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Determinants of renal function in preterm newborns

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Summary

Background: Nephrogenesis is active until 36 weeks' gestation and may be negatively affected by preterm birth. Moreover, potentially kidney-detrimental events usually occur during the first phases of post-natal life of preterm infants. Little is known about potential endogenous biomarkers other than serum creatinine (sCr), such as serum cystatine C (CysC) or beta-trace protein (BTP) in the assessment of renal function in preterm newborns.

Aims: The main aims of the study were to evaluate CysC and BTP levels in preterm newborns born at a gestational age (GA) <32 weeks, and to assess the impact of pre-natal and post-natal potentially kidney-detrimental factors on kidney function, evaluated according to different estimated glomerular filtration rate (eGFR) formulas.

Subjects and methods: newborns with GA<32 weeks were enrolled in our NICU in the period September 2015-December 2017. Blood samples were obtained on the 3rd day of life (T0) and when they reached a GA of 36 weeks (T36), and sCr, CysC, BTP and urea levels were measured. eGFR was calculated according to 9 existing formulas (Schwartz 2009, Brion, Schwartz 2012, Zappitelli, Filler, Dorum, Trieber, Zappitelli-combined) at T36. For each subject, pre-natal and post-natal kidney injury risk scores were calculated by the number of potentially pre-natal (intrauterine growth restriction, maternal pre-eclampsia, use of nephrotoxic medications during pregnancy, positive family history for arterial hypertension) and post-natal (born small for gestational age, need for invasive and non-invasive ventilation, bronchopulmonary dysplasia, sepsis, use of nephrotoxic antimicrobic agents, ibuprofen or surgery for patent ductus arteriosus, necrotizing enterocolitis, intra-ventricular hemorrhage) kidney-detrimental factors.

Results: We collected data from 71 newborns at T0 (31 with GA≤28 weeks) and from 53 subjects at T36 (25 with GA≤28 weeks). At T0, newborns with GA≤28 weeks had higher sCr levels than those with GA>28 weeks (p=0.016). At T0, sCr was negatively correlated with GA (R= -0.315, p=0.009), whereas both CysC and BTP were not influenced by GA. At T36, newborns with GA≤28 weeks had lower sCr, BTP and higher urea levels than those with GA>28 weeks (p=0.007, p=0.005 and p=0.029, respectively). At T36 eGFR values calculated by the four formulas using only CysC were not different in newborns with GA≤28 weeks and >28 weeks. Conversely, eGFR values estimated by other formulas were higher in subjects born at a lower GA. The pre-natal score did not correlate with eGFR calculated with any formulas. We found a direct correlation between the post-natal score and eGFR estimated according to the sCr-based formulas by Schwartz 2009 (R=0.345, p=0.027) and Brion (R=0.312, p=0.044). However, these correlations did not persist when adjusted for urea levels at T36 and GA. Conversely, no correlations were found between the scores and eGFR according to the other formulas.

Conclusions: eGFR formulas using CysC are not influenced by GA. Post-natal score shows a direct correlation with eGFR according to sCr-based formulas, which does not persist after adjustment for GA and urea levels, suggesting that the underlying confounder may be the nutritional status of preterm newborns. Indeed, more premature subjects receive a more aggressive nutritional regimen, as suggested by the higher urea levels found in newborn with GA≤28 weeks at T36.

Riassunto

Razionale: La nefrogenesi si completa a 36 settimane di gestazione e può essere negativamente influenzata dalla nascita pretermine. Inoltre, nelle prime fasi della vita dei neonati pretermine spesso si verificano eventi potenzialmente nefrolesivi. Ancora scarse sono le conoscenze sull'impiego di marcatori endogeni alternativi alla creatinine sierica (sCr), quali cystatine C (CysC) o beta-trace protein (BTP), nella valutazione della funzione renale dei nati pretermine.

Obiettivi: Obiettivi principali dello studio sono stati valutare i livelli di CysC e BTP in neonati pretermine con età gestazionale (GA) <32 settimane, e verificare l'impatto di fattori pre- e post-natali potenzialmente nefrolesivi sulla funzione renale, valutata come estimated glomerular filtration rate (eGFR) calcolato con formule diverse.

Soggetti e metodi: nel periodo Settembre 2015-Dicembre 2017 sono stati arruolati presso la nostra Unità di Terapia Intensiva Neonatale i nati di GA <32 settimane. Campioni ematici sono stati raccolti in terza giornata di vita (T0) e all'età di 36 settimane (T36) per il dosaggio di sCr, CysC, BTP e urea. L'eGFR è stato calcolato in base a 9 formule esistenti (Schwartz 2009, Brion, Schwartz 2012, Zappitelli, Filler, Dorum, Trieber, Zappitelli-combined) al T36. Per ogni soggetto sono stati calcolati punteggi di rischio di danno renale pre- e post-natale sulla base del numero di fattori potenzialmente nefrolesivi prenatali (ritardo di crescita intrauterina, pre-eclampsia materna, uso di farmaci nefrotossici in gravidanza, familiarità per ipertensione arteriosa) e post-natali (nato piccolo per l'età gestazionale, necessità di ventilazione invasiva e non-invasiva, broncodisplasia, sepsi, uso di antibiotici nefrotossici, uso di ibuprofene o intervento cardiochirurgico per dotto arterioso pervio, enterocolite necrotizzante, emorragia intra-ventricolare).

Risultati: Abbiamo raccolto i dati di 71 neonati a T0 (31 con GA ≤28 settimane) e di 53 a T36 (25 con GA ≤28 settimane). A T0, i neonati con GA ≤28 settimane avevano livelli di sCr più elevati dei nati sopra le 28 settimane (p=0.016). A T0, sCr correlava inversamente con la GA (R= -0.315, p=0.009), mentre CysC e BTP non erano influenzate dalla GA. A T36, i neonati con GA ≤28 settimane avevano livelli più bassi di sCr e BTP e più alti di urea (p=0.007, p=0.005 and p=0.029, rispettivamente). A T36 i valori di eGFR calcolato con le quattro formule che utilizzano solo CysC non differivano nei nati sotto o sopra le 28 settimane. Al contrario, i valori di eGFR stimati dalle altre formule erano più elevati nei soggetti nati a GA inferiori. Il punteggio prenatale non correlava con eGFR calcolato secondo nessuna formula. Invece, il punteggio post-natale correlava positivamente con l'eGFR stimato con le formule basate sulla sCr, la Schwartz 2009 (R=0.345, p=0.027) e la Brion (R=0.312, p=0.044). Tuttavia, tali correlazioni non persistevano quando pesate per i livelli di urea a T36 e per la GA. Al contrario, il punteggio non correlava con l'eGFR stimato con tutte le altre formule.

Conclusioni: Le formule per eGFR che utilizzano CysC non risultano influenzate dalla GA. Il punteggio di rischio post-natale correla direttamente con l'eGFR calcolato con formule basate sulla sCr, ma tale correlazione scompare quando pesata per GA e livelli di urea, suggerendo che il confondente sotteso a tale correlazione potrebbe essere lo stato nutrizionale. Infatti, i soggetti più gravemente prematuri ricevono un regime nutrizionale più aggressivo, come testimoniato dai maggiori livelli di urea a T36 nei neonati con GA ≤28 settimane.

1. INTRODUCTION

Preterm birth and nephrogenesis

The survival of preterm newborns has dramatically improved in the recent decades, with babies born as young as 25-week gestation having up to the 80% chance of survival.[1] Preterm birth has many potential detrimental effects on developmental programming, and the kidney is particularly vulnerable to these effects. In the human, the first nephrons are formed by 9 weeks of gestation and nephrogenesis is completed between 32 and 36 weeks of gestation, with the majority of nephrons being formed in the third trimester of pregnancy.[2] Any impact on nephron number at the very beginning of life may have adverse consequences for life-long renal health.[3] Emerging epidemiological studies have linked preterm birth with altered renal function in childhood and adulthood.[4] In addition, preterm birth seems to be associated to an increased risk of developing arterial hypertension later in life.[5,6] In a baboon model,[7] whose timing of nephrogenesis and kidney morphology closely resembles that of humans, a marked increase of the kidney weight to body weight ratio was shown in preterm baboons, compared to control full-term baboons. This is likely a compensatory response to the increased functional demands on the kidney after preterm birth. There was evidence of ongoing nephrogenesis in the outer renal cortex of preterm baboon kidneys at postnatal day 21, with a clearly visible nephrogenic zone. Consistent with this, there was an increase in the number of glomerular generations formed in preterm kidneys after birth, and an increase in the total number of nephrons, albeit at the lower end of the normal range observed in term kidneys. Of particular concern, a high number of abnormal glomeruli was observed in some of the preterm kidneys. These abnormal glomeruli displayed a relatively immature form with scant capillarization, a cystic Bowman's space, and they were only observed in the outer renal cortex, suggesting that the recently formed glomeruli or those formed in the extrauterine environment were vulnerable to preterm birth. Not all kidneys exhibited abnormal glomeruli with the proportion of abnormal glomeruli per kidney ranging from 0.2% to 18.3%. Given the gross abnormalities it is considered unlikely that these glomeruli would ever be functional. Therefore, the preterm baboons with a high proportion of abnormal

glomeruli would have a marked reduction in the endowment of functioning nephrons at the beginning of life. To determine whether these abnormalities are also present in the kidneys of preterm human infants, a study was performed in autopsied kidneys of preterm infants who were born between 24 and 35 weeks of gestation and lived for 2–68 days after birth.[8] The kidneys from the preterm infants were compared with postconceptional age-matched control infants who had died acutely in utero. Similar to the preterm baboon kidneys, there was evidence of ongoing nephrogenesis in preterm kidneys. The number of glomerular generations was significantly increased in preterm kidneys compared with stillbirth controls. However, the width of the nephrogenic zone and the proportion of glomeruli in the most immature state of differentiation were significantly decreased in the preterm kidneys. Taken together, these findings suggest that there may be an accelerated postnatal renal maturation following preterm birth. It is not possible to determine whether the accelerated development results in the early cessation of nephrogenesis. Similarly to the preterm baboon kidneys, there were marked glomerular abnormalities in the outer renal cortex in some, but not all, kidneys from the preterm infants (ranging from 0% to 13%). In addition, in the normally formed glomeruli there was a significant increase in size, indicative of glomerular hypertrophy and thus hyperfiltration. The variability in the proportion of abnormal glomeruli in the outer renal cortex between preterm infants suggests that there may be differences in haemodynamics, and/or other factors in the postnatal environment of the infant (such as exposure to nephrotoxic drugs, oxygen supplementation, mechanical ventilation and co-morbidities) which may negatively impact glomerulogenesis.[3] In this regard, there is a major haemodynamic transition at the time of birth when blood pressure and heart rate are markedly elevated[9] and blood flow to the kidneys is increased.[10] Hence, it is possible that the developing capillaries of immature glomeruli are not prepared for the haemodynamic transition at birth and the glomeruli formation may be adversely affected by such a damage. Indeed, it has been shown an injury to the aortic wall as a result of preterm birth[11] and it may be supposed that a similar injury occur in preterm glomerular capillaries.

