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Étude clinique des modifications du profil métabolique urinaire secondaires à une anomalie congénitale de l'écoulement des urines par Résonance Magnétique Nucléaire et analyse métabolomique

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Résumé

Les dilatations des voies urinaires de diagnostic anténatal (DVU) peuvent être transitoires, ou correspondre à une anomalie significative de l'écoulement des urines pouvant entrainer une dégradation de la fonction rénale. L'identification de biomarqueurs urinaires pourrait contribuer à différencier précocement les uropathies des dilatations transitoires.

La métabolomique permet l'identification de toutes les molécules de bas poids moléculaire présentes dans un échantillon biologique et la mise en évidence d'une signature métabolique associée à un événement physiopathologique.

L'objectif principal de cette thèse est l'identification de marqueurs urinaires pronostiques chez les nouveaux nés présentant une DVU de diagnostic anténatal, par spectroscopie RMN et analyse métabolomique.

Soixante-dix nouveau-nés ayant une DVU unilatérale et 90 témoins ont été inclus dans cette étude prospective. Tout d'abord, nous comparons la précision de différentes classifications échographiques pour l'évaluation du risque d'intervention chirurgicale. Ensuite, nous montrons l'absence de différence significative entre les profils métaboliques urinaires des nouveau-nés ayant une DVU et ceux des témoins. Dans une troisième partie, nous démontrons l'influence significative de l'âge, du poids et de la taille sur le profil métabolique urinaire des nouveau-nés. Enfin, nous montrons l'évolution du profil métabolique urinaire avec la croissance chez les nourrissons ayant une DVU.

Ce travail permet une meilleure compréhension de la physiopathologie des DVU et de la maturation métabolique des nouveau-nés. Il contribue à identifier des biais potentiels dans les études métaboliques en néonatologie.

Title

Investigation of urine metabolic profiles in newborns with prenatally diagnosed unilateral urinary tract dilatation using ^{1}H NMR spectroscopy and metabolomic analysis.

Abstract

The prenatal finding of unilateral Urinary Tract Dilatation (UTD) can be transient or represent a significant urinary flow impairment that would lead to progressive deterioration of renal function. Identifying urinary biomarkers could help to differentiate uropathy requiring surgical management from transient dilatation at an early stage.

Metabolic phenotyping studies provide untargeted quantification of all detectable low molecular-weight molecules by profiling without any a priori the metabolic signatures of biological samples in connection to physiopathological events.

The main objective of this study is to identify diagnosis and prognosis urinary markers for uropathy in newborns with prenatally diagnosed unilateral UTD using ^{1}H-NMR spectroscopy combined with multivariate statistical analyses.

A total of 70 newborns with unilateral UTD and 90 controls were included in this prospective study. First, the usefulness of different ultrasound grading systems in predicting the need for surgical intervention is evaluated. Then, we report the absence of significant difference between the urinary metabolic profiles of newborns with UTD and controls. In the third part, the influence of age, weight and height on the urinary metabolic profiles of healthy newborns is highlighted for the first time, and key-metabolites responsible for this evolution are identified. Finally, we demonstrate the influence of age on the urinary metabolic profiles of children with UTD.

This work allows a deeper understanding of the metabolic maturation of healthy newborns. It contributes to identify potential confounding factors for metabolomics investigations in neonatology. It represents a step toward a better comprehension of the physiopathology of UTD.

Mots clés

Metabolomique; Spectroscopie par RMN; Urine; Dilatation des voies urinaires; Echographie;

Keywords

Metabolomics; ^{1}H RMN spectroscopy; Urine; Urinary Tract Dilatation; Ultrasonography;

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Table of Contents

1. Metabolomics as tool for translational research in pediatrics and neonatology 15

1.1. Introduction to the metabolomic approach **15**

1.1.1. What is metabolomics ? *15*

1.1.2. A dynamic field of research in pediatrics and neonatology *17*

Applications of metabolomics in neonatology 17

Applications of metabolomics in pediatrics 18

1.2. Clinical context of the study: Urinary tract dilatations **19**

1.2.1. Prenatal diagnosis of urinary tract dilatations *19*

1.2.2. The unpredictable evolution of upper urinary tract dilatation after birth *21*

1.2.3. The biochemical approach to UTD: searching for a reliable biomarker *21*

1.3. Metabolomic methodology **25**

1.3.1. Analytical techniques *25*

1.3.2. NMR investigations of biofluid *26*

Sample preparation 26

NMR data acquisition 27

Metabolites identification 29

Bucketing 30

Data alignment 30

Normalization 31

Scaling 32

Descriptive Statistics 32

Unsupervised analysis 33

Supervised analyses 33

Models validation 34

Biomarkers identification and interpretation 35

2. Case-control metabolomic study of newborns with prenatally diagnosed unilateral urinary tract dilatation 36

2.1. Introduction **36**

2.2. Study protocol **37**

2.2.1. Study population *37*

2.2.2. Samples collection and storage . . . 37
2.2.3. Management of UTD . . . 38

2.3. Prognostic value of ultrasound grading systems in prenatally diagnosed unilateral urinary tract dilatation . . . **38**
2.3.1. Objective . . . 38
2.3.2. Method . . . 39
Statistical analysis . . . 39
2.3.3. Results . . . 40
2.3.4. Discussion . . . 44
2.3.5. Conclusions . . . 47

2.4. Comparison of urine metabolic profiles of children with UTD and controls . . . **47**
2.4.1. Objective . . . 47
2.4.2. Methods . . . 47
Study population . . . 47
Sample preparation . . . 48
1H-NMR spectroscopy of urine samples . . . 48
Data processing . . . 49
Multivariate analysis of urine metabolic profiles . . . 49
Identification of metabolites . . . 49
2.4.3. Results . . . 50
Descriptive Statistics . . . 50
Identification of metabolites . . . 51
Quality of the ^{1}H-NMR data . . . 56
Comparison between ^{1}H-NMR spectra of patients and controls . . . 57
Comparison between the surgical and the non-operative management groups . . . 57
2.4.4. Discussion . . . 59

3. Influence of growth parameters on newborns urinary metabolic profiles . . . 61

3.1. Influence of age, weight and height on urinary metabolome in healthy newborns . . . **61**
3.1.1. Newborns metabolic maturation . . . 61
3.1.2. Experimental section . . . 62
Study population . . . 62
Metabolomic analysis . . . 63
Quality of the ^{1}H-NMR data . . . 63

3.1.3. Metabolic signature associated with age *64*
3.1.4. Metabolic signatures associated with growth parameters. *68*
3.1.5. Influence of gender on the urine metabolomic profiles *73*
3.1.6. Limitations of the study *73*
3.1.7. Conclusions *74*

3.2. Influence of age, weight and height on the urinary metabolome of newborns with urinary tract dilatation **75**
3.2.1. Introduction *75*
3.2.2. Methods *75*
3.2.3. Metabolic signature associated with age in patients with UTD before 4 months . . . *75*
3.2.4. Influence of age on the urinary profile of patients with UTD until 18 months *79*
3.2.5. Influence of growth parameters and gender in patients *85*
3.2.6. Toward improvement of metabolomics investigations in newborns *86*

Conclusion **87**

References **89**

Appendices **109**
Appendix A: Embryology of the renal system *109*
Appendix B: Diagnosis and treatment of uropaties associated with prenatally diagnosed urinary tract dilatation *117*
Appendix C: Authorization from the Commission Nationale de l'informatique et des libertés *125*
Appendix D: Authorization of the Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé *126*
Appendix E: Authorization of the Comité de protection des personnes *127*
Appendix F: The standard for reporting diagnostic accuracy checklist (STARD) *128*
Appendix G: List of metabolites identified on ^{1}H-NMR spectra of newborns urine *130*
Communications *140*

Abbreviations

95% CI:	95% Confidence Intervals
3 HMG:	3-Hydroxymethylglutarate.
ALP:	Alkaline phosphatase
ANOVA:	Analysis of Variance
APD:	Antero-Posterior intrasinusal Diameter of the renal pelvis
AUC:	Area Under the Curve
Ca19-9:	Carbohydrate Antigen 19–9
CPMG:	Carr-Purcell-Meiboom-Gill experiment
CV-ANOVA:	Cross Validatory Analysis of Variance
CyC:	Cystatin-C
DRF:	Differential Renal Funtion
DMSA :	^{99m}Tc dimercaptosuccinic acid
DTPA :	^{99m}Tc di-ethylene-tertra-amine-penta-acetic acid
EDICIS :	Ethylene dicysteine
FID:	Free Induction Decay
GFR :	Glomerular Filtration Rate
γGT:	Gamma-Glutamyl Transferase
HCA:	Hierarchical Clustering Analysis
HO-1:	Heme Oxygenase-1
HSQC:	Heteronuclear Single Quantum Correlation
IP-10:	Interferon-γ-inducible Protein 10
IQR:	Interquartile Range
KIM-1:	Kidney Injury Molecule-1
LR-:	Negative Likelihood Ratio
LR+:	Positive Likelihood Ratio
MAG3:	Mercaptoacetyltriglycine
MCUG :	Micturating cystography
MIP-1α:	Macrophage Inflammatory Protein-1α
NAG:	N-acetyl-beta-D-glucosaminidase

NAP:	N-Acetylputrescine
NGAL:	Neutrophil Gelatinase-Associated Lipocalin
NOESY:	Nuclear Overhauser Effect SpectroscopY
NMR:	Nuclear Magnetic Resonance
NPV:	Negative Predictive Value
O-PLS:	Orthogonal Partial Least Squares / Orthogonal Projections to Latent Structures
OPN:	Osteopontin
OR:	Odds Ratio
PCA:	Principal Component Analysis
PLS:	Partial Least Squares / Projection to Latent Structures
PPV:	Positive Predictive Value
PQN:	Probabilistic Quotient Normalization
PUJ:	Pyelo-Ureteral Junction
QC:	Quality Control
RANTES:	Regulated on Activation Normal T-cell Expressed and Secreted
ROC:	Receiver Operating Characteristic
RR:	Risk Ratio
SFU:	Society for Fetal Urology
SRV:	Statistical Recoupling of Variables
STARD :	Standards for Reporting of Diagnostic accuracy
TGF-β1 :	Transforming Growth Factor Beta 1
TOCSY:	TOtal Correlation SpectroscopY
TSP:	3-trimethylsilyl-2,2,3,3-tetradeuteropropionic acid
UFI:	Urinary Flow Impairment
US:	Ultrasonography
UTD:	Urinary Tract Dilatation
UV:	Unit Variance
VUR:	Vesico-Ureteral Reflux

Introduction

The development of new experimental methods and tools in molecular biology enabled tremendous progress in genomics, transcriptomics and proteomics over the past decades. These holistic approaches aim to describe the content and operation of biological systems at a defined molecular level. These "omics" techniques are used in an untargeted, hypothesis free, top-down approach, without a priori regarding a selected molecule. They intend to delineate the complete assembly of genes, transcripts or proteins to study the overall response of a biological system to a physiological or pathological event. Metabolomics was introduced as the latest of these "omics" approach. Its purpose is to detect and quantify large numbers of small molecules, called metabolites, in a complex biological sample. As they represent the downstream expression of genome, transcriptome and proteome, the quantitative and qualitative pattern of metabolites in body fluids reflects the status of an organism at a specific time. The tremendous amount of data collected by these new technologies can be computed by multivariate statistical analyses to identify specific profiles associated with particular diseases or status. Thus, they represent a promising and dynamic field of translational research. Metabolomics is a precious tool to discover biomarkers with prognosis or diagnosis value, that could therefore be used in clinical practice.

In the field of pediatric urology, the diagnosis of Urinary Tract Dilatation (UTD) is particularly challenging. UTD may be a response to various events occurring throughout the developmental period, and does not necessarily imply significant urinary flow impairment (UFI). Identifying urinary biomarkers could help to differentiate uropathy requiring surgical management from transient dilatation at an early stage and prevent the progressive deterioration of renal function.

This thesis is dedicated to the study of urine metabolic profiles by ^{1}H NMR spectroscopy and metabolomic analysis, in newborns with prenatally diagnosed unilateral UTD.

The first chapter sets the background of the present study. First, we describe the general principles of metabolomics as tool for translational research. Then, the diagnosis challenge associated with UTD is presented. The different steps of the metabolomic approach are detailed in a third part.

