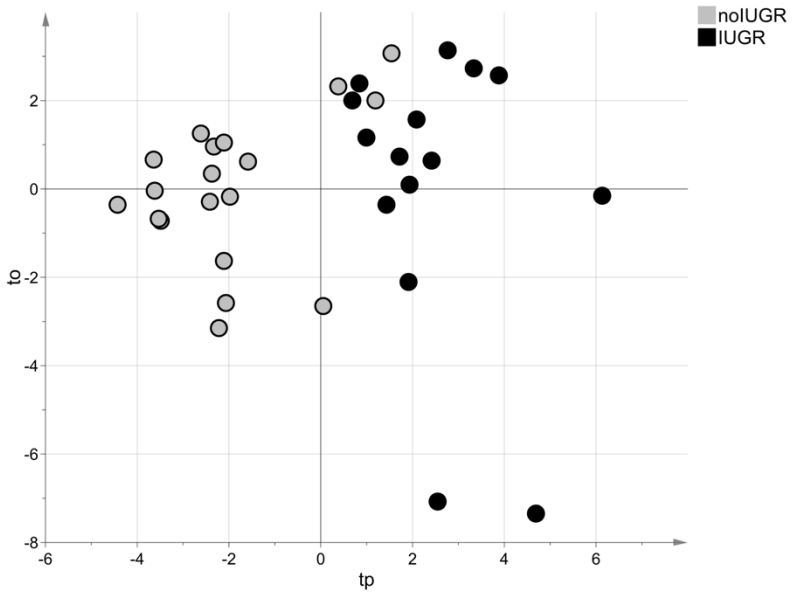

Figure 1: Score scatter plot of the ptPLS2-DA model for IUGR (black circles) versus non-IUGR group (grey circles) in the positive positive data set.

All the 34 variables earmarked by the stability selection process were included in the set of relevant variables selected by the t-test with a false discovery rate. (Table 6)