Physiology

The human kidney forms from three primordial "attempts" beginning with the primitive, but obligatory anlage, called the "pronephros" at about 3 weeks' gestation and lasting for only 3–5 days [12][13][14][15][16]. The pronephros provides the pattern of ciliated epithelial cells that are reminiscent of the proximal tubular epithelial cells that will compose a major part of the human nephron [13][14][15][16]. With the regression of the pronephros, the mesonephros emerges as a much more complex structure with nephrotomes and filtration bundles developing around the incursion of the Wolffian duct into the mesonephric mesenchyme. Subsequently, the mesonephric nephrons begin their involution and the Wolffian duct gives rise to the ureteric bud, which starts its incursion into the metanephric mesenchyme, giving rise to the metanephros, the third anlage and true human kidney. From the 5th gestational week, the ureteric bud begins sequential branching into the metanephric mesenchyme propagated by a complex system of signaling molecules emanating from both tissues [14][15][16]. By the 20th gestational week, the fetal urine comprises 80% of the amniotic fluid at a rate of approximately 800 ml per day (300 ml/kg fetal weight/day) [17][18]. Over 80 % of the nephrons form during the last trimester, with a rapid succession of "nephron generations" from the cortico-medullary junction extending up to the "nephrogenic blue zone" under the capsule [12][19]. From 22 to 36 weeks' gestation, 10–12 generations of nephrons form, similar to the rings of a tree [12][19]. With cessation of glomerulo-genesis, the nephrogenic zone disappears [14][15]. Thereafter, only tubular and vascular growth and interstitial tissue expansion occurs [14][15]. The glomerular epithelial cells are cuboidal in shape and the capillary loops are poorly perfused, receiving less than 2 % of the fetal cardiac output [17][18].

It appears that the epithelial cells decrease in density as the nephrons expand with the inner glomeruli becoming more "mature", while those of the outer cortex are the last to expand, making them the more vulnerable to adverse circulation ex-utero [16].

Nephron endowment

I. Nephron number

Based upon autopsy studies, it has been proven that human beings have on average approximately 900,000 nephrons per kidney [20] [21], and the number declines with age [22]. However, there is substantial inter-individual variability across different populations, even among healthy subjects. In 1934, Moritz and Hayman [23] used the acid maceration method to count glomeruli in 14 human kidneys, and they found an average glomerular number of 1,282,800 per kidney, with a range of 940,000 to 1,542,000. When the kidneys of four subjects with "chronic diffuse glomerular nephritis" were examined, the glomerular numbers ranged from 155,000 to 528,000 [23]. In 1992, Nyengaard et al. published the first study to use unbiased stereology to estimate glomerular number in a cohort of 37 healthy Danes. The average number of glomeruli in this group was approximately 600,000 per kidney ranging from 330,000 to 1.4 million [22]. Keller et al. subsequently assessed glomerular number in 20 kidneys from middle-aged German subjects killed in automobile accidents [24]. Among normotensive subjects, the mean number of glomeruli was 1.4 million (range 800,000 to 2 million), while hypertensive subjects had half as many glomeruli (700,000, range of 530,000–950,000) [24].

The largest cohort to date was described by Bertram et al. in 2003 and included 398 subjects with a variety of ethnic and racial backgrounds. The average number of glomeruli per kidney reported was 895,711, and there was a greater than tenfold variation in nephron number between subjects (ranging from 210,332 to 2,702,079). In this study, female sex, older age, race (lowest in the Australian Aborigines), and low birth weight were all associated with lower nephron number [20] [21]. All the previous studies assessed nephron number in adult subjects, raising the possibility that the wide variability in nephron number may be due to pathologic or premature loss of nephrons in some patients. Studies on younger subjects have found similar results, suggesting that substantial variation in nephron number is present from birth. For example, in a study of infants aged <3 months who died of congenital abnormalities or sudden infant death syndrome, Zhang et al. found that nephron number ranged from 246,181 to 1,106,062 per kidney [25]. Unfortunately, it must be emphasized that while it may be possible to determine "normal" nephron

number, without the ability to prospectively study nephron number in living persons, it is impossible to determine the nephron number required for good health and maintenance of long-term renal function.

II. Surrogates for nephron number

Given the intrinsic limitations of autopsy techniques for determining nephron number, surrogates have been searched in living subjects. Initial investigations found that kidney weight correlated with nephron number [22], so the radiologic measurement of kidney size as a surrogate for renal weight, and therefore nephron number, has been widely used. Ultrasound-based methods are appealing due to their wide availability and non-invasive nature, although they may be limited by large inter-observer variability.

The most significant limitation to radiology-based methods to estimate nephron number is that significant hypertrophy (mainly of the tubules) often occurs when glomerular number is reduced [26]. In the setting of oligonephronia, glomeruli enlarge to maintain the total amount of surface area for filtration while the tubules show a hypertrophic response to increased filtrate or effective renal plasma flow [27] [28]. For instance, in the above described study by Keller et al. [24], glomerular number was directly determined after kidney weights were ascertained in 20 subjects who died in automobile accidents, ten of whom were known to have arterial hypertension. While subjects with hypertension had approximately half as many nephrons as the normotensive controls, there was no difference in kidney weight between the two groups [24]. It has been estimated that only 10 % of the variation in nephron number is explained by a difference in renal size in adults [29]. For similar reasons, glomerular volume has also been proposed as a surrogate for nephron number, given the inverse relationship between the size of glomeruli and the nephron number [30]. Moreover, a clear association between body size and glomerular volume was shown, and certain ethnic groups (such as African Americans) tend to have larger glomerular volume in general, making this measure more difficult to implement in practice. Given the limitations of both radiologic and pathologic methods, investigators attempted to combine radiologic and pathologic data to obtain better estimates of glomerular number in vivo. Basgen et al. proposed that glomerular number could be estimated by measuring the cortical volume of the kidney [obtained by magnetic resonance imaging], then multiplying

by the fraction of the renal cortex made up of glomeruli divided by the mean glomerular volume (obtained by renal biopsy) [31]. While there was overall good agreement between this approach and the fractionator–dissector method in ten dog kidneys, the discrepancy between the two methods varied by up to 36 % for an individual kidney. This technique has subsequently been applied in renal transplant patients, with renal cortical volume measured by MRI and two percutaneous renal biopsy cores used to estimate glomerular number [32]. Although the results in experimental animals are promising, there has been no method to validate these findings in living patients yet.

Factors influencing nephron endowment

I. Prenatal factors

In humans, nephrogenesis begins at 9 weeks and continues through 32-36 weeks of gestation [33] [34]. Nephrogenesis is a finely orchestrated process that requires reciprocal induction of the dividing ureteric bud with the regenerating metanephric mesenchyme. Myriad factors—both genetic and environmental—can disrupt this delicate balance, leading to aberrant or abbreviated nephrogenesis.

a) Genetic and epigenetic factors

As understanding of the human genome expands, the list of genetic factors that affect nephrogenesis continues to grow. However, it is clear that the complex genetic control of nephrogenesis can be altered at many points, leading to similar phenotypes. Mutations affecting nephron number affect either the ureteric bud or metanephric mesenchyme. For instance, mutations of Six2 have been discovered in patients with renal hypoplasia [35]. Six2 is expressed in undifferentiated mesenchyme and, when inactivated, there is a reduced number of progenitor cells [36]. Mutations in the ureteric bud or its ability to branch also affect an individual's nephron mass. Infants with a common Ret variant [the RET(1476A) allele] have a kidney volume that is 10% smaller than controls [25]. Homozygous Ret knockout mice do not develop kidneys, while heterozygotes have a reduced nephron number [37]. Ret transcription is regulated by Pax2, and a specific Pax2 haplotype found in 18.5% of healthy newborns is associated with a 10% reduction in kidney volume [38]. Most of the single gene factors known to alter nephron number are gene

disruptions that lead to loss of function and reduced nephron number. However, genetic variants may also increase nephron number. El Kares et al. found that individuals with a common variant in ALDH1A, present in 20% of healthy Caucasians from Montreal, had a 22% increase in kidney volume as newborns [39]. Moreover, little is known about epigenetic factors involved in kidney development [40]. Histone deacetylases are involved in gene expression via chromatin modification. The deletion of class I histone deacetylases causes dysregulation of nephron gene expression [41]. In animal models of intrauterine growth restriction (IUGR), decreased methylation of a promoter ultimately results in increased apoptosis and reduced glomerular number [42]. More recently, it has been demonstrated that histone methylation may affect the cell fate of the metanephric mesenychme [43]. As earlier discussed, Six2, a renal specific transcription factor, is involved in nephrogenesis [44] and the binding of Six2, as well as of beta-catenin, at regulatory sites promotes nephron development.