The second chapter reports the results of a case-control metabolomic study of newborns with prenatally diagnosed unilateral UTD, undertaken in Hôpital Femme Mère Enfant and Institut des Sciences Analytiques of Lyon. First, the population and study

design are presented. In a second part, the usefulness of different ultrasound grading systems in predicting the need for surgical intervention is evaluated. In the third part, urinary metabolic profiles of newborns with UTD are compared with controls using ^{1}H-NMR spectroscopy combined with multivariate statistical analysis.

In the third chapter of this thesis the influence of age, weight and height on the urinary metabolic profiles of healthy newborns is highlighted, and key-metabolites responsible for this evolution are identified. Finally, we demonstrate the influence of growth on the urinary metabolic profiles of children with UTD.

This work demonstrates the evolution of urinary metabolic profiles during the first months of life in healthy newborns and newborns with prenatally diagnosed UTD. It allows a deeper understanding of the metabolic maturation of healthy newborns, contributes to identify potential confounding factors for metabolomic investigations in neonatology, and brings a new light on the physiopathology of UTD.

1. Metabolomics as tool for translational research in pediatrics and neonatology

1.1. Introduction to the metabolomic approach

1.1.1. What is metabolomics ?

The contributions of laboratory medicine in improving patient care have increasingly become essential in the past decades. Progress in molecular biology, especially in genome sequencing and high-throughput measurements, enabled system–level analyses in genomics, transcriptomics and proteomics. The "omics" technologies consist of holistic approaches on the molecules that make up a cell, tissue or organism, such as genes, transcripts, proteins and metabolites.[1] "Systems biology" seeks to integrate these different levels of information for a global understanding of biological systems.[2] *(Figure 1.1.1)*

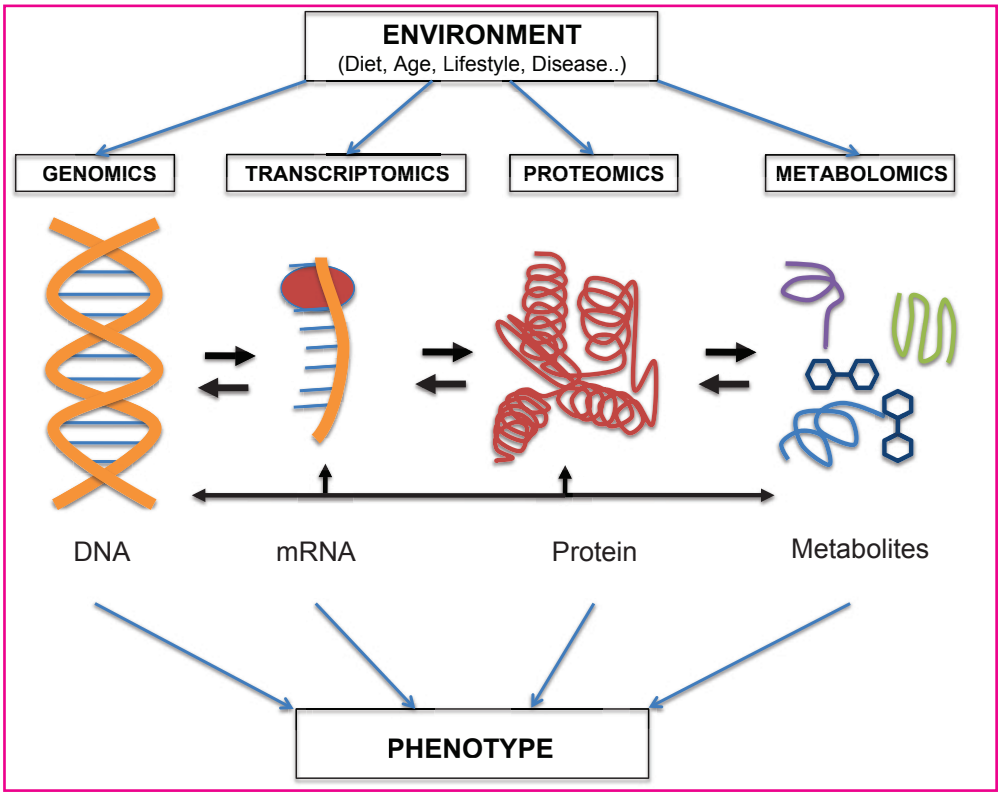


Figure 1.1.1: **The biological organization of "omics" sciences.** *According to systems biology, the flow of information in biology is organized as follows from the genome to the transcriptome, to the proteome and then the metabolome. However, the main biochemical components of each level of organization interact with others and thus can affect one another. Furthermore, the environment plays a crucial role on the genome not only by modifications (i.e. genetic mutations) but also on the expression and concentration of transcripts, proteins and metabolites.*

These perspectives are of extreme importance to improve diagnosis, prognosis and clinical outcome by the routine application of emerging molecular technologies.[3] However, even in combination, genomics and proteomics do not provide the range of information needed for an understanding of the integrated cellular function in living systems, since both ignore the dynamic metabolic status of the whole organism. Thus, metabolomics is aimed at the augmentation and complementation of the information provided by genomics and proteomics.[4]

Metabolites are molecules less than 1 kDa, including peptides, oligonucleotides, sugars, organic acids, ketones, aldehydes, amines, amino acids, lipids, steroids, and alkaloids. Because they represent the downstream expression of genome, transcriptome and proteome, they can closely reflect the phenotype of an organism at a specific time. Metabonomics is defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological events, drug treatments or genetic polymorphisms.[4] Metabolomics seeks an analytical description of complex biological samples, and aims to characterize and quantify all the small molecules in such a sample. In practice, the terms "metabolomics" and "metabonomics" are often used interchangeably.[5]

Metabolomic analysis methods can be applied to the study of various fluids (serum, plasma, urine, cerebrospinal fluid, bile, amniotic fluid, seminal fluid, saliva, cell culture supernatant...),[6-8] as well as biological tissues or full intact organisms (biopsies, cells, nematode Caernohabditis elegans…).[9]

Metabolomic analyses are generally built as case-control studies, comparing groups of samples coming from individuals with different physiopathological states.

The experimental metabolomic approach can be described in three main steps *(Figure 1.1.2)*. First, the simultaneous quantification of metabolites is achieved by means of analytical techniques, providing a metabolic profile for each sample. Metabolites present in the samples are identified. The metabolic profiles are then processed and reduced to numerical data. Finally, the raw data obtained are subjected to uni and multivariate statistical analyses to extract the significant differences in metabolite levels between groups, defining a metabolic signature.

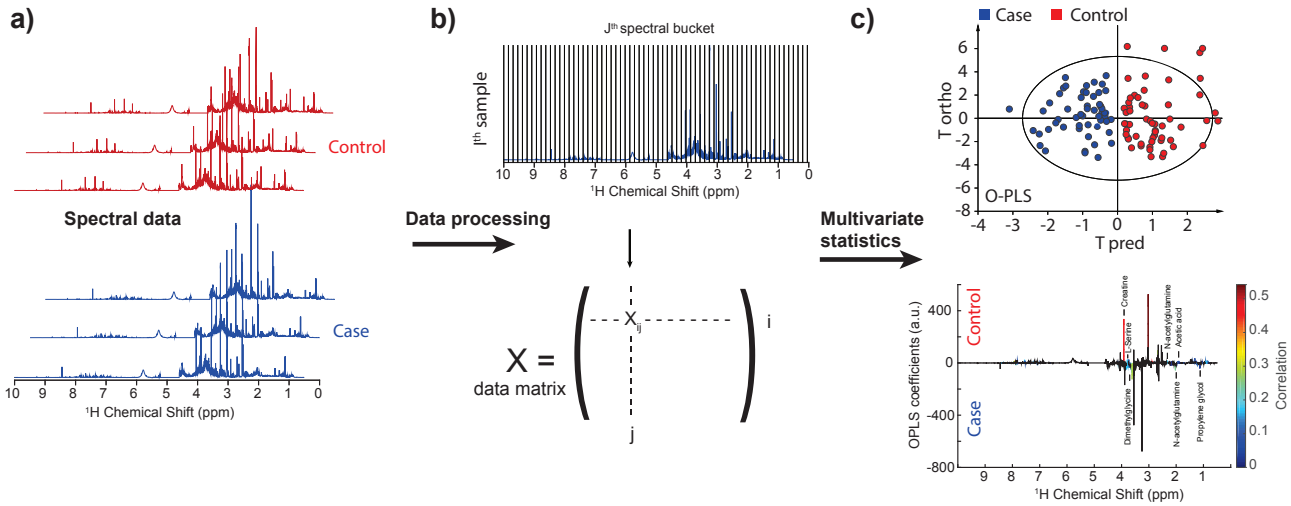


Figure 1.1.2: **The steps of experimental metabolomic approach.** *a) Acquisition of spectral data for cases and controls. b) Reduction of metabolic profiles to numerical data. c) Statistical analyses and identification of metabolic signatures.*

Applications of metabolomics in personalized medicine are very promising, including disease diagnosis, monitoring the effects of medical interventions including drugs or surgery, and analysis of biochemical pathways and their perturbations resulting from mutations, aging, diet, exercise, or lifestyle.[10] A growing number of studies focus on the applications of metabolomics in the fields of pediatrics and neonatology.[3,11-13]

1.1.2. A dynamic field of research in pediatrics and neonatology

Applications of metabolomics in neonatology

Several experimental and clinical studies investigated the influence of Intrauterine Growth Retardation and Low Birth Weight on metabolomic profiling in maternal venous peripheral blood, in blood from umbilical cord and in neonatal urine.[3,14-18] Low birth weight is defined as a birth weight of less than 2500 g. Based on the hypothesis that the risk of developing chronic diseases in adulthood is influenced by environmental factors acting during the periconceptual, fetal and infant phases of life,[19] a large body of research has focused on low birth weight and growth patterns as risk factors for metabolic diseases in adulthood.[20-24]

Other recent studies focused on specific changes in metabolic profiles associated with prematurity.[11,25-28] Specific urinary metabolic signatures associated with prematuri-

ty were reported. Moreover, the metabolomic analysis of plasma from full term and very low birth weight preterm infants revealed that most essential and non-essential amino acids are depleted in very low birth weight infants.[29] Metabolomics was also able to differentiate the urinary profiles of neonates with and without an acute kidney injury diagnosis and to identify metabolic developmental profiles correlated with gestational age.[27]

Perinatal asphyxia has also been studied by means of metabolomic analyses. Banupriya et al. demonstrated that the excretion rate of urinary proteins, urinary malondialdehyde and urinary uric acid increases with the severity of perinatal asphyxia and associated brain damage.[30] Another study reported differences in urinary metabolic profiles of newborns suffering from perinatal asphyxia, according to their clinical outcome.[31]

However, very few studies have investigated the evolution of metabolomic profiles of healthy newborns during the first months of life.[32]

Applications of metabolomics in pediatrics

The application of metabolomic approach in pediatrics is a recent and dynamic field of research. A few studies have investigated the metabolomic profiles of children treated for asthma, using exhaled breath[33-35] or urine.[36,37] A characteristic metabolic profile in the cerebral spinal fluid of children with influenza-associated encephalopathy has been reported.[38] Metabolomic analysis has showed increased urinary excretion of N-methyl-2-pyridone-5-carboxamide, N-methyl nicotinic acid, and N-methyl Nicotinamide in autistic children compared with their unaffected siblings.[39] Other metabolomic studies have focused on celiac disease,[40] cytomegalovirus infection,[41] diabetes,[42] sepsis,[43] and inborn errors of metabolism.[44,45]

Although metabolomics has enormous potential in the field of pediatric nephrology and urology, it has been used only in a very limited number of clinical applications so far. The diagnosis of Urinary Tract Dilatation (UTD) is particularly challenging, and the evolution of urinary metabolomic profiles in children with UTD is yet unknown.