ID	Compound	Neutral_ mass	m/z	Rt	model	CIAUC_95%	type
X1041	4.75_440.1786n	440,1786105	479,1597344	4,74953	MV	0.570482183314551 - 0.910219571071414	AGA > IUGR
X108	4.42_377.1470m/z		377,1470315	4,41848	UNI	0.745343501412559 - 0.995007375780423	IUGR > AGA
X1191	4.11_136.0528n	136,0527982	119,0501061	4,11247	MV	0.588492919722191 - 0.927296553962019	AGA > IUGR
X1196	5.40_463.1992m/z		463,1992104	5,40103	MV	0.572607462039292 - 0.92914692392562	AGA > IUGR
X1199	5.25_318.1319n	318,131945	341,1211657	5,24633	MV	0.575102154571383 - 0.933669775253179	IUGR > AGA
X1214	4.82_298.1433n	298,14327	345,1560707	4,81777	MV	0.664605214101416 - 0.977500049056478	IUGR > AGA
X1254	4.75_420.1637n	420,1637334	421,1541676	4,75493	MV	0.621882940877796 - 0.957064427543257	AGA > IUGR
X1531	5.38_615.2230m/z		615,2229931	5,38483	MV	0.563765089364383 - 0.916936665021581	IUGR > AGA
X1647	5.22_204.0902n	204,0902145	205,1216045	5,21932	UNI	0.664770224204602 - 0.963299951233995	AGA > IUGR
X1894	3.68_337.1643n	337,1643248	292,1661248	3,67535	MV	0.608963811820051 - 0.948930925022054	IUGR > AGA
X2012	5.21_377.2162n	377,2161809	416,180119	5,20850	MV	0.516294001827751 - 0.901249857821372	IUGR > AGA
X212	5.22_466.1497n	466,1497109	423,1694609	5,22472	MV	0.689033392149622 - 0.98114204644687	IUGR > AGA
X229	4.00_203.0818m/z		203,0818182	3,99558	MV	0.526467674105055 - 0.912128817123016	IUGR > AGA
X2330	5.54_377.2776n	377,2775513	360,274263	5,53953	MV	0.585861635846213 - 0.922910293978348	IUGR > AGA
X239	1.01_230.1143n	230,1142823	202,1188823	1,00860	MV	0.529232333912415 - 0.895329069596357	AGA > IUGR
X2570	6.67_371.2590m/z		371,2590004	6,66775	MV	0.672395035103945 - 0.969710228053949	AGA > IUGR
X263	2.74_181.0724m/z		181,0724285	2,73965	MV	0.528767274745282 - 0.888776584903841	IUGR > AGA
X2646	4.78_302.1267n	302,1267227	349,1661394	4,77655	MV	0.57385180458275 - 0.913867493662864	AGA > IUGR
X298	4.00_237.1117n	237,1117339	221,0924839	4,00098	MV	0.548838889098098 - 0.917827777568569	IUGR > AGA
X301	0.77_138.0432n	138,0431803	139,0505336	0,77273	MV	0.599019170605173 - 0.944840478517634	IUGR > AGA
X31	4.48_394.1678n	394,1678259	395,1751023	4,48133	MV	0.577040847269881 - 0.93173108255468	IUGR > AGA
X318	5.75_330.2181n	330,2180995	331,226674	5,75368	UNI	0.542065337558829 - 0.896531153669241	AGA > IUGR
X360	0.59_306.0628m/z		306,0627904	0,59100	MV	0.518869756305272 - 0.898674103343851	IUGR > AGA
X37	3.98_202.0747n	202,0746833	266,1141752	3,97937	MV	0.50446462634201 - 0.906061689447464	IUGR > AGA
X372	0.57_234.0148n	234,0147553	257,003976	0,57478	UNI	0.564226928729089 - 0.909457281797227	AGA > IUGR
X403	0.89_154.0501n	154,0501389	137,0460652	0,89162	MV	0.559951448291915 - 0.899697674515103	AGA > IUGR
X462	0.68_225.0874m/z		225,0873877	0,67747	UNI	0.60237889217608 - 0.927445669227429	AGA > IUGR
X473	3.99_355.1485n	355,1484532	296,1357532	3,99018	MV	0.591650318602061 - 0.938174242801448	IUGR > AGA
X550	0.54_225.8975n	225,897521	180,899321	0,53897	UNI	0.52497867790018 - 0.892565181748943	AGA > IUGR
X697	3.68_129.0793n	129,0793454	130,0810623	3,68077	MV	0.561166683981311 - 0.905499982685356	IUGR > AGA
X7	4.11_191.0586n	191,0585753	192,0480705	4,11247	UNI	0.588492919722191 - 0.927296553962019	AGA > IUGR
X904	4.11_349.1101n	349,1101322	350,1174229	4,11247	MV	0.588492919722191 - 0.927296553962019	AGA > IUGR
X966	5.16_423.1697m/z		423,1696686	5,15987	MV	0.670899639583198 - 0.964188079715048	IUGR > AGA
X981	6.80_592.4137n	592,4137072	656,4529832	6,79543	MV	0.551239245704628 - 0.90139233324274	IUGR > AGA

Table 6: List of relevant variables selected by the t-test with a false discovery rate.

We were able to annotate 13 relevant variables, all with confidence level 2 ("putatively annotated compounds") (Table 7).