A better understanding of genetic and epigenetic control of nephrogenesis would not only improve our ability to identify patients at a greater risk for their low nephron number, but could also identify novel therapeutic targets and strategies.

b) Birth weight

A low birth weight, either due to premature birth or intrauterine growth restriction, seems to negatively affect nephrogenesis. An autopsy study of 56 patients without known kidney disease suggested that every 1 kilogram increase in birth weight confers an average of 260,000 additional nephrons, with 18 % of the variability in nephron number explained by differences in birth weight alone [45]. Being birth weight determined by a complex interplay of genetic, nutritional, social, and obstetric factors, it is extremely challenging to disentangle the effects of a single variable from confounders. Many low birth weight infants are premature. However, birth weight seem to correlate with nephron endowment independently of gestational age. Manalich et al. performed a post-mortem evaluation of 35 infants born at >36 weeks of gestation and noted that weight at birth was directly correlated with glomerular number and inversely correlated with glomerular size [46]. Similarly, Hinchliffe et al. observed that nephron number was decreased both in a group of six fetuses who died during gestation and eight infants with IUGR who died in the first year of life when compared to controls at similar gestational ages [47]. It is the

asymmetric growth pattern seen in type II IUGR infants (with preservation of brain and heart sizes, but a disproportionate reduction in other organs, including the kidneys) that is most strongly associated with lower nephron number and an increased long-term risk of chronic kidney disease (CKD), as excessive postnatal growth can outstrip kidney function [48]. While infants with "low birth weight"—whether from premature birth or IUGR—may share a common phenotype of increased CKD risk, the causes of reduced nephron number may differ significantly between these groups, requiring distinct strategies or therapeutic interventions to protect nephrogenesis in early life in each of these populations.

c) Nutrition

Nutritional factors play an important role in nephron development, but for obvious reasons it is difficult to isolate the effect of particular vitamin, nutrient, or calorie deficiencies in observational studies in humans. Reliance on data from animal models is therefore necessary. Global caloric restriction has been best evaluated in sheep. Sheep given a diet composed of 50% of the calories from the 28th to 80th day of gestation then allowed to return to a full diet until the completion of gestation (147 days) were found to have a reduced number of nephrons. This restriction in calories occurs during a period of rapid placental growth, leading to offspring having 10–50% fewer nephrons as adults [49] [50]. Protein restriction also reduces nephron number, although this effect is influenced by both the degree of restriction and its timing. In studies with rodents, when mothers received a low protein diet throughout gestation but a normal amount of protein postnatally, their offspring had a 16–50% decrease in nephron number [51] [52] [53]. Similar findings occur even when protein restriction begins at the time of birth, as postnatal nephrogenesis occurs in rodents [54]. The reduction in nephron number seen in these models may be related to increased apoptosis and reduction in the number of progenitor cells [53]. In contrast, high protein diets do not seem to affect offspring nephron number [55]. Iron deficiency in rodents also reduces the nephron number in offspring. Lisle et al. [56] exposed pregnant rats to a low iron diet before conception and throughout gestation. Pups of these dams had a 23% reduction in glomerular number, although there was no difference in kidney weight between control and intervention groups. A proposed mechanism for this reduction is that iron deficiency leads to impaired hepatic mobilization of vitamin A. Vitamin A reduction, in turn, leads to decreased

ureteric bud branching, ultimately reducing nephron number [57]. Vitamin A modulates nephron number through the regulation of the RET receptor. Expression of RET in the ureteric bud tip is exquisitely sensitive to retinoic acid signals from adjacent mesenchymal cells, and vitamin A deficiency downregulates the RET receptor [58]. In rats, there is a strong linear association between plasma retinol and nephron number, such that 69% of the variation in nephron number is explained by differences in plasma retinol [59]. Additionally, postnatal vitamin A supplementation can reverse the effect of in utero protein restriction on nephron endowment in rodents [60]. While nutritional deficiencies generally decrease nephron number, there may be exceptions. For instance, when rats were fed a vitamin D-restricted diet from the time of conception through lactation, their offspring had a 20% increase in nephron number [61]. Two mechanisms have been proposed to explain these findings. First, active (1,25-OH) vitamin D stimulates cell differentiation and inhibits proliferation [62]. Alternately, since active vitamin D negatively regulates renin expression, increased levels of angiotensin II may stimulate nephrogenesis [63] [64,65,66].

d) Teratogens

The detrimental effects of fetal exposure to ethanol involving other organ systems are well described. Recently, Moritz et al. studied the effects of alcohol on the developing kidney and found that ethanol exposure during gestation reduces the number of glomeruli by 10–15% in both rodent and sheep models when compared to control animals [67,68]. Epidemiologic observations suggest that maternal tobacco exposure in the form of cigarette smoking during pregnancy affects fetal kidney volume in a dose-dependent manner. In a prospective cohort study in the Netherlands, the smoking habits of approximately 1,000 mothers were assessed during pregnancy and compared to the respective fetus renal volume. As cigarette smoke exposure increased, the size of the fetal kidneys decreased compared to a control group with no smoke exposure [69]. Another established teratogen is hyperglycemia, and both in vivo and in vitro studies have demonstrated the detrimental effects of hyperglycemia on nephron development. Metanephros development is impaired by hyperglycemia [70], and in one study rodent pups born to dams with diabetes had up to a 35 % reduction in nephron number compared to controls [71]. There is similar evidence in humans as well. Adults born to mothers

with type 1 diabetes have been found to have smaller increases in glomerular filtration rate (GFR) and effective renal plasma flow when subjected to amino acid infusion than subjects whose father had diabetes, suggesting reduced functional renal reserve [71] [72].

e) Medications

Corticosteroids are frequently administered to women with preterm labor due to their benefits in accelerating fetal lung maturity. However, there is evidence that steroids alter the expression of key genes involved in the regulation of branching morphogenesis [72]. Exposing metanephroi culture media to dexamethasone for 2 days decreases the number of ureteric buds, resulting in fewer nephrons even when the dexamethasone is removed [74]. The timing of exposure seems important: early steroid administration (occurring when the ureteric bud is just invading the metanephric mesenchyme) alters nephrogenesis and reduces final nephron number by 30% [73] [74]. Many medications that have nephrotoxic effects in fully developed kidneys appear to adversely affect developing kidneys as well. Drugs that disrupt the renin–angiotensin system lead to frank renal insufficiency [67], and cyclosporine reduces nephron number in animal models [75]. The effects of exposure to non-steroidal anti-inflammatory drugs (NSAIDs), such as cyclooxygenase-2 (COX-2) selective inhibitors and acetylsalicylic acid, have shown variable results. Although rodents treated with COX-2 inhibitors from either conception or birth over the first 3 weeks of life had reduced glomerular size, there was no reduction in glomerular size if exposure occurred only during gestation [76]. Exposure to acetylsalicylic acid had no effect on nephron number, but during this experiment the rodents were only treated from day 7 to day 20 of gestation [77]. In animal studies, in utero gentamicin exposure reduced glomerular number by 20%, and offspring developed glomerulosclerosis during one year of monitoring [78]. Interestingly, some nephrotoxic effects seem to be strain specific, such that a reduction in glomerular number occurs only in animal strains already susceptible to glomerulosclerosis [79]. Such findings may have implications for diverse human populations, although it is difficult to extrapolate these animal data to human exposures given the variability of dose, timing, and follow-up.

II. Postnatal factors

a) Prematurity

Since nephrons are formed until late gestation, in infants born prematurely kidney development presumably continues while in the neonatal intensive care unit. Therefore, it is unsurprising that this may result in aberrant nephrogenesis. Our understanding of the effects of prematurity comes from a limited number of autopsy series and animal studies. Several studies examining the kidneys of premature infants have been published in the last decades [8] [80] [81]. Although there are risks in applying the results of autopsy studies to living patients, several observations are informative. In 2004 Rodriguez et al. found lower radial glomerular counts and cessation of glomerulogenesis after 40 days following birth in extremely premature infants [82]. Sutherland et al. performed a similar study, with similar results [8]. While these authors found no difference in either the number of glomerular generations or total nephron number, there was accelerated renal maturation as demonstrated by a reduction in immature glomerular structures among the premature infants [8]. Both groups reported an increased frequency of abnormal glomeruli with increased mesangial tuft and capsular areas and larger glomeruli [8]. In animal models, the closest approximation to human prematurity is the baboon, as it has a relatively long gestation and, if delivered prematurely, can be exposed to hyperalimentation, mechanical ventilation, and nephrotoxic medications in an intensive care setting. Baboons delivered at 125 (of 185) days gestation and harvested at 3 weeks of age had larger kidneys due to enlarged glomeruli, although these animals also had decreased glomerular density, shrunken tufts, and cystic Bowman's space [7]. There are several limitations with alternative animal models. Smaller species, such as rats and mice, continue to develop nephrons for several days postnatally [83], and their body size precludes invasive methods to sustain life. Nonetheless, there is a recently described mouse model in which mice born 1–2 days prematurely develop hypertension, albuminuria, and fewer nephrons at 5 weeks of age [84]. Although the best evidence of the relationship between prematurity and nephron number is derived from autopsy and animal studies, renal volume (despite its imperfections) has been frequently examined in clinical studies as a surrogate of nephron number. In the youngest group studied, Kandasamy et al. examined 68 infants at a corrected gestational age of 38 weeks and found lower total renal volume in premature infants [85]. Yet when renal volume

was corrected for body surface area, the premature group had greater kidney volume than full term infants [85]. These results are in contrast to studies in older children. In a Greek cohort of infants followed for more than 2 years, premature infants had a smaller kidney length than their full term controls [86], while a Dutch cohort had smaller renal volume in young adulthood than term controls [87]. Moreover, it has to be pointed out that premature birth and consequent NICU staying exposes neonates to many factors, that could be potentially harmful for kidney development and health, such a nephrotoxic medications, mechanical ventilation with possible hyperoxia, hemodynamic instability, whose singular contribution to renal damage may be difficult to disentangle.

b) Acute kidney injury

There is now convincing evidence that the occurrence of acute kidney injury (AKI) is associated with the development of CKD later on. It has recently been proposed that AKI and CKD are simply the acute and chronic phases of a single clinical syndrome of decreased GFR [88]. Central to this hypothesis are the observations that AKI leads to nephron loss and tubulointerstitial fibrosis and that reduced nephron mass in turn impairs the ability to recover from subsequent injury and accelerates CKD progression [89]. Since current methods of counting nephrons preclude examining nephron number before and after AKI, there is presently no way to evaluate the extent to which AKI may occur disproportionately among individuals with an already decreased number of nephrons or if nephron loss is dependent on a particular type, duration, or developmental timing of injury.

c) Nephrotoxic medications

Aminoglycoside Antibiotics

Preterm infants are often exposed in utero and/or immediately after birth to antibiotics that could adversely affect nephron formation. Aminoglycosides are the most commonly administered antibiotics. Clinical studies have reported that postnatal gentamicin exposure leads to a marked increase in the fractional excretion of sodium [90] [91] and a decrease in GFR [92] [93] [94] [95] [96] [97] [98] as well as an increase in urinary protein excretion in preterm neonates. [95] [99] [100] Therefore, it is essential to determine whether postnatal administration of nephrotoxic antibiotics to the

preterm neonates may contribute to a reduced nephron endowment and consequently reduced renal function later in life.