1.2. Clinical context of the study: Urinary tract dilatations

1.2.1. Prenatal diagnosis of urinary tract dilatations

UTD is diagnosed by prenatal ultrasonography in 0.2 - 2% of all pregnancies.[46,47] Dilatation may occur at any level of the collecting system and may be localized to the renal pelvis and/or ureters on one or both sides. *(Figure 1.2.1)*

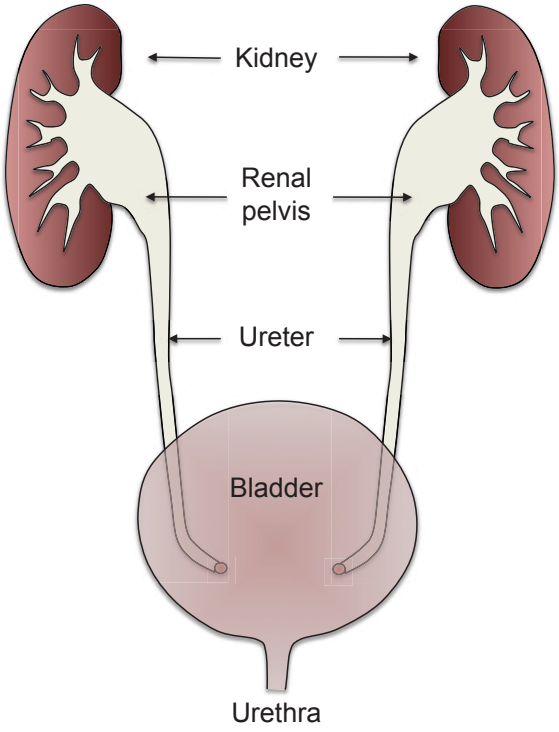


Figure 1.2.1: **Scheme of the urinary tract**

UTD may be a response to various events occurring throughout the developmental period. It does not necessarily imply significant urinary flow impairment (UFI), which may affect the renal development and the renal function. The terms “hydronephrosis” and “uretero-hydronephrosis” are often used when describing the upper urinary tract, but the term “dilatation” seems more accurate, and does not preclude any underlying etiology. Similarly, the term “obstruction” has no consensual definition and should be avoided, as the diagnosis of obstruction can only be made retrospectively.[48]

Prenatal ultrasonography allows detection of UTD, mainly during the second trimester of gestation. The embryology of the renal system is summarized in *Appendix A.*

Although the interpretation of dilatation remains controversial, it is an essential signal of potential postnatal urinary tract pathology (uropathy). Significant UFI that occurs during early kidney development affects renal morphogenesis, maturation and growth.[48] In the most severe cases this will ultimately entail progressive renal tubular atrophy and interstitial fibrosis with loss of nephrons.[49]

The renal pelvis is only readily seen by ultrasonography (US) once the Antero-Posterior Diameter (APD) exceeds 2 mm. *(Figure 1.2.2)* The threshold used for diagnosing UTD based on APD typically varies depending on the gestational age of the fetus, as there is no consensus on the dimensions of the renal pelvis that defines UTD.[46] However, it seems that APD of at least 5 mm in the second trimester and/or 7 mm in the third trimester warrants postnatal investigation.[48] Besides renal pelvis dilatation, prenatal US also gives other information about the renal parenchyma, the ureters and the bladder. Ureters are normally not seen by US during normal development. Visualization of the ureters on prenatal US can therefore be considered a ureteral dilatation. The aspect of the renal parenchyma is another precious indication that can point toward renal dysplasia. Loss of parenchymal differentiation, presence of cysts, or poor renal development may reflect a structural anomaly with impaired renal function.[50] A thickened and dilated bladder associated with a bilateral dilatation of the upper urinary tract can reflect posterior uretral valves, urethral atresia or complex cloacal malformations that are associated with a poor prognosis.[51] US can also reveal the association of UTD with other urological anomalies such as a duplex system, a horseshoe kidney or a ureterocele. An abnormal amniotic fluid volume is also an important sign, as fetal urine accounts for the majority of its production in the second and third trimesters of gestation. Severe loss of urine production may lead to an oligo-hydramnios, which may be the consequence of either a mechanical UFI or a deficient renal function as found in bilateral renal agenesis or placental failure.[51] Following identification of UTD in the fetus a detailed anomaly scan should be performed to exclude the presence of associated extra-renal malformations.

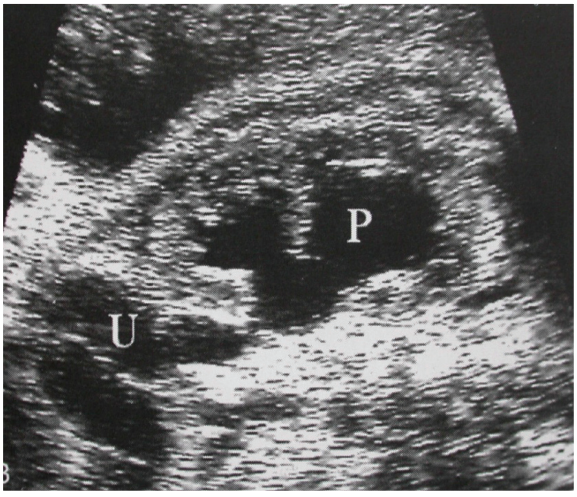


Figure 1.2.2: **Prenatal ultrasonography showing a dilatation of the renal pelvis (P) and the ureter (U)**

1.2.2. The unpredictable evolution of upper urinary tract dilatation after birth

When a UTD is diagnosed by prenatal US, another US must be performed after birth to confirm the presence of the UTD and to evaluate its severity. For unilateral UTD and unless posterior urethral valves are suspected, it is recommended that this first postnatal US be delayed for at least 48 h after birth to avoid underestimating the severity of dilatation.[48] Invasive diagnostic procedures are not required when APD is less than 10 mm and clinical surveillance alone is recommended.[52,53] On the other hand, it is recommended to investigate moderate (10 < APD < 15 mm) and severe (APD ≥15 mm) UTD to detect potential uropathies. The diagnosis of these uropathies can only be assessed by repetitive US and additional imaging including renal isotope studies and possibly micturating cystogram, with significant consequences on childhood radiation, parental anxiety and healthcare resources.[46] At birth, the neonate is sometimes placed on prophylactic antibiotics to prevent the risk of UTI until the cause of dilatation is elucidated.

1.2.3. The biochemical approach to UTD: searching for a reliable biomarker

Currently, serial ultrasound and invasive isotope studies are performed to guide surgical management of UTD, but the ability of these diagnostic modalities to accurately predict the need for surgery is questionable. Additionally, there remains debate regarding the parameters that indicate clinically significant obstruction.[54,55] There is a need to find more exact predictors of long-term development of decreasing renal function. Identifying urinary biomarkers could help to differentiate uropathies requiring surgical management from transient dilatation at an early stage. They may generate early measures of renal injury and functional impairment.[49]

The glomerular filtration rate (GFR) would be a good determinant of renal function and a predictor of postnatal function, but it is challenging to determine GFR accurately in the fetus and there are no normative data for this parameter in uncomplicated pregnancies.[56]

β2-microglobulin has been used as an indirect measure of fetal GFR. It is advantageous to use this antigen for GFR estimation because of its constant production by the fetus, its inability to cross the placental barrier, and its free filtration at the glomerulus.[57] The measure of fetal serum β2-microglobulin has good predictive value for postnatal

renal function. However, its usefulness as a marker is limited because fetal blood sampling carries the risks of bleeding, amniotic fluid leak, infection, and fetal death.

Other molecules have been identified as potential biomarkers, as their urinary concentration in newborns is modified by urinary obstruction. Obstructive nephropathy is not a simple result of mechanical impairment to urine flow but a complex syndrome resulting in alterations of glomerular, hemodynamic and tubular function caused by the interaction of a variety of vasoactive factors and cytokines that are activated in response to obstruction.[58-60]

Early fetal urinary tract obstruction is associated with an increased urinary concentration of tubular mesenchymal markers (transforming growth factor beta 1 (TGF-β1),[61,62] β-catenin, and vimentin), and a decrease in epithelial markers (aquaporin-2 and E-cadherin).[56]

The renal response to ureteral obstruction involves angiotensin-dependent renal vasoconstriction with consequent reduction in glomerular filtration rate, ischemia, hypoxia, and accumulation of reactive oxygen species. This results in tubular oxidative stress, increased production of renin, angiotensinogen, angiotensin, and TGF-β1, leading to proximal tubular atrophy and cell death.[55,63] These are also mediated by production of epidermal growth factor (EGF)[64-67] and increased production of cell adhesion molecules, kidney injury molecule-1 (KIM-1), and neutrophil gelatinase-associated lipocalin (NGAL).[68] *Table 1.2.2* summarizes the modifications of urinary concentrations of potential biomarkers of urinary obstruction in children with PUJ.

Table 1.2.2: **Modifications of urinary concentrations of potential biomarkers of urinary obstruction in children with PUJ. Adapted from Jackson et al 2017.**[69]

Children undergoing pyeloplasty are compared to healthy controls and/or conservatively managed children with PUJ (CMP).
The primary group is labeled PUJO for 3 studies in which the primary group includes children with unilateral UTD, either operated on or managed conservatively.
**Where applicable sensitivity (Se), specificity (Sp) and accuracy (Ac) of the test at best threshold value from receiver operating characteristic curve analysis is presented*
† To detect differential renal function (DRF) < 40% out of all hydronephrosis cases
‡ To detect pyeloplasty cases out of all hydronephrosis cases
§ To detect pyeloplasty cases out of all cases

ALP, Alkaline Phosphatase; Ca19-9, Carbohydrate antigen 19–9; CyC, cystatin-C; HO-1, Heme Oxygenase-1; γGT, Gamma-glutamyl Transferase; IP-10, Interferon-γ-Inducible Protein 10; KIM-1, Kidney Injury Molecule-1; MIP-1α, Macrophage Inflammatory Protein-1α; NAG, N-Acetyl-beta-D-Glucosaminidase; NGAL, Neutrophil Gelatinase-Associated Lipocalin; OPN, Osteopontin; RANTES, Regulated on Activation Normal T-cell Expressed and Secreted

Urinary protein (corrected for creatinine)—	Primary measured group	Comparators	Bladder urine protein l——evel	Sensitivity/ Specificity/ Accuracy *	Post-operative bladder urine (compared to pre-operative)	Ref
ALP	Pyeloplasty	CMP	Increased pre-operative	Se 91.4% Sp 100% Ac 94%	Decreased 12 months post-operative	70
Angiotensinogen	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 93.3% Sp 60% †		63
B2-microglobulin	PUJO*	Healthy control	Increased		Decreased 42 months post-operative	65
B2-microglobulin	Pyeloplasty	Healthy control	No change			71
Ca19-9	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 76% Sp 85% ‡	Decreased 3 months post-operative	72
Ca19-9	Pyeloplasty	Healthy control Hydrocele/ renal cyst	Increased pre-operative	Se 100% Sp 82.6% §	Decreased 3 months post-operative	73
CyC	Pyeloplasty	Healthy control	No change			71
EGF	PUJO*	Healthy control	Decreased (obstructed group only)		No change	65
EGF	Pyeloplasty	Healthy control	Decreased pre-operative		Increased	64
EGF	Pyeloplasty	Healthy control	Increased pre-operative	Se 70.4% Sp 69.2%	Decreased 3 months and 1 year post-operative	66
EGF	Pyeloplasty	Healthy control	No change			61
ET-1	Pyeloplasty	Healthy control VUR Renal stones	Increased pre-operative	Se 74.3% Sp 90% Ac 81.5%	Decreased 12 months post-operative	70
γ GT	Pyeloplasty	CMP	Increased pre-operative	Se 62.9% Sp 100% Ac 74%	Decreased 12 months post-operative	70
HO-1	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 72.2% Sp 78.1% †	Decreased 1 months post-operative	74
IP-10	Pyeloplasty	Healthy control	No change			66
KIM-1	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 100% Sp 71.4% †		75
MCP-1	Pyeloplasty	Healthy control	Increased pre-operative	Se 77.8% Sp 69.2%	Decreased 3 months and 1 year post-operative	66
MCP-1	PUJO*	Healthy control	Increased		Decreased 42 months post-operative	65
MCP-1	Pyeloplasty	Healthy control	Increased pre-operative			64
MCP-1	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 100% Sp 0% ‡	Remains high 3 months post-operative	76
MIP-1alpha	Pyeloplasty	Healthy control	Decreased pre-operative		Increased 1 year post-operative	66
NAG	Pyeloplasty	CMP	Increased pre-operative	Se 97.1% Sp 80% Ac 92%	Decreased 12 months post-operative	70
NGAL	Pyeloplasty	Healthy control	No change			71
NGAL	Pyeloplasty	Healthy control	Increased pre-operative			77
NGAL	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 100% Sp 28.6% ‡	Decreased 3 months post-operative	75
OPN	Pyeloplasty	Healthy control	No change			71
OPN	Pyeloplasty	Healthy control CMP	Increased pre-operative	Se 98.5% Sp 10.5% ‡	Remains high 3 months post-operative	76
RANTES	Pyeloplasty	Healthy control	No change			66
TGF-beta	Pyeloplasty	Healthy control	Increased pre-operative	Se 100% Sp 80% Ac 90.8%	Decreased 1 year post-operative	61
TGF-beta	Pyeloplasty	CMP	Increased pre-operative	Se 82% Sp 86%		78

Although the concentrations of these molecules concentrations in blood and/or urine are modified by urinary obstruction, further investigations are needed to use them as reliable tests to differentiate transient UTD from uropathies and to identify which patient would eventually need surgery. Common flaws of studies investigating potential biomarkers for uropathy are the modest numbers of patients and the lack of consensual definition of urinary obstruction. Moreover, some of the above studies aimed to differentiate between UTD and healthy controls, while others were designed to discriminate between UTD needing surgery and UTD that could be managed conservatively, which is clinically more relevant. For these reasons, none of these studied molecules are currently integrated in clinical practice.