varID	Compound	HMDB ID	Adducts	Formula	Annotation	Description	m/z	Rt (min)	IUGR	NO IUGR	class
X229	4.00_203.0818m/z	HMDB04185	M+H-CH2O2	C12H12N2O4	Level 2	5-Hydroxyindoleacetylglycine	203,0818	4,0	x		Glycine derivative
X1531	5.38_615.2230m/z	HMDB01081	M+H-C2H4O2	C25H42N2O19	Level 2	(N-acetylneuraminosyl(alpha2-6)lactosamine)	615,2230	5,4	x		Neuraminic acids
X1196	5.40_463.1992m/z	HMDB00313	M+H+Glucuronide	C18H22O3	Level 2	16b-Hydroxyestrone	463,1992	5,4		x	Estrogens and derivatives
X318	5.75_330.2181n	HMDB00920	M+H-2H2O, M+H	C21H30O3	Level 2	11a-Hydroxyprogesterone	331,2267	5,8		x	Gluco/mineralocorticoids, progestogins and derivatives
X1191	4.11_136.0528n	HMDB00209	M+H-CH2O2, M+H+NH4HCO2, M+FA+H, M+H-H2O	C8H8O2	Level 2	Benzeneacetic acid	119,0501	4,1		x	Benzene and substituted derivatives
X2570	6.67_371.2590m/z	HMDB00391	M+H-2H2O	C24H38O5	Level 2	7-ketodeoxycholic acid	371,2590	6,7		x	Bile acids, alcohols and derivatives
X697	4.11_191.0586n	HMDB00763	M+H-H2O, M+H, M+H-CH2O2	C10H9NO3	Level 2	5-Hydroxyindoleacetic acid	192,0481	4,1		x	Indole-3-acetic acid derivatives
X263	3.68_129.0793n	HMDB04226	M+H-CH2O2, M+H	C6H11NO2	Level 2	N4-Acetylaminobutanal	130,0811	3,7	x		Alpha-hydrogen aldehydes
X301	0.77_138.0432n	HMDB02730	M+H-H2O, M+H	C6H6N2O2	Level 2	Nicotinamide N-oxide	139,0505	0,8	x		Nicotinamides
X360	0.59_306.0628m/z	HMDB00229	M+H-COH	C11H15N2O8P	Level 2	Nicotinamide ribotide	306,0628	0,6	x		Nicotinamide nucleotides
X108	4.42_377.1470m/z	HMDB00244	M+H	C17H20N4O6	Level 2	Riboflavin	377,1470	4,4	x		Vitamin B2
X904	4.11_349.1101n	HMDB14559	M+H-NH3, M+K, M+H	C16H19N3O4S	Level 2	Ampicillina	350,1174	4,1		x	Drugs
X263	2.74_181.0724m/z	HMDB04400	M+H-H2O	C7H10N4O3	Level 2	5-Acetylamino-6-amino-3-methyluracil	181,0724	2,7	x		aromatic amide

Table 7: List of putative biomarkers of IUGR derived from annotation process.

The variables annotated were submitted to pathway over-representation analysis. Six pathways were found significantly perturbed: Figure 2 shows the impact of the perturbed pathways and their negative log p-value.

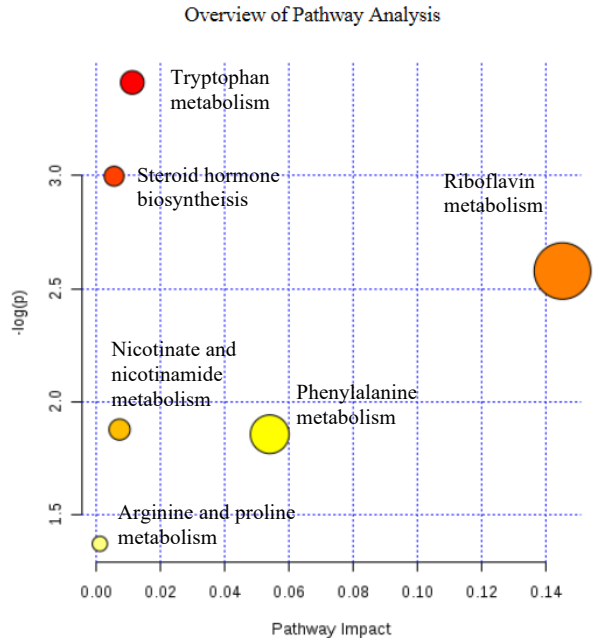

Figure 2: Over-representation pathway analysis: impact of each perturbed pathway versus its negative log p-value (-log(p)).

In a second step, we compared the urine samples of 9 infants with EUGR and 10 controls, collected at T0, T 21 and T 36. No models were able to discriminate between the two groups.

DISCUSSION

Growth and nutrition in the very early stages of life are topics of great interest, not only to neonatologists, but to all physicians. There is indeed growing evidence that inappropriate nourishment during this key period in life might impact on lifelong health of the individual.

IUGR and EUGR are two paradigmatic conditions, in which an inadequate intra- and extrauterine environment respectively, can hamper infant development and predispose to certain major diseases later in life, including metabolic syndrome, obesity, coronary heart disease, hypertension, dyslipidemia and type 2 diabetes, and chronic kidney diseases. Unfortunately, we still lack reliable biomarker capable to assess the suitability of growth and nutrition in preterm babies, so that these consequence can be prevented.

The first aim of our study was to compare the urinary metabolic profile between preterm neonates affected and not affected by IUGR at birth.