Nonsteroidal Anti-Inflammatory Drugs

Other commonly prescribed therapeutic medications in preterm neonates, particularly those born extremely preterm, include the NSAIDs, which are prostaglandin inhibitors, administered in order to close a patent ductus arteriosus.[101]. Nonsteroidal anti-inflammatory drugs, such as indomethacin and ibuprofen, are known to have detrimental effects for the kidney, particularly the developing one.[102] In the preterm neonate, administration of NSAIDs has been shown to delay the normal increase in GFR that occurs after birth and lead to a decrease in urine output.[98][103][104]

Hyperoxia

Postnatal exposure to hyperoxia in the preterm neonate is linked to the development of both bronchopulmonary dysplasia and retinopathy of prematurity; underlying each of these conditions is impaired angiogenesis.[105][106] Results of a recent study suggest, however, that microvascular rarefaction may not only be confined to the retina and lung, with children born very preterm (30 week gestation) demonstrating significantly reduced functional skin capillary density at 7 to 12 years of age.[107] The kidney is a highly vascular organ, with ongoing vascularization of the glomeruli occurring following preterm birth. During nephrogenesis in utero, the mammalian fetal environment is relatively hypoxic;[108][109][110] rat metanephric organ culture studies have shown low (1%-3%) oxygen concentrations to be optimal for both vasculogenesis and tubulogenesis.[111] At birth, preterm infants are exposed to atmospheric (21%) oxygen levels, and this physiologic hyperoxia may be further increased by the use of supplemental oxygen during the neonatal period. Therefore, it is conceivable that development of the glomerular capillaries will be adversely affected by high oxygen concentrations following preterm birth. To date, only one experimental study reports the effects of neonatal hyperoxia on the adult kidney in an animal model. Interestingly, Yzydorczyk et al [112] demonstrated that nephron endowment was reduced by 25% in adult rats (25-35 weeks of age) exposed to hyperoxia during the period of postnatal nephrogenesis. However, since the rats were also found to have increased blood pressure by 9 weeks of age, it cannot be determined from this study whether

the nephron deficit was the result of the exposure to hyperoxia or induced by prolonged hypertension.

Assessment of kidney function in newborns

Historically, **inulin** has been considered the "gold standard" for measurement of GFR in infants [113]. It is a naturally occurring polysaccharide that is a derivative of the Jerusalem artichoke and other legumes such as chicory. It meets the essential criteria for accurate measurement of GFR, including being freely filtered through the glomerulus and neither being secreted nor re-absorbed in its transit down the nephron [114]. Moreover, it must not be metabolized or protein bound after injection. Importantly, it must be safe and innocuous for clinical investigations in infants and children. During the early years of investigation into the renal function of infants, there were a number of studies that reported inulin clearances relative to gestational and post-natal age [115 116 117 118 119 120]. These studies were considered the foundation for the assessment of neonatal kidney function and development. Another direct method of GFR calculation is the one based on non-radioactive iothalamate [121]. Although it is partially protein bound and there is possible tubular secretion, it is inexpensive and, in the neonate, closely approximates inulin clearance. Iohexol is a nonradiopaque contrast agent used in the measurement of GFR in children with CKD, but it has not been validated in infants [122]. Despite the imperative for having a reliable assessment of GFR in the neonate, the performance of clearance studies, especially in sick infants, is unfeasible in clinical settings. As a consequence, endogenous markers of GFR have been studied and indirect estimating equations elaborated. To serve as an accurate marker of GFR, the plasma solute must be freely filtered across the glomerular membrane and neither secreted nor reabsorbed along the tubular network. It must be solely excreted via the kidney and the endogenous production rate must be constant.

I. Endogenous markers of kidney function

The most used endogenous biomarker for the assessment of neonatal kidney function is serum **creatinine**. Creatinine (Cr) is a metabolic product of creatine and phosphocreatine found in muscle and as such reflects muscle mass [123 124]. Cr

concentrations are insensitive to detection of mild to moderate reductions in GFR. There is substantial interindividual variability due to differences in muscle mass [125]. In childhood, serum Cr is influenced by age, gender and muscle mass [126]. So it was necessary to search alternative endogenous biomarkers of GFR, in particular low-molecular weight proteins. The idea of using endogenous small molecular weight proteins, such as CysC [127], b-trace protein (BTP) [128], and b-2 microglobulin (B2M) [129][130], as markers of GFR is not new [131]. Cysteine proteases are proteolytic enzymes involved in many pathological processes and are found in the lysosomes of cells. They are essential in normal cellular metabolism, being fundamental to intracellular protein turnover, degradation of collagen, and cleavage of precursor proteins [132]. Human **cystatin C** (CysC) is a low molecular mass protein (13,343 Da, 120 amino acids) belonging to the cystatin superfamily of reversible inhibitors of cysteine proteases of the papain and legumain families [133]. In contrast with Cr, CysC does not appear to be affected by body muscle mass, age, gender, inflammatory state, or nutritional conditions [134]. In two meta-analyses, CysC has been shown to be a superior marker for the detection of mildly impaired GFR in the so called creatinine-blind range [135][136]. As for creatinine, CysC is filtered freely through glomeruli. The estimation of GFR from CysC does not require information about age, sex, ethnicity, weight, or height. Hence, CysC is increasingly being used across the world. Based on the latest research, combining CysC and creatinine is the most cost-effective way to determine estimated GFR, both in adults and in children [137][138]. There are limited studies of CysC use to assess GFR in neonates [139][140][141]. Initial studies suggest that CysC does not cross the placenta [142], even if this data are controversial. Mothers have increased CysC concentrations toward the end of the pregnancy [143][144]. It is unclear how the placenta transports CysC. Nonetheless, the proportion of CysC reflected by maternal CysC concentrations is small. CysC is thus fundamentally different from serum creatinine as only minimal amounts cross the placenta, thereby reflecting mostly neonatal function [145].

Also **beta-2 microglobulin** (B2M) seems not to cross the placenta. Increased serum B2M concentrations have been associated with respiratory distress syndrome when compared with healthy control newborns, questioning the feasibility of B2M to serve as a marker of neonatal renal function [146].The efficacy of measuring neonatal GFR with **beta-trace protein** (BTP), a 23- to 29-kD enzyme consisting in 168 aminoacids, has not been extensively studied, but it may be a

good marker of maternal and neonatal renal function because BTP does not cross the placenta at all [147]. Ideally, age-independent z-scores of CysC or BTP concentrations should be established for postconceptual age using Box–Cox transformations and age-independent L, M, and S-scores [147]. However, both CysC and BTP assays are not routinely available in the hospital chemistry laboratory as standard assays and they are 10–15 times more expensive.

II. Normative charts for creatinine, cystatin C, BTP and eGFR

In the last years, experts have called for the adoption of normative charts for infants and children to be able to interpret measures of renal assessment similar to growth curves with the calculation of z-scores or percentiles relative to the normal pooled data. Traditionally, the dogma has been to dismiss elevated serum creatinine in preterm infants during the neonatal period as unreliable. However, there is growing concern among pediatric nephrologists and neonatologists that failure to recognize kidney injury during the neonatal period may result in a lack of surveillance for the detection of AKI and development of progressive kidney disease [148]. Table 1 provides the mean± standard deviation (SD) calculated for preterm and term infants at birth and at 3, 6, 12, 24 and 36 months post-conceptual age for serum creatinine, cystatin C, and eGFR.

Table 1. Reference values for serum creatinine, Cystatine C, BTP and GFR measured by inulin or estimated according to different formulas, in preterm and term newborn, and at 3, 6, 12, 24, 36 months.

	Preterm	Term	3 months	6 months	12 months	24 months	36 months
Scr mg/dl	0.7 ± 0.3	0.5 ± 0.1	0.4 ± 0.2	0.3 ± 0.2	0.3 ± 0.1	0.3 ± 0.2	0.3 ± 0.2
ScysC mg/l	1.42 ± 0.21	1.33 ± 0.20	1.20 ± 0.26	0.98 ± 0.22	0.85 ± 0.22	0.72 ± 0.12	0.72 ± 0.10
S_{BTP} mg/l	1.79 ± 0.56	1.27 ± 0.27		0.84 ± 0.35		0.68 ± 0.17	0.74^{*}
GFR_{INULIN} ml/min/1.73 m^2	44 ± 9	55 ± 8	60 ± 17	87 ± 22	96 ± 12	105 ± 17	111 ± 19
$eGFR_{cr}$ ml/min/1.73 m^2	24 ± 7	46 ± 10	63 ± 8	92 ± 10	105 ± 12	120 ± 17	113 ± 10
$eGFR_{cysC}$ ml/min/1.73 m^2	46 ± 10	54 ± 8	61 ± 10	78 ± 8	92 ± 12	112 ± 10	112 ± 8
$eGFR_{BTP}$ ml/min/1.73 m^2	46 ± 7	63 ± 13		94 ± 39		115 ± 29	106 ± 8

*Upper limit of range for adults
Scr: serum creatinine; ScysC: serum cystatin C; S_{BTP}: serum beta trace protein; GFR_{INULIN}: glomerular filtration rate measured by inulin; $eGFR_{cr}$: estimated GFR by creatinine; $eGFR_{cysC}$: estimated GFR by cystatin C; $eGFR_{BTP}$: estimated GFR by beta trace protein