It is unlikely that any single analyte will have sufficient power to become a "gold standard" test for developmental kidney injury, but several biomarkers in combination could provide the best accuracy and predictive value.

In this context, the use of "omics" analysis is promising to find potential biomarkers with diagnosis and prognosis value for UTD.

A proteomic study by Decramer et al. investigated the urinary proteome of 103 newborns with PUJ, showing that the analysis of 51 distinct polypeptides allowed to discriminate between healthy newborns, newborns with PUJ who required surgery and newborns with PUJ who did not require surgery.[79] Although a clear definition of obstruction and indications for surgery were lacking in this study, it opens interesting perspectives regarding the use of proteomics for biomarkers identification in newborns with UTD. However, a similar analysis of urine from older children requiring pyeloplasty failed to discriminate them from PUJ treated conservatively, showing that the test result based on an infantile proteome pattern cannot be extended to older patients.[80]

Although important efforts have been made for identification of useful biomarkers in UTD, pediatric urologists currently use none. The present work uses a metabolomic approach aiming to identify such biomarkers. The next chapter presents the general principles and successive steps of metabolomic analysis.

1.3. Metabolomic methodology

1.3.1. Analytical techniques

Multiple analysis techniques can be used in metabolomics. Among these, nuclear magnetic resonance (NMR) spectroscopy[81] and mass spectrometry (MS)[82] are the techniques of choice, sometimes used in a complementary manner for the most complete characterization of samples. MS has to be coupled with separation methods such as gas chromatography, liquid chromatography and capillary electrophoresis. Characteristics of both methods are summarized in *table 1.3.1.*

Table 1.3.1. **Main characteristics of NMR spectroscopy and MS**

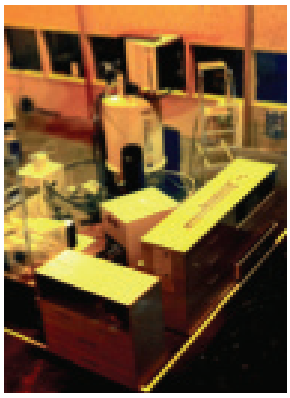
	NMR spectroscopy	Mass Spectrometry
Sample preparation	Minimal, non invasive, possible analysis of intact tissues, analysis can be automated	Samples have to be extracted into a suitable solvent, time consuming
Quantitation	Quantitative because the signal intensity is only determined by the molar concentration	Ion suppression limits quantitation
Sensitivity	Low, therefore less suited for analysis of trace compounds	High
Analytical reproducibility	High	Poor
Technique	Nondestructive	Destructive as metabolite identification is based on fragmentation patterns

Others analytical techniques may also be used for metabolic profiling, such as Fourier transform infrared spectroscopy, thin layer chromatography, phenotype microarrays and Raman spectroscopy.[83,84] Whatever the technique used, the result of the detection and quantification of metabolites is called a spectrum.

In this thesis, the acquisition of metabolic information is based on NMR spectroscopy experiments on urine.

1.3.2. NMR investigations of biofluid

NMR exploits the specific properties of the nuclear spins of a molecule when placed in a magnetic field, and allows the identification of the different nuclei (for example ^{1}H, ^{13}C, ^{31}P, ^{15}N) according to their characteristic nuclear resonance frequency. For clinical studies, proton NMR is widely exploited because the majority of known metabolites in biological samples contain hydrogen. This high throughput and quantitative technique is a rapid, robust and reliable method that presents a high reproducibility and requires a minimal and non-destructive sample preparation.[85] The obtained ^{1}H NMR spectra are the complex superposition of the spectra from all metabolites present in the fluid.[5]. NMR is also a quantitative method. The concentration of a metabolite can be calculated by comparing its peak intensity to an internal or external standard with a known concentration.



NMR is generally less sensitive than MS, and requires concentrations in the range of 1 to 10 µmol/L.[86] However, its sensibility can be improved by the use of longer analysis times, application of higher magnetic fields and the use of cryogenic probes.[87] In this work, NMR analyzes were performed on a spectrometer at 600 MHz equipped with a cryogenic probe. *(Figure 1.3.1)*

Figure 1.3.1: **600.55 MHz spectrometer equipped with a cryo-probe, and high-throughput sample changer**

Sample preparation

Sample preparation in liquid-state NMR is quite straightforward. NMR samples from biological fluids are prepared according to standard protocols.[88] For the large cohort studies, this step can be automated by the use of a robotic liquid handling technology, which has been developed to handle repetitive liquid transfer tasks.

In this study, urine samples were thawed at room temperature and centrifuged at 12,000 g at 4°C for 5 min. 540µl of the supernatants were mixed with 60µl of phosphate buffer in D2O (1.5 M, pH = 7.4) containing 1% of TSP (3-trimethylsilyl-2,2,3,3-tetradeuteropropionic acid) as internal standard (final TSP concentration = 0.77 mM) and 3 mM sodium azide to prevent microbial contamination and then transferred into 5 mm NMR tubes. Samples were kept at 4°C before analysis.

Quality Control (QC) samples were used to ensure the reproducibility of the NMR analyses within and between periods of manipulation called batches.[89] The QC samples are defined as being biologically identical to the samples of the study. Several types of QC samples can be exploited, commonly either pooled QC (a pool of small fractions of each sample, mixed together) or commercially available samples composed of standard mixtures of small molecules. In this thesis, urine QC samples were obtained by pooling a fraction of the first twelve urine samples analyzed. The pool was aliquoted and each aliquot processed as a regular sample, and analyzed throughout the run to assess the good stability of the NMR setup and reproducibility of the experiments.

NMR data acquisition

Each sample is introduced in the spectrometer, and the nuclear magnetic resonance response is obtained, called the free induction decay (FID). A Fourier transform is carried out to extract the frequency-domain spectrum from the raw time-domain FID. Repeated acquisitions are carried out to improve the signal-to-noise ratio.

A small set of complementary pulse sequences implemented following standardized protocols is commonly used for most NMR metabolomic applications.[90]

The 1D ^{1}H NOESY (Nuclear Overhauser Effect SpectroscopY) pulse sequence with water presaturation is the most popular acquisition scheme for collection of metabolites fingerprints from biological samples. The water presaturation allows for good water signal suppression and maintains a flat baseline. Indeed, water is highly concentrated in biological fluids, leading to the presence in the NMR spectrum of a broad band that overlaps with the resonances of certain metabolites and hence causes information loss. In many studies, the 1D ^{1}H NOESY experiment corresponds to the one and only spectrum that is recorded for each sample in a metabolomics cohort. The NOESY pulse sequence has the advantage of being a simple scheme, with minimal need for parameter optimization, while its implementation does not require any sophisticated hardware. The 1D ^{1}H NOESY pulse sequence consists in a series of three 90° radio-frequency pulses. *(Figure 1.3.2)* It can be the first increment of a two-dimensional NOESY experiment. The first pulse creates transverse spin magnetization. The second and third pulses, separated by a mixing time τm correspond to the NOESY filter, which improves the quality of water suppression for acquisition of metabolomic profiles in biofluids.

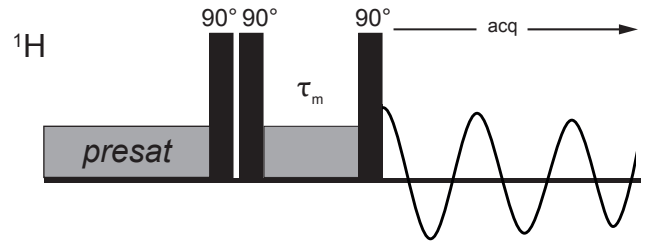


Figure 1.3.2: **Standard one-dimensional ^{1}H NOESY pulse sequence.** *The water presaturation allows for good water signal suppression and maintains a flat baseline. The first 90° radio-frequency pulse creates transverse spin magnetization. The second and third pulses, separated by a mixing time τm correspond to the NOESY filter, which improves the quality of water suppression.*

The CPMG (Carr-Purcell-Meiboom-Gill) experiment is also commonly used in metabolomic analyses of plasma or serum.[88] In these biofluids, protein and lipoproteins produce broad background signals that may possibly cover the presence of low-concentrated small metabolites. The main feature of the multiple spin-echo CPMG experiment pulse sequence is that it allows the resonances from larger molecules like proteins and lipids to relax before detecting the longer-lived resonances from small molecules. Thereafter, the baseline of these spectra is improved and contributions from weaker signals in the aromatic region are clearly visible.[87]

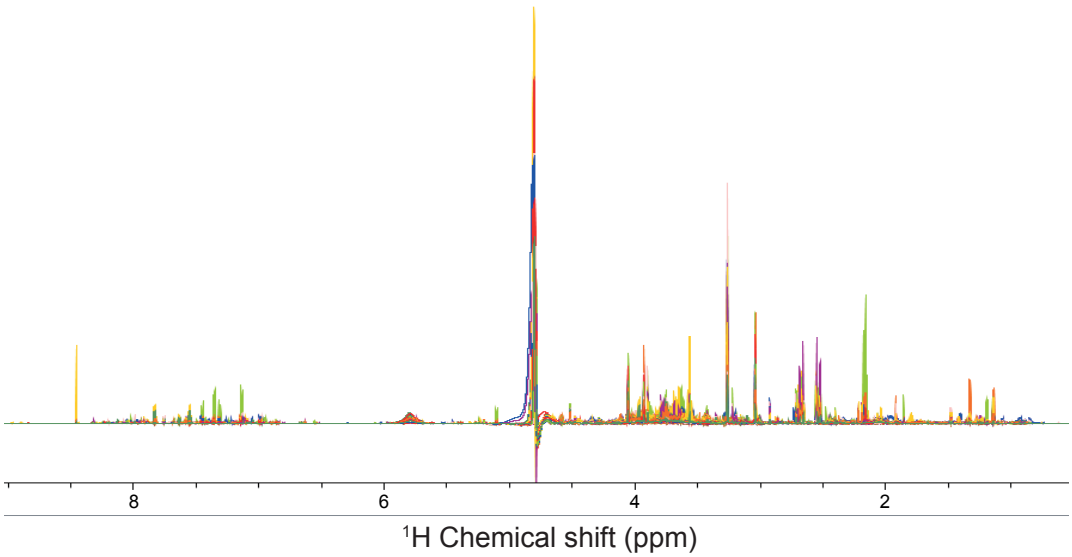


Figure 1.3.3: **Superimposition of 600 MHz ^{1}H-NMR NOESY spectra from 90 urine samples.** *Samples are colored differently, showing important differences in metabolic profiles.*

Proteins and lipoproteins being usually absent in urine, only standard ^{1}H 1D NMR NOESY pulse sequence with water presaturation was applied in this work. *(Figure 1.3.3)*

After acquisition, NMR spectra are phased, the baseline is corrected, and finally they are calibrated with an internal standard such as TSP, previously introduced into each sample.