The analysis of clinical data of the whole group of enrolled subjects did not show any difference in the incidence of the main complications of prematurity, such as need for surfactant therapy, BPD, intraventricular haemorrhage, sepsis, NEC, retinopathy between IUGR and AGA infants. This might be apparently in contrast with data in the literature which show poorer prognosis in IUGR infants. The effects of prenatal growth restriction condition are probably somehow "attenuated" by significant higher gestational age of the subjects diagnosed as IUGR.

The comparison through UPLC-MS of early urinary metabolome between a group of IUGR newborns and matched controls revealed an evident and interesting clustering of subjects (IUGR versus AGA infants).

Annotating the variables that most effectively discriminate between the two groups enabled us to identify some putative metabolic derangements that might be involved in this condition.

Within the metabolic pathways of tryptophan, two variables, that were annotated as 5-Hydroxyindoleacetylglycine and 5-Hydroxyindoleacetic acid, were able to discriminate between IUGR and AGA. Both these metabolites belong to the steps of conversion of tryptophan into serotonin and of serotonin into its final metabolites (Figure 3). Previous studies have shown increased tryptophan plasma concentrations in IUGR [48,109]. Tryptophan is an essential amino acid that can only be obtained through the diet, it is actively transported to the fetus via the placenta where it plays an important role in protein synthesis for growth and development. It has been highlighted that tryptophan is also metabolized in the placenta to produce functionally important metabolites, among the other serotonin. Serotonin is an important neurotransmitter, that plays a number of key processes in neurodevelopment, including cell proliferation, neuronal differentiation and migration and synaptogenesis. As known, intrauterine growth retardation is associated with increased risks for

permanent neurological disabilities. A study on baboons has shown reduced expression of serotonergic proteins and mRNAs, as well as fewer serotonergic neurons in IUGR animals [110]. In another research growth retarded rat fetuses exhibited marked changes of the monoamine metabolism in the brain: basal levels of serotonin and its main metabolites were low while an inappropriate acceleration of the serotonin synthesis rate took place during hypoxia [111]. These findings, although preliminary, suggest to focusing future investigations on the association between IUGR and disrupted placental tryptophan and serotonin metabolic pathway [112].

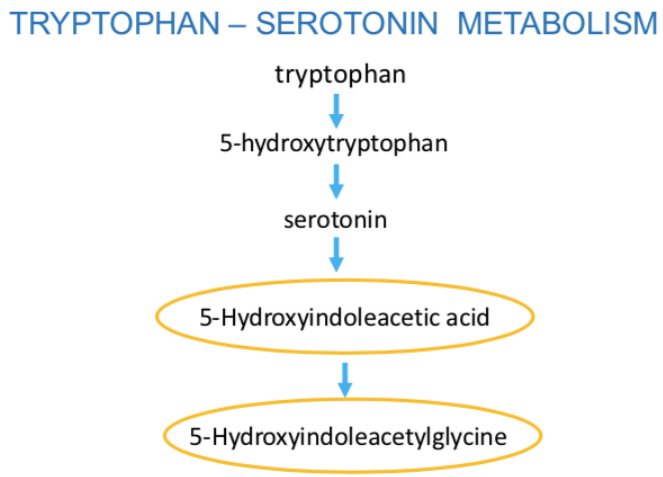

Figure 3: Tryptophan-Serotonin metabolic pathway

In addition, we found increased level of riboflavin in the IUGR group. Riboflavin or vitamin B2 is a water-soluble essential micronutrient with a key role in maintaining human health. Like the other B vitamins, it supports energy production by acting in fats, carbohydrates, and proteins metabolism. Vitamin B2 is also required for red blood cell formation and respiration, antibody production, and for regulating of human growth and reproduction [113]. With the aim to prevent vitamin deficiency, parenteral nutrition multivitamins, as well as preterm infant formula for VLBW infants, are both supplemented with riboflavin (vitamin B_2). In particular, parenteral nutrition provides great daily riboflavin per weight and plasmatic concentrations are often elevated in VLBW infants during early postnatal life. In a study by Porcelli et al., serial plasma and urine riboflavin concentrations measurements were performed in VLBW infants. After short period (24 hours) of riboflavin free nutrition, both plasma and urine riboflavin decreased to normal values, suggesting that a lower daily intake would maintain plasma riboflavin close to normal [114]. Our finding of higher levels of vitaminB2 in urine of IUGR than controls, suggests an excessive intake or reduced elimination in the former. The serum metabolomic profiling of these subjects would probably clarify this issue. Even if there is no evidence for riboflavin toxicity in humans due to excessive intakes, because of its lower water solubility and because what exceeds the absorption is usually excreted via the kidneys, no data

are available in preterm infants. Hence, a more personalized management of nutritional intake in IUGR population should possibly be achieved.