Assessment of kidney size in newborns

I. Total kidney volume in the assessment of kidney function

Preterm birth imposes immediate and potential long-term alterations in kidney size and function. Infants born before 36 weeks' gestation during active nephrogenesis will have a decreased number of filtering nephrons and the GFR will be proportionate to the number of nephrons being perfused. The GFR will also be a function of the gestational age since renal size in utero follows gestational growth curves [149]. In a small autopsy study of term neonates (n = 15) glomerular number correlated to kidney weight [23]. This is consistent with adult autopsy studies in various populations, suggesting that nephron endowment can be estimated by kidney size [22 33 150 151 152 153 154 155]. Even more compelling is that kidney size individualized to body surface area (BSA) closely correlates with kidney function across all age groups[156 157 158]. Individual kidney volume can be measured using the equation for an ellipsoid: volume = length ×width × depth × 0.523 and then left and right kidney volumes can be summated for the TKV (ml) [159 160]. Ultra-sonographers can estimate these measurements from standard renal ultrasound images. There is a normal Gaussian distribution of TKV individualized to BSA (TKV/m2=ml/m2) when measured by non-invasive renal ultrasound from birth to adulthood [158]. A cross-sectional study of renal ultrasounds in 624 healthy German children from birth to 18 years of age showed an average TKV/m2 to be 132 ml/m2 with the 10th and 90th percent confidence interval being 90 and 171 ml/m2, respectively [158]. In a Canadian cohort of 136 full-term healthy Caucasian infants <3 months of age, an identical curve was generated with the mean TKV/m2=132±29 ml/m2 [25]. The Generation R study of over 6000 Dutch children followed prospectively from birth used TKV as a marker of kidney health [161 162]. When the Gaussian distribution of TKV/m2 of the normal population of 624 infants, children and adults was superimposed on that of 60 preterm infants at birth, the remarkable observation was that they were similar [158]. This corroborates previous studies that nephron mass and presumed nephron endowment is normally distributed and similar in preterm infants at birth.

The implications are that, if active nephrogenesis is present in post-natal life, then the preterm infant could potentially achieve a nephron endowment similar to that

of a term infant. It also suggests that individuals whose TKV/ m2 falls below the 10th percentile have an inherent risk for "oligonephropathy" and should be monitored for hypertension and progression to CKD. In seeking more accurate and alternative estimates of GFR in infants and children, it has become evident that TKV should be considered in the baseline and longitudinal assessment of neonatal kidney function and has implications for the individual's renal longevity [163] [164].

Consequences for adult life

According to the recent data from the Vermont Oxford Network, ~90% of infants born weighing 501 to 1500 g survive to NICU discharge, and ~60% of survivors leave the NICU without any major neonatal morbidity. [165] Although there has been a great deal of research into the neurodevelopmental outcomes of premature infants, the impact of prematurity or low birth weight (LBW) on other organ systems is less well understood. There is now evidence that premature and LBW infants who survive the NICU still face serious risks to their long-term kidney health. David Barker is credited with the observation that many "adult" diseases may in fact have their origins in fetal life. [166] [167] To survive in a stressful or nutrient-poor environment, a fetus must make "choices" about how to use scarce resources in a way that maximizes the likelihood of survival in early life, even at the expense of greater susceptibility to chronic illnesses and increased mortality in adulthood. This kind of developmental programming among LBW infants [168] [169] has been associated with problems including obesity, [170] hypertension, [171] insulin resistance, [172] and coronary artery disease. [173] Nephrologist Barry Brenner first applied Barker's theory to the development of CKD. He hypothesized that either a congenital or acquired reduction in nephron number could explain why some individuals are more prone to hypertension and CKD. [174] Brenner proposed that persons with a decreased nephron number can initially maintain a normal GFR as individual nephrons enlarge to increase the total surface area available for renal work. Over time, however, this adaptive response becomes harmful. Increased glomerular surface area leads to sodium retention and systemic hypertension, and glomerular hyperfiltration disrupts renal autoregulatory mechanisms, generating intraglomerular hypertension and proteinuria. [175] [176] [177] These processes eventually

cause nephrons to become sclerotic and senescent. This in turn leads to additional decline in nephron number and greater hyperfiltration in remnant nephrons, culminating in more rapid nephron dropout and perpetuating renal injury in a vicious cycle [178].

I. Prematurity and CKD

To date, no prospective, population-based studies confirm the association between prematurity or LBW and CKD. Although it is difficult to disentangle the effects of confounders such as socioeconomic status in retrospective studies, a systematic review of 31 cohort or case-control studies found a 70% increase in adulthood CKD for LBW infants. [179] Although this meta-analysis excluded studies consisting exclusively of extremely LBW or very premature infants, other more inclusive analyses showed similar results. A national registry-based study including all infants born in Norway from 1967 to 2004 found an overall relative risk of 1.7 for the development of end-stage renal disease (ESRD) for infants with birth weight <10th percentile compared with those with birth between the 10th and the 90th percentile. [180] Clinical signs of oligonephropathy among patients born prematurely may be detectable in childhood. [19][181] Two recent case series (each including 50 infants born at, 30–32 weeks' gestation) found that children born prematurely had smaller kidneys and higher blood pressure compared with term controls, even though their GFR remained normal. [89][182] Microalbuminuria, an early indicator of kidney disease and a risk factor for future cardiovascular morbidity, [183] is also common among children aged 8 to 11 years who were born prematurely or with LBW. [184]

II. AKI and CKD

Acute kidney injury in the NICU is not uncommon. Although its overall incidence is difficult to determine given the lack of multicenter studies and of a unique definition of AKI, the existing studies report an extremely variable incidence of AKI among preterm and LBW infants, ranging between 12.5% and 71%. Moreover, as the majority of pediatric patients with AKI are discharged from the hospital with a normal serum creatinine, [185] the long term significance of their renal injury may not be fully appreciated. It was long taught that AKI is reversible. [186] This may be true for AKI caused purely by volume depletion, but it is now clear

that intrinsic forms of AKI cause cumulative and irreversible damage. In animal models of acute tubular necrosis, renal regeneration is not complete: there is permanent reduction in vascular density and compromised oxygen delivery. [187] [188] Regeneration of tubular epithelial cells can result in sustained fibroblast activation, leading to progressive fibrosis even after the initial insult has subsided. [189] There is now ample epidemiologic evidence associating AKI with the development of CKD, leading some authors even to suggest that the rising incidence of AKI may be partly responsible for the nationwide increase in CKD and ESRD. [190]

2. STUDY AIMS

The aims of the study were: to evaluate the performance of endogenous biomarkers of kidney function other than serum creatinine, i.e. cystatine C and beta-trace protein, in preterm newborns; to evaluate kidney volume measured by ultrasonography as a nephronic mass surrogate in preterm newborns; and to assess the impact of pre-natal and post-natal potentially kidney-detrimental factors on kidney function of preterm newborns.

3. SUBJECTS AND METHODS

We performed an observational longitudinal study in the Neonatal Intensive Care Unit (NICU) of Maggiore della Carità University Hospital, Novara, a tertiary level perinatal center. We enrolled all the preterm babies with a GA ≤32 weeks born between September 2015 and December 2017. Exclusion criteria were the presence of prenatally detected kidney anomalies and complex syndromes or congenital anomalies.

Preterm babies were enrolled at birth and assessments were performed at two time points: T0, on the 3rd day of life, and T36, when they reached a GA of 36 weeks. The timing of T0 was chosen on the basis that it is known that serum creatinine values are influenced by maternal ones in the first 48-72 hours after birth. Babies who were discharged or moved to other hospitals or died before 36 weeks GA only had the T0 assessment.

Anamnestic data

From medical records, we collected for each subject the following data: gender, GA, birth weight and birth weight category (AGA, if birth weight was appropriate for gestational age; SGA, if small for gestational age, with a birth weight lower than 10th centile for sex and GA; LGA, if large for gestational age, with a birth weight higher than 90th centile), single/twin pregnancy, delivery mode, mother's and father's age at the time of delivery, use of medications during pregnancy -in particular potentially nephrotoxic drugs-, positive family history for arterial hypertension (within the second degree of kinship), history of intrauterine growth restriction (IUGR) or maternal pre-eclampsia, maternal serum creatinine value at the time of delivery. GA was determined by fetal ultrasound and projected date of delivery by last menstrual period. INeS charts [191] were used to evaluate birth weight category. Nephrotoxic drugs administered to the mothers included betamethasone [192], non-steroidal anti-inflammatory drugs [193], anti-hypertensive drugs [194], anti-microbial agents [195].

Clinical course data

From our NICU dataset, we collected for each subject the following data: the need for invasive ventilation and its duration, the need for non-invasive ventilation and its duration, the length of need for O2-support, the development of bronchopulmonary dysplasia (BPD), the development of sepsis, the use and duration of antibiotic therapy – especially nephrotoxic drugs (gentamicin, vancomycin) -, the presence of patent ductus arteriosus (PDA), needing a pharmacological (ibuprofen) or surgical approach, the development of necrotizing enterocolitis (NEC), the development of intra-ventricular hemorrhage (IVH), the length of NICU staying.

The durations are expressed as number of days. The Yes/No items were used to create two kidney injury risk scores (each "Yes" answer accounting for 1 point), prenatal and postnatal ones, given by the sum of all the "Yes" answers to the presence of potential risk factors for kidney injury (Table 2). A total score was finally calculated by the sum of the pre-natal and post-natal kidney injury risk scores.

Table 2. Items considered in pre-natal and post-natal kidney injury risk scores: each Yes answer accounts for 1 point.

Pre-natal kidney injury risk score	Post-natal kidney injury risk score
Intrauterine growth restriction (IUGR)	Born small for gestational age (SGA)
Maternal pre-eclampsia	Need for invasive ventilation
Nephrotoxic medications during pregnancy	Need for non-invasive ventilation
Positive family history for arterial hypertension	Bronchopulmonary dysplasia
	Sepsis
	Use of nephrotoxic antimicrobic agents
	Ibuprofen for patent ductus arteriosus (PDA)
	Surgery for patent ductus arteriosus (PDA)
	Necrotizing enterocolitis (NEC)
	Intra-ventricular hemorrhage (IVH)

Anthropometric measurements

Weight and length were measured at both T0 and T36. Birth weight was measured using an electronic weighing scale integrated into the incubator until the babies were taken in the incubators, and then by an electronic infant weighing scale. The length measurement (head to toe) was carried out with the infant lying supine and with the body, hips, and knees straightened; measurements were taken twice and then averaged. Body surface area (BSA) was calculated according to Haycock formula: BSA (m2) = 0.024265 x L (cm) $^{0.3964}$ x weight (kg) $^{0.5378}$.