Metabolites identification

Each chemically distinct hydrogen nucleus in each metabolite in a biological sample exhibits an NMR signal at a characteristic resonance frequency, which is measured as a chemical shift (with units of parts per million) relative to a standard compound such as TSP. The ^{1}H NMR spectrum of a sample is the superimposition of all the peaks corresponding to different metabolites. *(Figure 1.3.4)*

A large number of signals can be directly annotated on the basis of their chemical shifts, J coupling constant and the multiplicity of the resonance peaks using knowledge

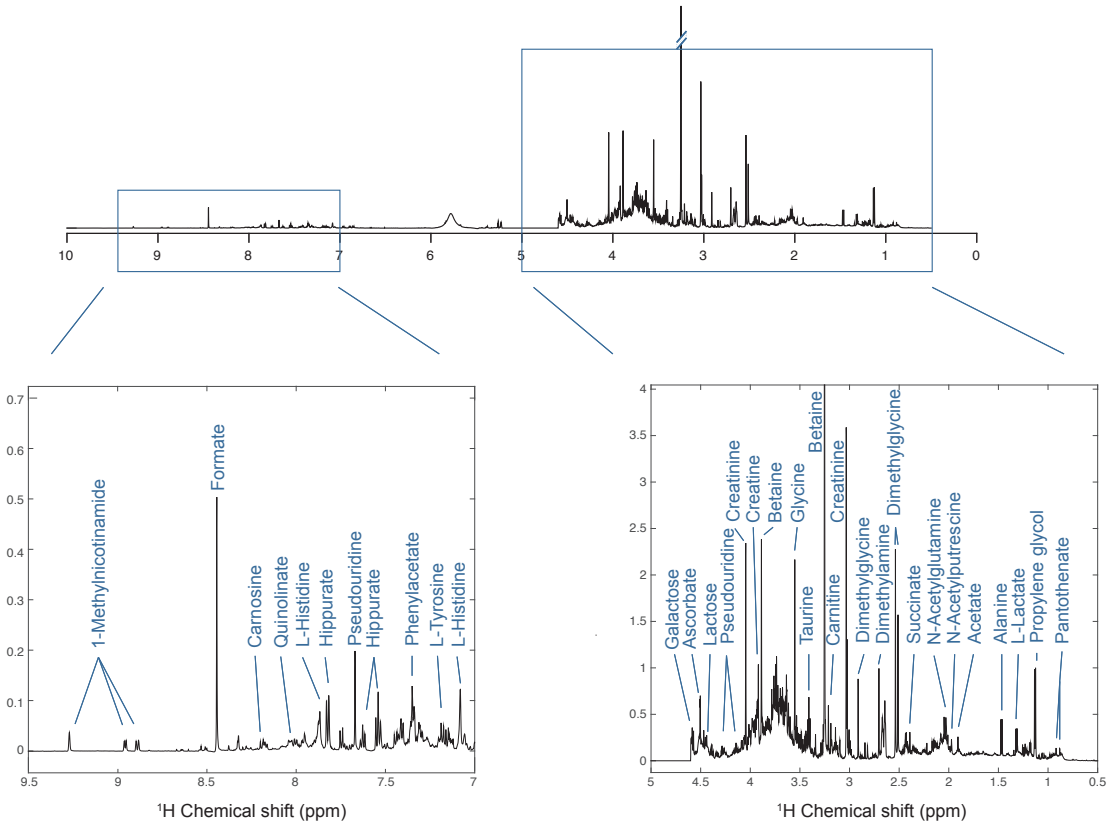


Figure 1.3.4: **Example of a 600 MHz 1H-NMR NOESY urine spectrum.**
A large number of metabolites are identified on the basis of their chemical shifts using knowledge from on-line international databases.

from on-line international databases (Madison Metabolomics Consortium Database,[91] Human Metabolic Database,[92] Biological Magnetic Resonance Bank,[93]…) and proprietary databases (ChenomxTMNMR Suite, ChenomxINc, Edmonton, Canada; AMIX SpectraBase, Bruker GmbH, Rheinstetten, Germany).

In addition, 2D-NMR experiments are used for increasing the signal dispersion and for elucidating the connectivity between nuclei, thereby enabling the identification of compounds present in the studied metabolic profile and solving some problems associated with the recovery of signals. These include ^{1}H-^{1}H TOCSY (TOtal Correlation SpectroscopY) experiments which provide spin-spin coupling connectivities, giving information on which hydrogen atoms in a molecule are close to one another, ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation), which investigates heteronuclear coupling and J-resolved NMR experiments, which yield information on the multiplicity and coupling patterns of resonances, facilitating molecule identification. The acquisition times of 2D experiments being much longer than the standard 1D pulse sequences (some minutes for 1D spectra against several hours for 2D spectra), they are usually acquired on only some representative samples in large cohort studies.

Bucketing

After obtaining NMR spectra, the raw data go through a pre-treatment step to reduce the data in a format suitable for statistical analysis. Bucketing is a simple automated method of signal integration (e.g. sum of intensities of the NMR signal) of the spectra into buckets (typically 0.001 ppm) over the chemical shift range (0-10 ppm).[94] Each spectrum is reduced to about 10,000 variables. During this step, the residual water signal is excluded. Data are regrouped in a matrix, called matrix X, where each row correspond to one of the samples, and columns corresponds to buckets.

Data alignment

Small changes in pH or osmolarity and intermolecular interactions can affect the absolute and relative peaks positions between samples. Therefore, alignment of the data can be necessary to ensure that each column of the matrix correspond to the same metabolite before statistical analyses. Several algorithms have been proposed to remove misalignment of NMR signals a posteriori.[87,95] In this thesis the alignment method developed by Savorani et al., called Icoshift is used.[96] *(Figure 1.3.5)*

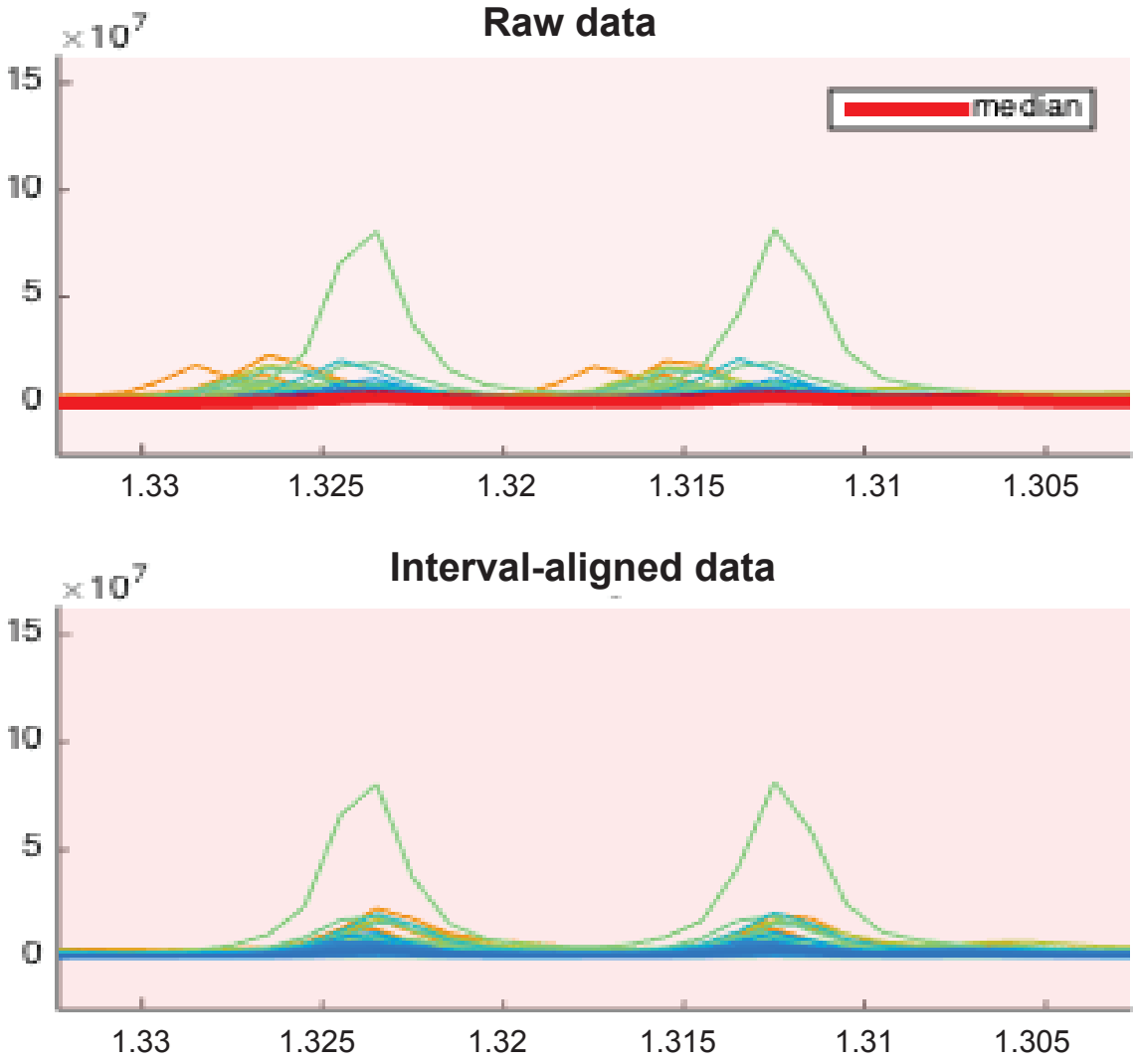


Figure 1.3.5: **Overview of the Icoshift algorithm results.** *On the top spectra, peaks are not aligned because of changes in pH or osmolarity between samples. On the bottom spectra, data has been aligned using Icoshift method.*

Normalization

Normalization is a mathematical correction applied to experimental data in order to cancel out effects of dilution between samples and to ensure that all observations are directly comparable.

The simplest form of internal normalization, called constant-sum normalization, is where each spectrum is normalized such that its integral is 1. Its purpose is to reduce or minimize the effects of dilution between samples. More complex methods of normaliza-

tion have been described, such as Probabilistic Quotient Normalization,[97] Cyclic Loess Normalization,[98] Contrast Normalization,[99] Quantile Normalization,[100] Linear Baseline Normalization,[100] Li-Wong Normalization,[101] and Cubic-Spline Normalization.[102]

The PQN (Probabilistic Quotient Normalization) was used in this work. This method starts with an integral normalization of each spectrum, followed by the calculation of a reference spectrum such as a median spectrum. Next, for each variable of interest the quotient of a given test spectrum and reference spectrum is calculated and the median of all quotients is estimated. Finally, all variables of the test spectrum are divided by the median quotient.[97] PQN better compensates for different dilutions between samples than integral normalization and vector lenght normalization. It is therefore suited for urine metabolomic analysis, as urine concentration is highly variable and affected by degree of hydratation.

Normalization may also be accomplished by computational means using internal standard (TSP).[103,104]

Scaling

The scaling methods are aimed at adjusting the variance of the different metabolites, as highly disparate intensities and variances between variables would force most forms of multivariate analysis to focus on a small set of intense signals.

Unit Variance (UV) scaling is a simple method using the standard deviation of the data as a scaling factor. One centers the data by subtracting from each feature its mean feature intensity across spectra. This will result in a fluctuation of the data around zero, thereby adjusting for offsets between high and low intensity features. From the centered data the standard deviation of each feature is obtained and data is divided by this scaling factor. Auto Scaling renders all features equally important, but can inflate measurement errors.[103]

Another scaling method was used in this work, the Pareto scaling.[105] After the data is mean-centered, it divides each value by the square root of the standard deviation of the variables of the column. This method gives to the variable a variance equal to its standard deviation thus high concentration metabolites do not dominate the multivariate models. Its effect is less intense than UV scaling and the scaling of the data is such that it remains close to their original values.[106] However, very large fold changes may still show a dominating effect.

Descriptive Statistics

Metabolomic analyses are generally aimed at comparing groups of samples to identify the metabolic signature associated with a specific physiopathological state. Demographic and clinical characteristics of the studied individuals are collected and stored in a matrix called Y. These variables can be either quantitative (age, height, weight, etc…) or qualitative (gender, disease, etc…). Descriptive statistics are usually the first step of the statistical analysis. Quantitative data are compared using the Student t-test if the variables are normally distributed, and the Kruskal-Wallis test if the data are not normally distributed. Qualitative data are compared using the Chi-square test or the Fisher exact test. The significance threshold is set to 0.05 for all these tests.

Unsupervised analysis

Multivariate analyses are performed to provide meaningful information on the sample classification and identify specific metabolic signatures. By extracting and displaying the systematic variation in the data based on projection methods, they reduce the variability of the data and combines complex interactions. The first step of this analysis relies on unsupervised methods, such as principal component analysis (PCA),[107] which measure without a priori the intrinsic variation within the data set. PCA is applied by summarizing the data into much fewer variables called scores, which are weighted averages of the original variables. Each Principle Component (PC) is a linear combination of the original data parameters and each successive PC explains the maximum amount of variance possible, not accounted for by the previous PCs. Unsupervised analyses help to identify "outlier" samples, resulting from an error in sampling, preparation or spectral acquisition, which will be excluded from the rest of the analysis. Less common unsupervised methods like Hierarchical Clustering Analysis (HCA)[108] and self-organizing maps can also be used.[109]

Supervised analyses

The next multivariate analyses, such as PLS regression methods (Partial Least Squares or Projection on Latent Structures)[110] and O-PLS (Orthogonal Partial Least Squares or Orthogonal Projection on Latent Structures),[111] aim at uncovering metabolic signals that are associated with the phenotype under study. These supervised methods are used to build models for the robust classification of samples and derived group-specific metabolic phenotypes.