Also, steroid hormone biosynthesis perturbation emerged from the metabolomic profile comparison between IUGR and non-IUGR subjects. In particular two metabolites, 11alfa-Hydroxyprogesterone and 16b-Hydroxyestrone, belonging to progesterone and estrogens metabolism respectively, were lower in IUGR than controls. There are increasing concerns that an altered endocrine environment may impair the growth of the fetus and there is evidence of reduction in circulating levels of estrogen and progesterone in women with preeclampsia, a complication of pregnancy that is frequently associated to IUGR [115].

Noteworthy, 11alpha-Hydroxyprogesterone has been reported to be a strong inhibitor of 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD) in animal models, an enzyme that catalyzes the conversion of cortisol to cortisone (Figure 4). Cortisol, because of its molecular similarity to aldosterone, is able to bind to the mineralcorticoid receptor, with a similar affinity; since levels of circulating cortisol are much higher than aldosterone, in order to prevent over-stimulation of the mineralocorticoid receptor, 11β-HSD converts the biologically active cortisol to the inactive cortisone, which can no longer bind the mineralocorticoid receptor [116]. It is well known that in IUGR infants the hypothalamo-pituitary-adrenal axis is activated as an adaptive response to stress. Analyses on cord blood samples have revealed higher cortisol levels in cases of IUGR [24].

Exposure of the fetus to excess of glucocorticoids has been demonstrated to reduce fetal growth.

11β-HSD type 2 is highly expressed within the placenta at the maternal-fetal interface, thus limiting the passage of glucocorticoids to the fetus. It has been proposed that down-regulation of 11β-HSD2 could be related to fetal growth restriction [117].

The decrease in urinary 11alpha-Hydroxyprogesterone might be due to high sequestration by the enzyme or represent a compensatory mechanism to reduce excessive levels of circulating cortisol.