Biochemical parameters

Venous or arterial blood samples were collected at T0 and T36 for clinical reasons, and serum creatinine (sCr), cystatin C (CysC), beta-trace protein (BTP) and urea were measured.

sCr: was measured on ADVIA Chemistry 1800 or ADVIA Chemistry XPT with an enzymatic assay. This method is considered more specific and reliable than all the Jaffé modified methods. The analytical variability coefficient was < 3%.

Cys C: was measured with BN II/ BN ProSpec® by amplified latex immunophelometry.

BTP: was measured on serum sample with N Latex BTP system. N Latex BTP is BTP specific.

Urea: was measured on ADVIA Chemistry 1800 or ADVIA Chemistry XPT using the glutamate dehydrogenase lined enzyme assay system. The analytical variability coefficient was < 2.5 %.

Estimated glomerular filtration rate (eGFR) was calculated according to 9 existing formulas, including creatinine in 2 cases, CysC in 5 cases, BTP in 1 case and combining sCr and CysC in 1 case. The applied formulas were:

1. **eGFR Schwartz 2009:** 0.413 x lenght/sCr [196]
2. **eGFR Brion:** 0.33 x lenght/sCr [197]
3. **eGFR Schwartz 2012:** 70.69 x (CysC) $^{-0.931}$ [198]
4. **eGFR Zappitelli:** 75.94 x (CysC) $^{-1.170}$ [199]
5. **eGFR Filler:** 91.62 x (CysC) $^{-1.123}$ [200]
6. **eGFR Dorum:** 74.835 x (CysC) $^{-0.750}$ [201]
7. **eGFR Treiber:** [(TKV/BSA)/CysC]/1.73 [202]

8. **eGFR Benlamri:** $10^{(1.902+(0.9515 \text{ x LOG}(1/\text{BTP})))}$ [203]

9. **eGFR Zappitelli-combined:** $(43.82 \text{ x } e^{0.003 \text{ x L}}) / (\text{CysC}^{0.635} \text{ x sCr}^{0.547})$

[204]

Urinary parameters

Only at T36 a urine sample was collected, due to the difficulty to collect an adequate urine volume at T0. Urine samples were centrifuged to remove particulates and assayed immediately or aliquoted and stored frozen. On urine samples creatinine (uCr), albumin (uAlb), protein (uProt), beta2microglobulin (uB2M) and KIM-1 were measured. These values were normalized to uCr concentration.

uCr: was measured on ADVIA Chemistry 1800 or ADVIA Chemistry XPT with an enzymatic assay, which is considered more specific and reliable than all the Jaffé modified methods. The analytical variability coefficient was $< 3\%$.

uAlb: was measured on ADVIA Chemistry 1800 or ADVIA Chemistry XPT using an immunoturbidimetric assay system. The analytical variability coefficient was $<5.5\%$.

uProt: was measured on ADVIA Chemistry 1800 or ADVIA Chemistry XPT using a dye-binding (pyrogallol red) assay. The analytical variability coefficient was $<5\%$.

uB2M: was measured on LIAISON® analyzer using an immunoassay kit.

KIM-1: was measured by a human ELISA kit (BioVendor, Laboratorni Medicina a.s., Brno, Czech Republic), as recommended by the manufacturer.

uAlb/cCr was considered pathological if >0.3, uProt/uCr if >0.5, uB2M/uCr if >100, and uKIM-1/uCr if >0.0036 [Pennemans V, Rigo JM, Faes C, Reynders C, Penders J, Swennen Q. Establishment of reference values for novel urinary biomarkers for renal damage in the healthy population: are age and gender an issue? Clin Chem Lab Med. 2013Sep;51(9):1795-802.].

Kidney ultrasonography

At T0 and T36 a kidney ultrasonography (US) was performed to evaluate kidney size. Philips HD7XE Ultrasound System with a compact (small footprint) curved linear 5–8 MHz frequency transducer was used. To avoid inter-observer bias

during scanning and measuring, all ultrasound scans of the kidneys were performed by a single neonatologist, skilled in renal US.

Renal length, anteroposterior diameter (width), and transverse diameter (depth) were measured for both kidneys. Kidney volume (KV; ml) was calculated by the equation for an ellipsoid:

volume = length x width x depth x $\pi/6$.

Total kidney volume (TKV) was calculated by the sum of left and right kidney volumes, and its ratios to weight, length and BSA were calculated too.

Statistical analysis

Data were expressed as mean ± SD, for continuous variables, and as number and percentage (%), for categorical variables.

For some analyses, subjects were divided into two subgroups according to gestational age (≤ and > 28 weeks). Comparisons between groups were performed using Kruskal-Wallis ANOVA and Mann-Whitney U test, as appropriate. Comparisons between paired data (T0 vs T36) were performed by Wilcoxon signed rank test. Correlations between continuous variables were evaluated by Spearman rank order correlation.

A p value of < 0.05 was considered statistically significant. All analyses were performed using SPSS version 21.0 (IBM, New York, NY, USA).

4. RESULTS

We enrolled 71 newborns (M:F=37:34), admitted from December 2015 to January 2018 to Maggiore della Carità Hospital NICU, born between 24 and 32 weeks of gestational age (mean GA 28.5±2.16). Out of them, 31 (43.7%) had a gestational age ≤ 28 weeks. For all the 71 enrolled newborns anamnestic, anthropometric, biochemical and US data were collected at T0. At T36 clinical course, anthropometric, biochemical, urinary and US data could be collected for 53 patients, since 5 newborns (7%) were dead and 13 (18.3%) were back-transferred before 36 weeks GA to other peripheral second-level Neonatology Unit.

Anamnestic data

The delivery mode was vaginal eutocic delivery in 13 newborns (19.4%), vaginal dystocic delivery in 1 (1.5%), and caesarean section in 53 (79.1%). Sixty-one neonates were singletons (85.9%) and 10 were twins (14.1%). Prenatal data were drawn from medical records: a history of intra-uterine growth restriction (IUGR) was reported in 12 neonates (16.9%), pre-eclampsia of the mother in 20 (28.2%), administration of at least one potential nephrotoxic drug in 54 mothers (76.1%). In 25 newborns (35.2%) a positive family history (within the second degree of kinship) for arterial hypertension was reported. Seven newborns (9.9%) were classified as SGA. Mean mother's age at the delivery was 33.9 ± 5.5 years, and mean father's age was 36.5 ± 6.9 years.

Mean serum creatinine level of the mother at the time of delivery was 0.64 ± 0.25 mg/dl (range 0.30-1.41), being significantly higher in pre-eclamptic mothers (0.82 ± 0.26 vs 0.51 ± 0.13 mg/dl, p<0.0001).

Mean creatinine level of the newborns measured in the first day of life was 0.92 ± 0.22 mg/dl (range 0.36-1.63), without difference according to gender or gestational age. It was positively correlated with maternal creatinine levels at the time of delivery (R=0.593, p<0.0001).

Anthropometric data

Anthropometric data at T0 and T36 are shown in Table 3.

At T0, all anthropometric parameters were significantly lower in newborns with GA≤28 w (p<0.0001 for each), whereas at T36 they were not different in the two GA groups.

No differences were found in any of the anthropometric measures according to gender at T0, whereas at T36 males had higher weight, height and BSA (p=0.03 for each).

Table 3. Anthropometric data in the overall population and in subjects with GA ≤ or >28 weeks, at T0 and T36.

	T0			T36		
	Overall (n=71)	GA≤28 w (n=31)	GA>28 w (n=40)	Overall (n=53)	GA≤28 w (n=25)	GA>28 w (n=28)
Weight (g)	1084±380 (490-2236)	829±215[*] (490-1450)	1281±363[*] (580-2236)	1863.7±358.3 (950-2795)	1861±373 (1360-2795)	1866±350 (950-2460)
Length (cm)	36.4±4.1 (28-46)	33.5±3.2[**] (28-40)	38.6±3.4[**] (30-46)	41.8±2.7 (36-48)	41.8±2.5 (37-48)	41.8±2.8 (36-47)
BSA (m2)	0.104±0.024 (0.062-0.169)	0.088±0.015[§] (0.062-0.128)	0.117±0.216[§] (0.069-0.169)	0.149±0.018 (0.097-0.196)	0.149±0.018 (0.126-1.196)	0.148±0.019 (0.097-0.180)

[*], [**], [§] p<0.0001

Clinical course data

Clinical course data are shown in Table 4.

The pre-natal and post-natal kidney injury risk scores were evaluated from some anamnestic and clinical course data, and the total kidney injury risk score was calculated. In the overall group, the mean pre-natal, post-natal and total scores were 1.5±0.9, 3.6±1.6 and 5.1±2.1, respectively. Comparing the group of subjects with GA ≤28 weeks and >28 weeks, the pre-natal score was similar (1.4±0.9 vs 1.6±0.9, NS), whereas the post-natal and the total scores were significantly higher in the subjects with lower GA (post-natal score 4.5±1.6 vs 2.9±1.3, p<0.0001, total score 5.8±2.1 vs 4.5±1.8, p=0.02).

Table 4. Clinical course data of the 53 subjects at T36, in the overall group and according to GA.