PLS models maximize the covariance between scores in X (predictor) and Y (outcome) spaces and in this way are able to account for both systematic variation in the metabolic profiles and correlations between the metabolic data and the outcome. PLS is often used in "discriminant analysis" mode to classify metabolic profiles according to a discrete set of categories (e.g., disease cases and controls).[112]

Results of the multivariate analyses are usually visualized as score plots, where each point stands for the projection of a single sample on the main principal components and as loading plots, which represent the contribution of the metabolic variables on the principal components.

O-PLS and O2-PLS are methods to separate the variations within the dataset into 2 parts. The first part, the "predictive" or "joint" variation, models variation that is common to both the X (predictor) and Y (outcome) matrix. For example, this could be variation in the metabolic profile that is linearly predictive of the disease class of an individual. The second part, the "orthogonal" or "unique" variation, models influences that are not linearly related to Y (the outcome). This captures any extraneous factors not related to the outcome, whether technical or biological, such as batch effects, gender, etc... Separation of the model into these 2 parts allows a more parsimonious explanation of the data because only the predictive part needs to be interpreted to understand the metabolic factors influencing the outcome.[113] However, OPLS can generate excellent class separation on the score plot even with random data. Validation is mandatory to ensure the model robustness before interpretation of the results.

Models validation

Once a discrimination model is obtained, numerous statistical tools are available to ensure its robustness and validate its performance. The models quality can be evaluated based on the residuals (R^2X, R^2Y) and the models predictive ability parameter (Q^2). R^2X and R^2Y are defined as the proportion of variance in X and Y matrixes explained by the model and indicate goodness of fit. Q^2 is defined as the proportion of variance in the data that can be predicted by the model. The Q^2 value is estimated by crossvalidation procedure, which determines the number of significant component in the model.[114] The highest the Q^2, the more the model can be considered as predictive. In model systems, Q^2 are considered as high typically when > 0.7 or 0.8. It is different when studying biofluids, which have high inter-individual variability. A predictive model in such a study is generally considered statistically robust if its Q^2 value is ≥ 0.3.

Resampling under the null hypothesis is another tool to validate the model per-

formance. This method consists in calculating all possible values of R^2 and Q^2 under random permutations of the Y matrix. The number of random permutations is usually set to 1000. The decrease of the goodness-of-fit R^2 and Q^2 parameters when correlation between original model and random models decreased, indicated the good quality of the model.[115,116]

Calculation of the level of statistical significance by CV-ANOVA can be also used. This test is based on an ANOVA (analysis of variance) assessment of the crossvalidatory (CV) predictive residuals of a model.[117]

External validation of the model by the blind projection of a new independent cohort on the model is the best method to evaluate its predictive ability, although it is rarely available and resource consuming. Eventually, the diagnosis performance of the model can also be evaluated with a ROC (Receiver Operating Characteristic) analysis.[118,119]

Biomarkers identification and interpretation

The results of the supervised multivariate analysis can be further processed to identify metabolites significantly associated with the physiological or pathological state under study. To derive statistically significant individual biomarkers, multiple univariate tests are used on all metabolites peaks simultaneously. The Student t-test or ANOVA test are the most commonly used for this purpose. However, the tremendous amount of variables necessitates that an equivalent number of tests are performed. Thus, it is mandatory to apply multiple testing corrections to compensate the false discovery rate. The different possibilities are the Bonferroni method, the Benjamini and Yekutieli method[120] and the Benjamini and Hochberg method.[121]

In this thesis, the statistical recoupling of variables (SRV) method is used to identify key metabolites being potential biomarkers.[122] The statistical relationships between consecutive variables of the X matrix allow aggregating them into clusters following the highest direction of covariance/correlation ratio, thus corresponding to NMR peaks. Neighboring clusters can then be merged into superclusters to recover NMR multiplets. These superclusters correspond to NMR variables of interest. SRV acts as an automated variable-size bucketing procedure coupled with an efficient noise-removing filter. Based on the statistical total correlation spectroscopy, it is possible to establish a two-dimensional (2D) pseudospectrum defined as a correlation map between the superclusters.[123] These new variables are then submitted to ANOVA analysis with multiple testing corrections of the p-values, and variables significantly associated with the variable of interest can be represented on a loading plot. This method allows

the identification key metabolites associated with different groups of samples. Metabolic profiles can be highlighted with respect to a particular characteristic of a sample group, such as the disease, the pathology severity or the response to therapy.

Identified metabolites can be regrouped according to the metabolic pathways in which they are involved using on-line tools such as MetPA, which is dedicated to the analysis and visualization of metabolomic data within the biological context of metabolic pathways. MetPA combines several advanced pathway enrichment analysis procedures along with the analysis of pathway topological characteristics to help identify the most relevant metabolic pathways involved in a given metabolomic study.[124]

2. Case-control metabolomic study of newborns with prenatally diagnosed unilateral urinary tract dilatation

2.1. Introduction

In the first part of this thesis, we introduced the metabolomic approach as tool for translational research in neonatology. We described the diagnosis challenge raised by the prenatal finding of a unilateral UTD, that can be transient or represent a significant urinary flow impairment that would lead to progressive deterioration of the renal function.[46] We then reported the current lack of reliable biomarkers in UTD. Metabolomics has huge potential for the identification of such biomarkers.

Although plasma, serum, amniotic fluid, cord blood or stool can be used for metabolomic analysis, urine is particularly suited in newborns and children due to its non-invasive method of collection. Urine biochemical composition is influenced by a variety of factors such as genotypes, diseases, nutritional state and lifestyle.[85,125] Moreover, urine is the biofluid whose compositon is the most likely to be modified in uropathies.

The main objective of this study was to identify prognosis factors in newborns with prenatally diagnosed unilateral UTD, to identify patients at risk for surgical intervention at an early stage.

In chapter 2.2, we will describe the design of this study including newborns with prenatally diagnosed unilateral UTD and healthy newborns as controls. The method

of sample collection and storage will be described in chapter 2.2.2, and the management of UTD in chapter 2.2.3.

Chapter 2.3 focuses on the accuracy of different ultrasound grading systems in predicting the need for surgical intervention in children with prenatally diagnosed unilateral UTD. Although strictly medical, this part of the thesis helps to better characterize the population studied by metabolomic analysis.

In chapter 2.4, we will report the comparison of urinary metabolic profiles of newborns with UTD and healthy controls, using ^{1}H NMR spectroscopy and metabolomic analysis.

2.2. Study protocol

2.2.1. Study population

Eighty-two consecutive patients under 4 months old prenatally diagnosed with unilateral UTD in Hôpital Femme Mère Enfant, Bron, France, were prospectively included from January 2011 to February 2015. Inclusions criteria were: Prenatally diagnosed unilateral UTD, normal bladder and APD ≥10 mm on the first postnatal US, and age ≤ 3 months. Twelve patients were excluded, 5 for dysplastic kidney and 7 for duplex systems. Seventy patients were included in the analysis. All patients were followed-up according to the usual protocols of the pediatric urology department.

Ninety newborns consulting in our institution for a benign condition (constipation, inguinal hernia, parental anxiety…) were included as controls during the same period. Exclusion criteria for controls were: prematurity (gestational age of less than 38 weeks), history of metabolic, nephrologic or urologic disease, and history of surgery. The study design did not include any follow-up of control patients.

This study was approved by the local ethics committee, *(Appendices C, D and E)* and financed by the Hospices Civils de Lyon in the context of a "Programme Hospitalier de Recherche Clinique". Written informed consent was obtained from the parents of all study subjects.

2.2.2. Samples collection and storage

One urine sample was collected non-invasively from each patient and control during a medical consultation within 120 days from birth. A second sample was collected between 7 and 18 months of age from with UTD. This second sample was only

available in 49 patients at the time of the present study. The genitals were cleaned thoroughly with warm water and soap and dried with sterile gauze. A sterile bag was placed on the genital area until micturition. Samples were stored in sterile tubes at -80°C before acquisition of the NMR data.

2.2.3. Management of UTD

Patients were divided in 2 groups.

- Group 1: Unilateral UTD with APD ≥10 mm, and homolateral ureter diameter < 6 mm.

- Group 2: Unilateral UTD with APD ≥10 mm, and homolateral dilatation of the ureter (diameter ≥ 6 mm).

Patients were managed according to the usual protocol of the pediatric surgery department. Patients underwent a clinical follow-up every 3 to 4 months, an US performed every 3 to 4 months and a renal isotope renography within 6 months of life. A micturating cystogram was performed in children from group 2 (ureteral dilatation ≥ 6mm) with an abnormal isotope study.

Indications for surgery were: recurrent febrile urinary tract infections, ipsilateral relative renal function on isotope study < 40%, ipsilateral flank pain and increasing APD of more than 20 % on serial US scans.

Clinical and imaging data were recorded prospectively.

2.3. Prognostic value of ultrasound grading systems in prenatally diagnosed unilateral urinary tract dilatation

2.3.1. Objective

Different classifications are used to evaluate the severity of the UTD in the aim of distinguishing children who require follow-up and surgical intervention from those who do not. Measurement of APD and the Society for Fetal Urology (SFU)[126] grading systems were most commonly used before creation of the Urinary Tract Dilatation (UTD) grading system introduced in 2014 by a multidisciplinary consensus.[46] The usefulness of this new grading system in predicting clinical outcomes has yet to be evaluated.

In this chapter, the accuracy of APD, SFU and UTD grading systems in predicting the need for surgical intervention in children with prenatally diagnosed unilateral UTD are compared.[127]

2.3.2. Method

The severity of the dilatation was evaluated prospectively on the first postnatal US performed in our institution at one month of age, using APD and SFU grading systems. *(Table 2.3.1)* Dilatation was graded retrospectively according to UTD grading system by an experienced pediatric urologist blinded to the clinical outcomes. Event of interest was need for surgery for uropathy before age 24 months.

	UTD P grading system	SFU grading system
Grade 1	APD between 10 and 15 mm only central calyceal dilatation	Visualized renal pelvis
Grade 2	APD ≥ 15 mm or dilated ureter or peripheral calyceal dilatation	Hydronephrosis and a few calices identified
Grade 3	Abnormal parenchymal thickness or abnormal parenchymal appearance or abnormal bladder appearance	Hydronephrosis and all calices are seen
Grade 4	-	Hydronephrosis and abnormal parenchymal thickness

Table 2.3.1. UTD and SFU grading systems. SFU: Society for Fetal Urology. UTD P: Urinary Tract Dilatation Postnatal

Statistical analysis

Continuous data were reported as medians (interquartile range, IQR). Data were compared using the Fisher exact test for categorical variables and Kruskal-Wallis test for continuous variables (APD and Follow-up time were not normally distributed). The Bonferroni correction was applied to adjust for multiple comparisons within each grading system. Adjusted p-value < 0.05 was considered statistically significant. Diagnostic accuracy of APD and the SFU and UTD grading systems was assessed by sensitivity, specificity, Predictive Positive Value (PPV), Negative Predictive Value (NPV), positive and negative likelihood ratio (LR+, LR-), Odds Ratio (OR), Risk Ratio (RR) with 95% Confidence Intervals (95% CI) and Receiver operating characteristic (ROC) plots. A

formal comparison of the number of correctly classified cases, reflecting differences in sensitivity and specificity, was made by comparing area under the ROC curves (AUC). AUC were compared using the method described by De Long *et al.*[128]

For patients who did not require surgery, the probability of persistent dilatation was calculated using the Kaplan Meier method.

Data collection and statistical calculations were performed using SPSS (version 23.0; SPSS, Inc, Chicago, IL) software.

There was no missing data. Completed checklists of standards for reporting of diagnostic accuracy (the STARD initiative) are shown in *Appendix F.*[129]

2.3.3. Results

A total of 70 children with unilateral urinary tract dilatation (left sided in 38 and right sided in 32) were included in the analysis.

The first postnatal US was performed at a median age of 4 days (IQR 3-6). Analysis was performed on the first US made in our institution at a median age of 43 days (IQR 34-69). Median follow-up was 39.7 months (IQR 27.4-48.0). Of the patients 33 (47%) patients required surgery (for PUJ anomalies in 30, megaureters in 2 and VUR in 1) before age 24 months. No patients required surgery after age 24 months. Indications for surgery were: increasing APD greater than 20 % (range 22% to 92 %) in 14 (42 %) cases, ipsilateral relative renal function < 40 % in 6 (18%), relative renal function < 40% and recurrent febrile urinary tract infections in 1 (3 %). Additional indications for surgery were increasing APD plus febrile urinary tract infections in 3 (9 %) cases, increasing APD plus alteration of the relative renal function in 8 (24 %) and all 3 indications in 1 (3 %). The median age at surgery was 4.8 months (IQR 3.8-6.4).