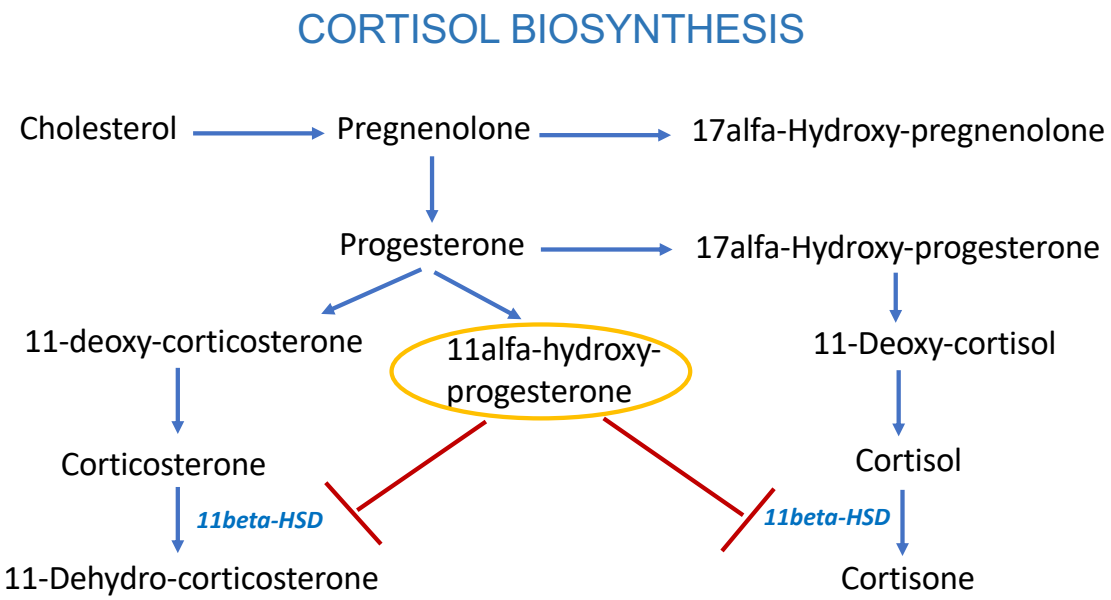

Figure 4: Cortisol biosynthesis

In addition, our analysis detected some perturbations in nicotinamide metabolism as demonstrated by higher levels of Nicotinamide Ribotide (NMN) and Nicotinamide N-oxide in IUGR. Particularly Nicotinamide Ribotide is an important precursor for NAD biosynthesis through the reaction NMN + ATP <=> Nicotinamide adenine dinucleotide (NAD) + PPi, catalysed by the enzyme nicotinamide mononucleotide adenyltransferase [113]. NAD is a molecule that plays a key role as a cofactor in cellular redox reactions. IUGR is well known to be related to placental hypoxia and consequent oxidative/reductive stress, that can contribute to NADH/NAD^+ redox imbalance [118]

A significant difference in excretion of bile acids (putative metabolite: 7-Ketodeoxycholic acid) was also detected. Bile acids are steroid acids found predominantly in bile. The distinction between different bile acids is minimal, depends only on presence or absence of hydroxyl groups in different positions. They modulate bile flow and lipid secretion, are essential for the gut absorption of fats and vitamins and have been implicated in the regulation of many enzymes involved in cholesterol homeostasis. Through the enterohepatic circuit, bile acids recirculate from liver, bile ducts, small intestine to portal vein [113]. In a study conducted by Kimura et al., large amounts of unusual bile acids, such as unsaturated ketonic and 7beta-hydroxylated bile acids, were detected during infancy, especially during the first month of life. The authors assumed that bile acid synthesis and metabolism in the liver of developing infants are significantly different from that occurring in adults [119]. Urinary concentrations of total bile acids in preterm infants have been found to be even higher than term neonates, probably because of an overproduction, or more likely to an impaired enterohepatic circuit [120]. The impact of abnormal bile acids metabolism in IUGR infants have never been

investigated. Nonetheless, on the basis of our results we believe it deserves further research, especially in relation to enteral feeding intolerance, a typical complication of IUGR.

Other three putative compounds were distributed in different way among the two groups and were annotated as Benzeneacetic acid, N4-Acetylaminobutanal and Sialyl-N-acetyllactosamines.

Benzeneacetic acid or phenyl acetate, a product of phenylalanine metabolism, was lower in IUGR. It has been demonstrated that excess of phenylalanine in the body can be disposed of through a transamination process leading to the production of phenylpyruvate. The phenylpyruvate can be further metabolized into a number of products including phenyl acetate (Figure 5). Phenylalanine blood levels were found to be higher in IUGR than non-IUGR in some previous metabolomic studies [57, 58, 63, 64] and lower in other [60]. Even if we didn't detect significant difference in phenylalanine levels we can speculate that in IUGR transamination reaction of phenylalanine might be impaired.

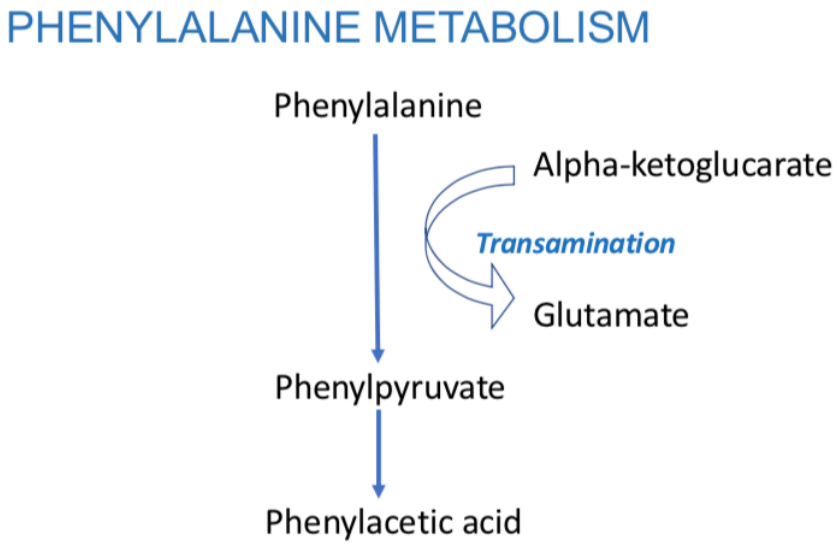

Figure 5: Phenylalanine metabolic pathway

N4-Acetylaminobutanal is an intermediate of the urea cycle and metabolism of amino groups, the product of the conversion of N-Acetylputrescine by enzyme monoamine oxidase.N-Acetylputrescine is commonly excreted in normal human urine, is produced by the breakdown of amino acids in living and dead organisms and is toxic in large doses. Both N4-Acetylaminobutanal and Benzeneacetic are also known microbial products, thus suggesting a possible perturbation in the microbiota of these newborn. A comparison between microbiota in IUGR neonates and controls could provide new insights on this field [113].