	Overall (n=53)	GA≤28 w (n=25)	GA>28 w (n=28)	p
Need for invasive ventilation (n, %)	30 (56.6%)	17 (68%)	13 (46.4%)	NS
Duration of invasive ventilation (days, mean±SD)	8.1±13.9	12.4±15.9	4.4±10.8	0.02
Need for non-invasive ventilation (n, %)	48 (90.6%)	25 (100%)	23 (82.1%)	0.02
Duration of non-invasive ventilation (days, mean±SD)	17.5±15.9	29.1±15.4	7.2±6.6	<0.0001
length of need for O2-support (days, mean±SD)	22.8±44	37.3±51.7	9.9±31.4	0.007
BPD (n, %)	14 (26.4%)	12 (48%)	2 (7.1%)	0.001
Sepsis (n, %)	9 (17%)	6 (24%)	3 (10.7%)	NS
Length of antimicrobial therapy (days, mean±SD)	17.7±14.6	24.3±17.2	11.8±8.6	0.002
Use of nephrotoxic antibiotics (n, %)	50 (94.3%)	24 (96%)	26 (92.9%)	NS
PDA treated with ibuprofen (n, %)	19 (35.8%)	13 (52%)	6 (21.4%)	0.02
PDA treated with surgery (n, %)	5 (9.4%)	4 (16%)	1 (3.6%)	NS
NEC (n, %)	5 (9.4%)	4 (16%)	1 (3.6%)	NS
IVH (n, %)	12 (22.6%)	9 (36%)	3 (10.7%)	0.03
Length of NICU staying (days, mean±SD)	65.2±34.5	83±35	50.7±26.7	<0.0001
Parenteral nutrition (n,%)	40 (75.5%)	25 (100%)	15 (53.6%)	<0.0001

Biochemical and US data

Biochemical and US data at T0 and T36 data are shown in Table 5.

No differences were found for any of the biochemical and US data according to gender at T0, whereas at T36 TKV was significantly higher in males than in females (p=0.03).

At T0, newborns with GA≤28 weeks had higher sCr levels and TKV/weight than those with GA>28 weeks (p=0.016 and p=0.023, respectively). At T36, newborns

with GA≤28 weeks had lower sCr, BTP and higher urea levels than those with GA>28 weeks (p=0.007, p=0.005 and p=0.029, respectively).

All the other biochemical and US data were not different according to gestational age both at T0 and T36.

Table 5. Biochemical and US parameters at T0 and T36, in the overall population and according to GA.

	Overall			GA≤28 w			GA>28 w		
	T0 (n=71)	T36 (n=53)	p	T0 (n=31)	T36 (n=25)	p	T0 (n=40)	T36 (n=28)	p
sCr (mg/dl)	0.83±0.22	0.30±0.07	<0.0001	0.88±0.22	0.27±0.07	<0.0001	0.79±0.22	0.33±0.05	<0.0001
CysC (mg/l)	1.57±0.33	1.46±0.29	NS	1.62±0.41	1.38±0.35	0.008	1.54±0.25	1.55±0.19	NS
BTP (mg/l)	1.484±0.464	1.299±0.381	NS	1.339±0.418	1.128±0.306	NS	1.583±0.474	1.48±0.374	NS
Urea (mg/dl)	22.3±1.4	9.8±6.9	NS	25±12.9	12.5±7.9	NS	21.3±11.3	7.36±4.84	NS
TKV (ml)	11.66±4.57	16.59±5.24	<0.0001	10.27±4.09	17.69±5.84	0.001	12.67±4.69	15.6±4.56	0.002
TKV/height (ml/cm)	0.314±0.104	1.223±0.433	<0.0001	0.302±0.113	1.275±0.558	0.001	0.323±0.097	1.17±0.27	<0.0001
TKV/weight (ml/g)	0.011±0.004	27.405±10.326	<0.0001	0.012±0.004	28.337±12.96	0.001	0.009±0.002	26.519±5.238	<0.0001
TKV/BSA (ml/m^2)	109.46±34.34	342.32±122.87	<0.0001	114.44±42.85	355.04±156.66	0.001	105.87±26.69	330.24±81.4	<0.0001

eGFR values estimated according to nine previously described formulas are shown in Table 6. No differences were found in newborns with GA≤28 weeks and >28 weeks calculating eGFR by the four formulas using only CysC. Conversely, eGFR values estimated by other formulas were higher in subjects born at a lower GA.

Table 6. eGFR values at T36 calculated by nine different formulas, in the overall population and according to GA. All eGFR are expressed in ml/min/1.73 m^2.

	T36			
	Overall (n=53)	GA≤28 w (n=25)	GA>28 w (n=28)	p
eGFR Schwartz 2009	59.5±15.1	65.7±17.1	53.2±9.5	0.012
eGFR Brion	47.5±12	52.5±13.6	42.5±7.6	0.012
eGFR Schwartz 2012	51.8±12	55.7±15	47.6±5.3	NS
eGFR Zappitelli	51.9±15.7	56.9±19.6	46.3±6.5	NS
eGFR Filler	63.5±18.3	69.3±22.9	56.9±7.6	NS
eGFR Dorum	48.8±17.2	54.3±21.6	42.6±6.8	NS
eGFR Treiber	45.2±16.5	51.1±19.7	37.9±6.4	0.04
eGFR Benlarmi	66.9±18	75.3±18	58±13.4	0.005
eGFR Zappitelli-combined	80.1±20.9	88.7±23.8	69.4±9.5	0.009

Urinary data

Urinary parameters are reported in Table 7.

No differences were found for all urinary parameters according to GA groups.

Considering the percentages of pathological ratios, we found that uAlb/uCr was pathological in 13.6% of overall newborns (19% in newborns with GA≤28 w vs 8.7% in those with GA >28 w, NS), uProt/uCr was pathological in 76.7% of overall newborns (80% in newborns with GA≤28 w vs 73.9% in those with GA >28 w, NS), uB2M/uCr was pathological in 75% of overall newborns (78.9% in newborns with GA≤28 w vs 71.4% in those with GA >28 w, NS), and uKIM-1/uCr was pathological in all the newborns.

Table 7. Urinary parameters according in the overall group and according to GA at T36.

	T36		
	Overall (n=53)	**GA≤28 w (n=25)**	**GA>28 w (n=28)**
uAlb/uCr	0.13±0.12 (0.01-0.58)	0.12±0.1 (0.01-0.39)	0.16±0.14 (0.02-0.3)
uProt/uCr	0.96±0.68 (0.18-3.8)	0.9±0.78 (0.18-3.8)	1.02±0.57 (0.25-2.31)
uB2M/uCr	261.4±217.6 (18.2-1052.6)	220.4±145.3 (18.2-472.9)	306.6±273.8 (18.3-1052.6)
uKIM-1/uCr	0.084±0.046 (0.026-0.203)	0.085±0.042 (0.032-0.157)	0.084±0.049 (0.026-0.203)

Correlation analyses

At T0, sCr values were positively correlated with CysC and BPT levels (R=0.415, p=0.01 and R=0.274, p=0.04, respectively). CysC and BTP were not correlated. Neither sCr nor CysC nor BTP correlated with urea. At T0, sCr was negatively correlated with GA (R= -0.315, p=0.009), whereas both CysC and BTP were not influenced by GA.

T0 levels of sCr, CysC and BTP did not correlate with either maternal sCr levels or anthropometric parameters at birth, even when adjusted for GA.

At T0, sCr levels were negatively correlated with TKV, TKV/length and TKV/BSA (R= -0.344, p=0.008, R= -0.333, p=0.01 and R= -0.258, p=0.049, respectively). Conversely, urea levels at T0 were positively correlated with TKV (R=0.615, p=0.04). Neither CysC nor BTP showed significant correlation with any of the US parameters.

TKV at T0 was positively correlated with GA (R=0.427, p=0.001), birth weight, length and BSA (R=0.6, p<0.0001 for each).

At T36, sCr values were positively correlated with CysC and BPT levels (R=0.527, p=0.001 and R=0.494, p=0.003, respectively), similarly to T0. CysC and BTP were directly correlated (R=0.531, p=0.001). Neither sCr nor CysC nor BTP correlated with urea at T36. Levels of sCr, CysC, BTP and urea did not correlate with body weight, length and BSA at T36. Biochemical parameters and kidney volumes were not correlated at T36. The T36 levels of sCr, CysC and BTP were all directly correlated with GA (R=0.469, p=0.001; R=0.317, p=0.046; and R=0.482, p=0.002, respectively).

Among urinary parameters, uProt/uCr was positively correlated with both BTP and urea at T36 (R= 0.404, p=0.02, and R=0.503, p=0.03, respectively). uProt/uCr was also positively correlated with TKV/weight (R=0.346, p=0.039) and TKV/BSA (R=0.330, p=0.049). uKIM-1/uCr was positively correlated with TKV/weight (R=0.509, p=0.018), TKV/length (R=0.453, p=0.039) and TKV/BSA (R=0.492, p=0.023).

eGFR according to any formula did not correlate with anthropometric parameters at T36. eGFR was negatively correlated with GA (R= -0.389, p=0.01 for Schwartz2009's formula, R= -0.389, p=0.01 for Brion's formula, R= -0.371, p=0.048 for Treiber's formula, R= -0.424, p=0.01 for Zappitelli-combined formula, and R= -0.458, p=0.003 for Benlarmi's formula; eGFR estimated according to all the other formulas tended to be negatively correlated, p=0.05), but the correlation did not persist when adjusted for weight, length, and urea levels at T36.

Both post-natal and total kidney injury risk scores were negatively correlated with GA (R=-0.552, p<0.0001, and R=-0.296, p=0.012, respectively).

Anthropometric measures at T36 are influenced by the kidney injury risk scores. Indeed, weight at T36 was negatively correlated with pre-natal, post-natal and total scores (R= -0.533, p<0.0001; R= -0.337, p=0.02; and R=-0.389, p=0.005, respectively), also when adjusted for GA. Likewise, length at T36 was negatively correlated with pre-natal and total scores (R= -0.422, p=0.003; and R=-0.434, p=0.002, respectively), also when adjusted for GA. Similarly, BSA at T36 was negatively correlated with pre-natal, post-natal and total scores (R= -0.518,

p<0.0001; R= -0.357, p=0.015; and R=-0.480, p=0.001, respectively), also when adjusted for GA.

No correlation was found between the kidney injury risk scores and kidney volumes.