Main baseline clinical characteristics of the surgical and non-operative management groups are summarized in *table 2.3.2.* Mean APD, proportion of abnormal renal parenchymal thickness and proportion of peripheral calyceal dilatations were significantly higher in children who required surgical procedures.

Table 2.3.2. **Patient demographics and baseline characteristics**

	Non-operative management	Surgery	p value*
No. patients	37	33	
No. males (%)	32 (86)	25 (76)	0.36
No. females (%)	5 (14)	8 (24)	
No. dilated ureter ≥ 6 mm (%)	11 (30)	6 (18)	0.28
No. At least one febrile urinary tract infection (%)	4 (11)	5 (15)	
No. differential renal function < 40 % (%)	0 (0)	16 (48)	
Median % differential renal function (IQR)	48 (46-50)	40 (33-47)	
No. > 20 % Increase of APD on serial US (%)	0 (0)	26 (79)	
Median mm APD (IQR)	14 (12–17)	26 (22-30)	< 0.001
No. APD ≥ 20 mm (%)	3 (8)	28 (85)	< 0.001
No. peripheral calyceal dilatation (%)	29 (78)	33 (100)	0.006
No. abnormal parenchymal thickness (%)	6 (16)	25 (76)	< 0.001
No. abnormal parenchymal appearance (%)	1 (3)	2 (6)	0.60
Median months follow-up (IQR)	39 (25-46)	42 (28-48)	0.34
No. SFU grading system (%)			
SFU I	0 (0)	0 (0)	
SFU II	8 (22)	0 (0)	
SFU III	23 (62)	8 (24)	
SFU IV	6 (16)	25 (76)	
No. UTD grading system (%)			
UTD P1	12 (32)	0 (0)	
UTD P2	19 (51)	8 (24)	
UTD P3	6 (16)	25 (76)	

** p-value calculated using Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables. Statistical significance p < 0.05*

IQR: Inter Quartile Range

APD: Antero-Posterior intrasinusal Diameter of the renal pelvis

US: Ultrasonography

SFU: Society for Fetal Urology

UTD: Urinary Tract Dilatation

Performance of various thresholds for APD and the UTD and SFU grading systems is summarized in *table 2.3.3.* All grading systems were significantly associated with likelihood of surgery. APD ≥ 20 mm had the best diagnosis accuracy and allowed correct classification of 86.96% of cases. Postnatal UTD grade 3 or higher and SFU grade 4 or higher had an accuracy of 80%. Comparison of sensitivity and specificity of the 3 measures for these selected thresholds failed to reach significance.

Table 2.3.3. **Diagnostic accuracy of various thresholds for APD, UTD and SFU grading systems in predicting the need for surgery**

	Patients per group	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Correctly classified cases (%)	LR+	LR-	OR (95% CI)	RR (95% CI)	p-value*
UTDP≥2	12/58	100.00	32.43	56.90	100	64	1.48	0.00	-	2.32 (1.73-3.12)	< 0.001
SFU≥3;	8/62	100.00	21.62	53.23	100	59	1.28	0.00	-	2.14 (1.64-2.79	0.012
UTDP grade 3; SFU grade 4;**	39/31	75.76	83.78	80.65	79.49	80	4.67	0.29	16.15 (4.95-52.67)	4.11 (1.97-8.57)	< 0.001
APD											
≥12mm	9/61	96.97	22.22	53.33	88.89	56	1.25	0.14	10.29 (1.26-86.33)	1.93 (1.37-2.71)	0.21
≥14mm	18/52	90.91	38.89	57.69	82.35	64	1.49	0.23	6.36 (1.63-24.87)	1.94 (1.32-2.86)	0.02
≥16mm	32/38	84.85	72.22	73.68	83.87	78	3.05	0.21	14.56 (4.39-48.29)	3.19 (1.83-5.54)	< 0.001
≥18mm	36/34	81.82	80.56	79.41	82.86	81	4.21	0.23	18.64 (5.56-62.51)	4.02 (2.04-7.92)	< 0.001
≥20mm	40/30	81.82	91.67	90.00	84.62	87	9.82	0.20	49.50 (11.31-216.65)	8.46 (2.87-24.96)	< 0.001
≥22mm	44/26	75.76	97.22	96.15	81.40	87	27.27	0.25	109.37 (12.85-930.85)	21.16 (3.08-145.40)	< 0.001

**p-value calculated using Fisher's exact test and Bonferroni correction to adjust for multiple comparisons*
***All patients graded SFU4 were also UTDP grade 3, because of abnormal renal parenchyma*

95% CI: 95% Confidence Interval
PPV: Positive Predictive Value
NPV: Negative Predictive Value
LR+: Positive Likelihood ratio
LR-: Negative Likelihood ratio
OR: Odds Ratio
RR: Risk Ratio
APD: Antero-Posterior Diameter of the renal pelvis
UTD: Urinary Tract Dilatation
UTDP: Urinary Tract Dilatation Postnatal grading
SFU: Society for Fetal Urology

Grading systems were compared using the ROC curves. *(Figure 2.3.1)* AUC was interpreted as the probability that a randomly selected patient who required surgery had a higher grade than a randomly selected patient who did not require surgery. AUC was 0.88 (95% CI 0.79-0.97) for APD, 0.84 (95% CI 0.74-0.93) for the UTD grading system and 0.82 (95% CI 0.72-0.92) for the SFU grading system. All AUCs were significantly greater than 0.5 (p-value < 0.001 for the 3 tests) but the differences between AUC were not statistically significant.

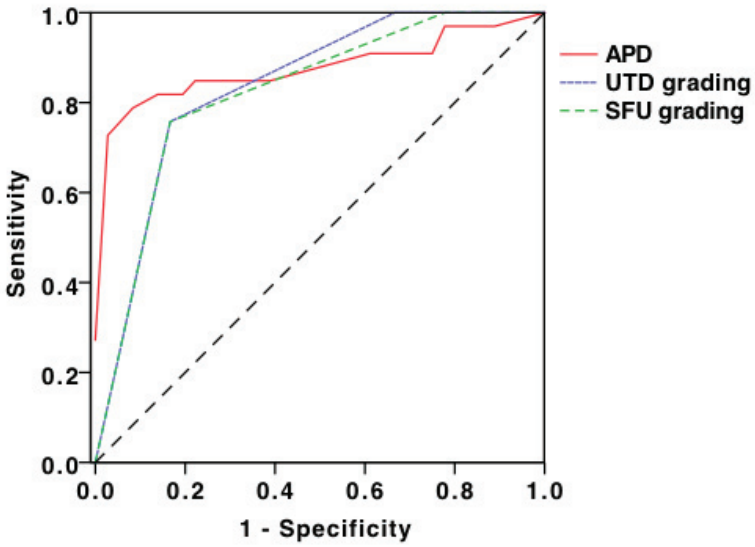


Figure 2.3.1:

ROC comparing APD, UTD and SFU grading systems as indicators of need for surgery

APD: Antero-Posterior Diameter of the renal pelvis
SFU: Society for Fetal Urology
UTD: Urinary Tract Dilatation

Patients with abnormal parenchymal thickness had a significantly larger mean APD (24 mm) than those with normal parenchymal thickness (16 mm) (p-value = 0.0001).

Factors significantly associated with differential renal function less than 40 % were APD (p-value = 0.004), postnatal UTD grade 3 (p = 0.001), SFU grade 4 (p-value = 0.001) and abnormal parenchymal thickness (p-value = 0.001).

A total of 37 patients did not require surgery after a median follow-up of 39 months (IQR 25-46) and a minimum follow-up of 24 months. Of these patients 23 had a transient dilatation with an APD of less than 10 mm at the last visit. Median time to resolution of UTD was 23.6 months (95% IC 15.6-31.6). *Figure 2.3.2* shows the decreasing probability of persistent dilatation over time calculated by the Kaplan Meier method.

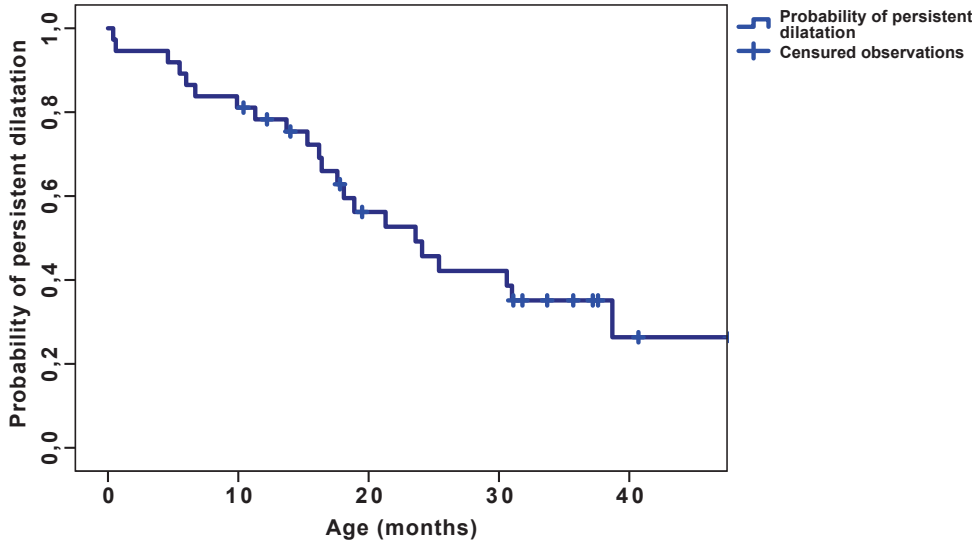


Figure 2.3.2: **Probability of persistent dilatation over time calculated by the Kaplan Meier method on 37 newborns with prenatally diagnosed unilateral UTD managed conservatively.**
The renal pelvis was considered as not dilated whenever APD was < 10mm.

2.3.4. Discussion

Unilateral UTD is a common condition and a major diagnosis issue. In this context it is crucial to validate US classification systems to differentiate between uropathies requiring surgical management and cases of transient dilatation at an early stage.

This study has several limitations. The number of patients is small, and UTD grading was done retrospectively, as the system was only created in 2014.[46] In addition, obstructive uropathies often require surgery during the first months or years of life, although patients submitted to surveillance only may require surgery at a later stage, which support the need for longer follow-up. Despite these limitations, this prospective cohort study is the first to highlight significant results comparing ATD and the SFU and UTD grading systems.

These results are consistent with recent publications regarding the correlation between APD and clinical outcomes. Dias *et al.* observed that the use of APD with a threshold of 20 mm had a sensitivity 80.0% of and a specificity of 92.7% in predicting need for surgery.[153] More recently Arora *et al.* reported a sensitivity of 88.5% and a specificity of 73.5%.[154] A sensitivity of 81.8% and a specificity of 91.7% using the same threshold were found in this series, although we chose to investigate only unilateral UTD

whereas bilateral UTDs were included in the two previous studies. Coplen *et al.* calculated a sensitivity of 72.6% and a specificity of 82.1% and Bouzada *et al.* a sensitivity of 82.4% and a specificity of 87.9% for fetal APD greater than 15 mm.[132,133] In a large retrospective study Passerotti *et al.* observed that severe hydronephrosis, parenchymal thinning, kidney echogenicity and ureter dilatation on the first postnatal US were significantly associated with uropathy.[134] Abnormal parenchymal thickness was associated with increased APD, altered ipsilateral relative renal function, and greater risk of requiring surgery in this series. In a meta-analysis by Lee *et al.* prenatal hydronephrosis was a predictor of uropathy but the risk of vesico-ureteral reflux was similar for all degrees of prenatal hydronephrosis.[135] In the present series only one patient was operated on for VUR. Therefore, the main usefulness of APD and the SFU and UTD grading systems is in predicting obstructive uropathies such as PUJ anomalies and obstructive megaureter.

The UTD classification system includes APD and abnormal parenchymal thickness, which are prognostic factor for surgery. This system was introduced in 2014 by a multidisciplinary consensus to establish a unified description of UTD that could be applied both prenatally and postnatally.[46] Hodhod *et al.* reported its usefulness for predicting surgical intervention with better reliability than the SFU classification system.[136] The validity of UTD in predicting the need for surgery with good sensitivity and specificity but with slightly less accuracy than APD measurement was confirmed in the present study.