3-Sialyl-N-acetyllactosamine and 6-Sialyl-N-acetyllactosamine are oligosaccharides found in human milk, that have been proved to inhibit enteric pathogens in vitro and in vivo. They are widely distributed among tissues and are involved in biological biosynthesis and degradation of the body glycoproteins, glycolipids, and glycans. 3-Sialyl-N-acetyllactosamine is one of the predominant oligosaccharides in human urine as a free form [121]

Lastly, the urinary metabolome analysis of our group of subjects evidenced the presence of drugs and drugs metabolites. In particular ampicilline was lower in IUGR infants, while 5-Acetylamino-6-amino-3-methyluracil (AAMU), one of caffeine major metabolites [113], was higher. This could reflect distinct metabolism processes or elimination mechanisms of drugs in IUGR. Once again metabolomics proves to be a useful tool to detect interpersonal differences in physiological and pathological conditions. Ideally, in the future, it will allow to set up individualized care in terms of nutritional support and pharmacological therapies titration.

For what concerns newborns who experience EUGR, even if we did not find any variables able to discriminate between the two groups, some remarks could be made. First of all, the absence of differences in metabolites profiles at birth leads us to speculate that there is no predisposition to development of post-natal growth restriction. This condition is probably multifactorial, secondary to the many variables that can complicate preterm newborn course, and that may impact on different metabolic pathways. As expected, from the analysis of the whole sample of infants born AGA, it emerged that those who experienced EUGR were smaller at birth and had suffered more from BPD, patent ductus arteriosus, intraventricular haemorrhage and late onset sepsis. They took longer time to reach full enteral feeding and spent more days on parenteral nutrition. In addition, they received higher amount of post-natal steroids, spironolactone-hydrochlorothiazide and fentanyl during NICU stay. Our results further confirm the complexity and multicausality of EUGR pathogenesis.

A strength of our work is the comparability of the groups in terms of gestational age, pregnancy history and main post-natal comorbidities, thus allowing us to state that the observed differences are due to the condition of interest, namely IUGR.

A limit of our study is the small sample size and the lack of validation. Nevertheless, further investigations are ongoing including metabolic profiling of urine in negative ionization mode and a targeted metabolomic analysis on plasm samples collected from the same recruited neonates. Next steps will also comprise the recruitment of a validation population to confirm the results.

It would be interesting in the future to explore the metabolomic profile of the premature infants also in relation to enteral feeding tolerance and type of feeding (human milk versus formula), possibly with the integration of emerging evidence from gut microbioma research.

CONCLUSIONS

IUGR and EUGR are two conditions in which the fetus/newborn is unable to achieve its genetically determined potential size with potentially severe short- and long-term health consequences. If on one hand, urine metabolomics profiling of our cohort has failed to detect any metabolite capable of discriminate between EUGR and non-EUGR infants, on the other hand, it has showed a clear clustering between IUGR and non-IUGR. Notably, we found derangements in metabolism of tryptophan-serotonin and biosynthesis of steroid hormones that might deserve more thorough investigations in the future.

Metabolomics proves to be a useful tool to explore biochemical pathways that regulate physiological and pathological processes in the newborn, potentially providing novel putative biomarkers able to guide nutritional management and growth monitoring in the NICU setting. Studying individual metabolomic profiles may indeed enable us to discriminate “good growth” from “bad growth”, the latter pointing the metabolism towards an obesogenic profile, especially in newborns who have experienced IUGR. Metabolomics could also help to detect otherwise unknown nutritional deficiencies in preterm infants lacking the third-trimester accrual of several macro- (proteins, lipids) and micro-nutrients (calcium, iron, phosphate). Obtaining such information with a metabolomic approach could support neonatologists to further improve the quality and appropriateness of newborn infants’ growth.

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