We evaluated the impact of kidney injury scores on eGFR estimated according to different formulas. The pre-natal score did not correlate with eGFR calculated with any formulas. We found a direct correlation between the post-natal and total scores and eGFR estimated according to Schwartz 2009 (R=0.345, p=0.027 for both) and Brion's formulas (R=0.312, p=0.044 for both). However, these correlations did not persist when adjusted for weight, length and BSA at T36. The correlations did not persist also when adjusted for urea levels at T36 and GA. Conversely, no significant correlations were found between the scores and eGFR according to the other formulas.

5. DISCUSSION

In our study, data from a cohort of preterm babies admitted to NICU born between 24 and 32 weeks of GA, were collected in the 3rd day of life and at a post-menstrual age of 36 weeks.

The first aim of the study was to evaluate in preterm newborns the performance of endogenous biomarkers of kidney function, serum cystatine C and beta-trace protein, in addition to traditional serum creatinine measurement. We found that CysC and BTP levels measured in the 3rd day of life in our NICU population are comparable to the reference ranges reported in literature for premature infants of similar gestational age[140].

In our population we found that at T0 only sCr values were negatively correlated with GA, whereas CysC and BTP were not. None of the biomarkers were influenced by gender or anthropometric parameters at T0. Our results are consistent with previous data by Armangil et al in a cohort of preterm newborns with a mean GA slightly higher than ours [205]. Similarly, Elmas et al reported that CysC values measured on the 3rd day of life were independent of gestational age, birth weight and gender [206]. The lack of correlation we found between BTP and GA in our cohort was previously described by Filler et al both in preterm and term newborns [207].

Creatinine levels on the 1st day of life largely reflects maternal levels, making this marker useless in clinical practice to assess alterations in kidney function of the newborn and supporting the evidence that creatinine crosses the placenta [208]. Soon after birth, there is a strong correlation between maternal and newborn creatinine which is lost subsequently: in fact in our study we found no correlation between maternal Cr and newborn's Cr on the third day of life.

At T36, sCr and BTP levels were lower in subjects with lower GA. One could hypothesize that the lower sCr values may be the hallmark of a worse nutritional state and of a reduced muscle mass. Actually, the higher urea levels in infants with GA ≤28 weeks seem to contradict this hypothesis. Therefore, it could be argued that the reduced levels of sCr and BTP found in subjects with lower GA may indicate a better kidney function. In our cohort CysC values are independent of GA at T36. All the three biomarkers are not influenced by anthropometric measures at T36. Intuitively, as a consequence of the lower sCr and BTP levels, in the group of

infants born before 28 weeks GA also the eGFR calculated by formulas using sCr or BTP (Schwartz 2009, Brion, Treiber, Benlarmi and Zappitelli-combined) were significantly higher. Interestingly, we found the unexpected result that eGFR was negatively correlated with GA, apparently indicating that subjects born at a lower GA have a better renal function at T36. However, the correlations did not persist when adjusted for weight, length, and urea levels at T36. Therefore, we may hypothesize that the confounder underlying this association actually is the nutritional status of preterm newborns. Indeed, in subjects born before the 28 weeks of GA anthropometric measures at T0 were significantly lower than in those with GA>28 weeks, but these differences did not persist at T36. This may be probably due to the fact that more premature subjects receive a more aggressive and careful nutritional regimen, as suggested by the higher urea levels found in newborn with GA≤28 weeks at T36. This hypothesis may also be supported by the significantly higher percentages of subjects receiving parenteral nutrition in the group born before 28 weeks of GA. Indeed, all newborns with GA≤28 weeks received parenteral nutrition, whereas only half of the subjects with GA >28 weeks did.

Furthermore, it has to be highlighted the high variability in eGFR calculated according to the nine formulas, both in the overall population and into the GA groups. Abitbol et al [149] showed in preterm and term infants that Cr-based equations consistently underestimated GFR, whereas CysC and combined equations were more consistent with reference inulin clearance studies.

In our study, kidney function at T36 was also evaluated in terms of urinary excretion of proteins, potentially aiming to differentiate a glomerular (albumin) from a tubular (B2M, KIM-1) damage. We found no differences in any of the urinary markers according to GA. However, values higher than upper limits may be noted for total urine protein, albumin, B2M and KIM-1, all corrected for urinary creatinine values. And when pathological ratios are considered, over three fourth of overall newborns showed pathological levels of proteinuria, that may be mainly due to the tubular components, as we can see by the fact that only 13.6% of subjects had pathological albuminuria, whereas normalized B2M and KIM-1 values were abnormal in 76.7% and 100% of newborns, respectively. Therefore, preterm birth and NICU staying seem to impact more on tubular than on glomerular function.

Up to now, only a few studies have investigated the occurrence of proteinuria following preterm birth. A high variation in urine albumin levels among preterm neonates has been shown, with the highest levels in those with a lower gestational age at birth and those that were clinically unstable [209] [210] [211]. Urinary levels of B2M have also been shown to be significantly higher in the preterm than in term newborns throughout the first month of life [211] [212]. Also pathological proteinuria has been previously reported in preterm neonates, possibly associated with renal immaturity and/or acute kidney injury.[213]

The second aim of our study was to evaluate kidney volume measured by ultrasonography as a nephronic mass surrogate. The main limitation to this aim was the lack of a gold standard, because we did not know the actual nephron number of our patients, being it possible only in post-mortem evaluations. Nonetheless, at T0 we found that sCr levels were negatively correlated with kidney volumes. In the recent paper by Ardissino et al [214], the Authors hypothesized that sCr measured in the first 2-4 days of life, representing the period of maximum physiological perinatal dehydration, could be a reliable indirect measure of nephron endowment in full-term newborns. Likewise, our results confirm such hypothesis in a cohort of preterm newborns, assuming that kidney volume is considered a proxy measure of nephron number. However, this seems no more true at a post-menstrual age of 36 weeks, when the essential unmasking effect of dehydration is missing and the link between sCr and kidney volume does not persist.

We found that, at T0, TKV was positively correlated with both GA and anthropometric measures. A smaller kidney volume in neonates with lower gestational age was also reported by Kandasamy et al [85]. They also reported that preterm babies <32 weeks GA showed a higher TKV pro body weight at 38 weeks compared to ones at term, suggesting that premature infants might display a subsequent compensatory kidney hypertrophy. Conversely, we did not found differences in TKV/weight at T36 according to GA. Previous studies [215] [216] have shown that low birth weight is associated with reduced kidney size. Spencer et al [216] estimated an increase in kidney volume of about 15ml/1,73 m2 for every kilogram increase in birth weight. However, we did not found differences in kidney volume according to the history of IUGR or SGA, probably due to the low number

of IUGR and SGA subjects in our cohort.

Finally, we aimed to assess the possible impact of pre-natal and post-natal potentially kidney-detrimental factors on kidney function of preterm newborns. Both prenatal and postnatal factors may impair kidney maturation and nephron endowment in preterm newborns, since many events potentially impairing renal function may occur during the pregnancy and the post-natal clinical course of premature infants. In our cohort, we found higher post-natal and total kidney injury risk scores in newborns with lower GA, as intuitively foreseeable in more premature babies with a longer NICU staying. Therefore, we expected that subjects born at a lower GA and facing more potentially kidney-detrimental events in post-natal life would have an impaired kidney function at T36, as estimated by eGFR. Surprisingly, we found a direct correlation between post-natal and total scores and eGFR estimated according to Schwartz 2009's and Brion's formulas. However, these correlations did not persist when adjusted for weight, length and BSA. Conversely, no significant correlations were found between the scores and eGFR according to the other formulas. Indeed, it has to be pointed out that the Schwartz 2009's and the Brion's formulas rely on sCr values, that are known to be influenced by nutritional status and muscle mass. Actually, in our cohort, sCr at T36 was significantly lower in subjects with lower GA. Moreover, such correlations did not persist also when adjusted for urea levels at T36, probably for the same reasons already explained for the inverse correlation between eGFR and GA. eGFR at T36 does not seem to be influenced by prenatal factors. Moreover, neither prenatal nor post-natal factors influenced kidney volumes at T36 in our cohort.

Limitations

The post-natal variables considered as potentially kidney-detrimental are basically hallmarks of a more rough and troubled NICU course. Therefore, they probably impair other functions of the preterm organism, and it would be difficult to entangle the additive or synergic effects of these alterations. Moreover, due to the limited sample number, we could not assess the singular contribution of all the considered risk factors on renal function.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

As almost 13 million infants worldwide are born prematurely each year, it is important to identify those at higher risk for developing CKD due to congenital or acquired low nephron number. Therefore, it would be important to find in preterm newborns the best biomarker, both in terms of sensitivity and specificity, to identify early signs of AKI, as well as of a chronically reduced kidney function.

Among the new endogenous biomarkers, CysC seems to be particularly promising, as it is not influenced by GA, gender or anthropometric parameters soon after birth and by gender, anthropometric parameters and nutritional status at 36 weeks of post-menstrual age, when nephronic maturation is supposed to be completed. However, its measurement is quite expensive and not widely spread. On the other hand, sCr is a low-cost and highly available marker, and it would be useful as a surrogate of nephron endowment, in the absence of US evaluation of kidney volume, at least soon after birth. Therefore, we may conclude that the overall best marker of renal function does not exist, but the best one could be chosen according to the preterm patient's characteristics (i.e. GA, post-natal age and others) and according to the neonatologist's clinical query (acute kidney injury, chronic kidney disease).

Finally, in the perspective of the best care for the preterm newborn, it has to be highlighted that the potentially damaging events occurring in pre-natal life have little if no impact on future kidney health. Conversely, post-natal clinical course influences kidney function, but overall importance has to be attributed to the nutritional status of preterm infants. Indeed, subjects born at a lower GA but receiving more intensive and targeted nutritional interventions have a better renal function than subjects with higher GA, who receive parenteral nutrition less frequently.

Future studies are needed to understand which practices during NICU staying need to be changed to preserve future kidney function, and to evaluate which nutritional strategies would be more protective of renal health.

Only a long-term follow-up would be able to assess if a mild but precocious impairment of renal function would influence kidney function and predispose to

the development of arterial hypertension and other non-communicable diseases in later-on life. [217] [218]

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8. APPENDIX I: Published and accepted papers