Before the advent of the UTD classification system, the SFU grading system was the most widely used in literature as reported in a meta-analysis by Sidhu et *al.*[137] The SFU system emphasizes peripheric calyceal dilatation and parenchymal thickness but does not directly include APD. The prognostic value of SFU grading system has been described previously.[54] In the present study APD had better prognostic accuracy than the SFU grading system consistent with two recent retrospective studies. Dias *et al.* reported on 371 newborns with prenatal hydronephrosis, including 92 patients with moderate and severe hydronephrosis, and observed that APD on the first postnatal US was slightly more accurate than SFU in predicting the need for surgical intervention.[130] Rianthavorn and Limwattana used a comparable ROC analysis, and reported an area under the curve of 0.86 for APD greater than 16 mm and 0.81 for SFU grade 4.[138] We had comparable results although we chose to exclude mild dilatations (APD less than 10 mm) as their tendency to resolve has been widely demonstrated.[54,139,140] In addition to better accuracy, APD has higher inter-rater reliability than SFU as reported by Vemulakonda *et al.*[141]

Moreover, current training in SFU grading is mostly unstructured, and inaccurate grading is common even among trained radiologist.[142] The main difficulty is in distinguishing between SFU grade 3 and 4 as parenchymal thinning can be segmental or diffuse, leading to modest inter-rater reliability.[143]

The classification developed by Onen in 2007 is similar to the SFU classification system. Onen grade 1 includes SFU grades 1 and 2, and SFU grade 4 is divided into Onen grade 3 and 4 according to a criterion of 50% parenchymal loss.[144]

Kim *et al.* reported that the Onen system has increased intra-rater reliability but decreased inter-rater reliability compared to the SFU grading system.[145]

APD is a reliable measure that is simple and commonly used. One of the downsides of this classification is that APD can be affected by degree of bladder distension, hydration and position of the patient when US is performed.[46] Moreover, this measure does not take peripheral calyceal dilatation or parenchymal thickness into account, both of which are predictive factors for surgery included in the UTD and SFU grading systems. The optimal APD threshold to determine further investigations has yet to be determined. A low threshold would lead to unnecessary invasive diagnostic procedures, whereas a high threshold could lead to underestimation of the potential renal risks. In this series APD $\geq$ 20 mm had the best predictive performances.

We found that APD and the UTD and SFU grading systems have good accuracy in predicting the need for surgery. Considering these results, we recommend use of APD and parenchymal thickness as predictive tools to guide further imaging investigations. Clinical surveillance alone is sufficient for mild dilatations (APD < 10 mm). [52-54,137,139,140] Identifying uropathies in a large birth cohort with a long follow-up Andrés-Jensen *et al.* recently suggested a postnatal APD of 12 mm as a cut-off value for active follow-up, as surgery was only needed in 1% of patients when APD was assessed at 10 or 11 mm.[146] Indications for renal scintigraphy should be questioned in asymptomatic children with unilateral UTD, APD < 20 mm and normal parenchymal thickness. On the other hand, close clinical and radiological follow-up is mandatory for patients with APD > 20 mm or abnormal parenchymal thickness as they are likely to require surgery in the first years of life.

2.3.5. Conclusions

This prospective cohort study confirmed the prognostic value of APD and the UTD and SFU grading systems in newborns with prenatally diagnosed unilateral UTD. APD is reliable for predicting the need for surgery and appears to be the simplest measure. APD and abnormal parenchymal thickness are the most important US criteria for identification of children at risk for needing surgery.

Identifying a urinary biomarker for UTD could improve the prognosis accuracy when used in combination with US grading, and guide further investigations and treatments.

2.4. Comparison of urine metabolic profiles of children with UTD and controls

2.4.1. Objective

Our objective was to identify diagnosis and prognosis urinary markers for uropathies in children with prenatally diagnosed unilateral UTD. The urinary metabolomic profiles of newborns with UTD were compared with controls using ^{1}H NMR spectroscopy combined with multivariate statistical analysis. The potential correlation of specific urinary metabolic profiles with clinical outcomes was also investigated.

2.4.2. Methods

Study population

The study population is described in chapter 2.2.1. Urine samples from 70 patients with prenatally diagnosed unilateral UTD and 90 controls were analyzed. Controls were newborns under 4 months of age consulting at the Hôpital Femme Mère Enfant, Bron, France for a benign condition (abdominal discomfort, inguinal hernia, parental anxiety), included during the same period (from January 2011 to February 2015). Exclusion criteria were prematurity (gestational age of less than 38 weeks), history of metabolic, nephrologic or urologic disease, and history of surgery. Demographic characteristics of controls are also detailed in chapter 3.1.2.

Mean age, weight and height of patients and controls were compared using the Student t test, and proportion of male and female was compared using Chi square test.

Sample preparation

Samples collected during the medical consultation within 120 days from birth were analyzed in randomized order. An additional series of quality control (QC) urine samples (15 samples) was included in the analysis to evaluate the reproducibility of the NMR data acquisition. The QC samples were obtained by pooling a fraction of the first 12 urine samples. The pool was aliquoted and each aliquot processed as a sample. QC samples were regularly analyzed throughout the run.

Urine samples were thawed at room temperature and centrifuged at 12,000 g at 4°C for 5 min. 540µl of the supernatants were mixed with 60µl of phosphate buffer in D_2O (1.5 M, pH = 7.4) containing 1% of TSP 3-trimethylsilyl-2H4-propionic acid as internal standard (final TSP concentration = 0.77mM) and 3 mM sodium azide to prevent microbial contamination and then transferred into 5 mm NMR tubes. Samples were kept at 4°C before analysis.

^{1}H-NMR spectroscopy of urine samples

All NMR experiments were carried out on a Bruker Avance III spectrometer operating at 600.55 MHz (proton resonance frequency) equipped with a cryo-probe, and high-throughput sample changer that maintained the samples temperature at 4 °C until actual NMR acquisition. All samples were analyzed in one batch during 14 days. For each session, Automatic 3D shimming was performed once on a QC sample. Prior to NMR data acquisition, automatic tuning and matching, frequency locking on D_2O and 1D automatic gradient shimming was performed on each sample. The temperature was regulated at 300 K throughout the NMR experiments. Standard ^{1}H 1D NMR NOESY pulse sequence with water presaturation was applied on each sample to obtain corresponding metabolic profiles. 256 transient free induction decays (FID) were collected for each experiment into 48074 data points over a spectral width of 20 ppm. The acquisition time was set to 2 s with a relaxation delay of 4 s. The NOESY mixing time was set to 100 ms. The 90° pulse length was automatically calibrated for each sample at around 14.24 µs. In addition, 2D NMR experiments (^{1}H-^{13}C HSQC, ^{1}H-^{1}H TOCSY and J-Resolved) were recorded on a subset of samples to achieve structural assignment of the metabolic signals.

Data processing

All FIDs were multiplied by an exponential function corresponding to a 0.3 Hz line-broadening factor, prior Fourier transformation. ^{1}H-NMR spectra were phased, baseline corrected and referenced to the TSP signal at -0.016 ppm using Topspin 3.2 (Bruker GmbH, Rheinstetten, Germany). Residual water signal (4.6–5.2 ppm) was excluded. Spectra were divided into 0.001 ppm-wide buckets over the chemical shift range [0; 10 ppm] using the AMIX software (Bruker GmbH). Spectra were normalized to Probabilistic Quotient Normalization and Pareto-scaled prior to analysis. Spectra were aligned using the module Icoshift[96] in Matlab (The Math-works Inc., Natick, MA).

Multivariate analysis of urine metabolic profiles

Data were exported to SIMCA-P 14 (Umetrics, Umea, Sweden) for statistical analysis. Principal Component Analysis (PCA) was performed on all samples including QC samples to assess the quality and homogeneity of the dataset.[107] Models were calculated using the Orthogonal Projections to Latent Structures (O-PLS) method to investigate variations in the dataset correlated with age, weight, height and gender.[111] Each individual newborn's age, weight, height and gender were used as the one-component Y matrix for the corresponding model.

The number of components for each model was optimized using cross validation. The models quality was evaluated based on the residuals (R2X, R2Y) and the models predictive ability parameter (Q2). R2X and R2Y are defined as the proportion of variance in X and Y matrixes explained by the model and indicate goodness of fit. Q2 is defined as the proportion of variance in the data that can be predicted by the model.

The models were tested for overfitting using y-table permutation testing (n = 1000) permutation. Significance of the models was further assed by an ANOVA based on the cross-validated predictive residuals (CV-ANOVA) with a significance threshold set to 0.05.[117]

Identification of metabolites

Metabolites were assigned from the mean NOESY and 2D spectra (^{1}H-^{13}C HSQC, ^{1}H-^{1}H TOCSY and ^{1}H J-resolved) using data available from literature, international reference database (Human Metabolic Database,[92] Biological Magnetic Resonance Bank,[93]

Madison Metabolomics Consortium Database[91]) or proprietary databases (Chenomx NMR Suite, Chenomx Inc., Edmonton, Canada; AMIX SpectraBase, Bruker GmbH, Rheinstetten, Germany).

2.4.3. Results

Descriptive Statistics

The Chi square test did not show any significant difference in the proportion of male and female between the patients and controls groups (*p*-value = 0.26). *(Table 2.4.1)*

Table 2.4.1: **Proportion of male and female in the study population.**

	Patients	Controls	Total
Male	57 (81%)	66 (73%)	123 (77%)
Female	13 (19%)	24 (27%)	37 (23%)
Total	70	90	160

The mean age was 1.47 months +/-0.74 in the patients group, and 1.69 months +/-0.73 in the control group. The result of the Student t test did not show any significant difference with a *p*-value of 0.99.

The mean weight was 5.04 kg +/- 1.01 in the patients group, and 5.05 kg +/-0.10 in the control group. The result of the Student t test did not show any significant difference with a *p*-value of 0.99.

The mean height was 56.45 cm +/- 3.95 in the patients group, and 56.25 cm +/-4.07 in the control group. The result of the Student t test did not show any significant difference with a *p*-value of 0.76.

Identification of metabolites

The mean ^{1}H-NMR NOESY spectrum from the 160 urine samples collected within 120 days from birth is shown in *Figure 2.4.1*, with annotations of key metabolites.

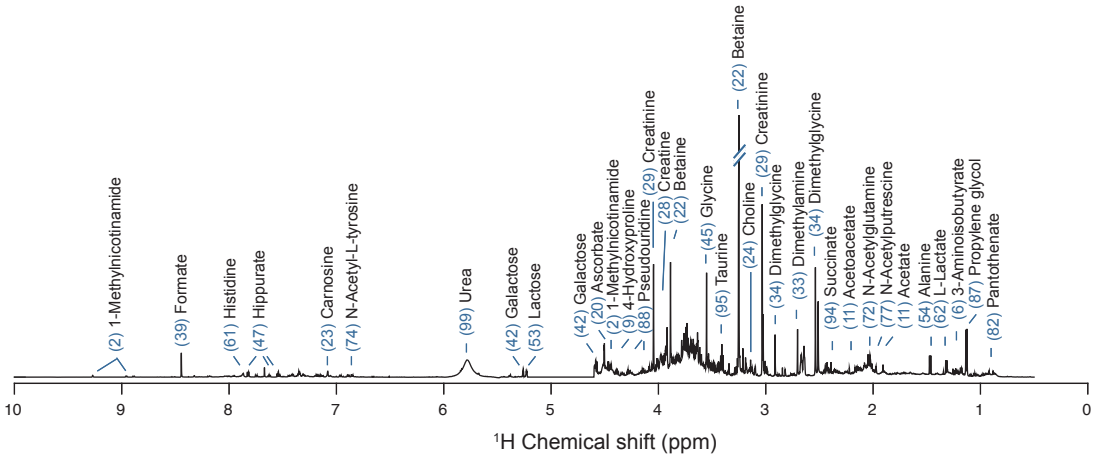


Figure 2.4.1. **Mean 600 MHz ^{1}H-NMR NOESY spectrum from all 160 newborn urine samples represented with selected metabolites annotations.** *The numbers in parentheses refer to the following list of identified metabolites.*

In total 100 metabolites were identified from ^{1}H NOESY spectra, supplemented by information from 2D ^{1}H-^{1}H TOCSY *(Figures 2.4.2 and 2.4.3)* and ^{1}H-^{13}C HSQC NMR experiment *(Figures 2.4.4 and 2.4.5)*.

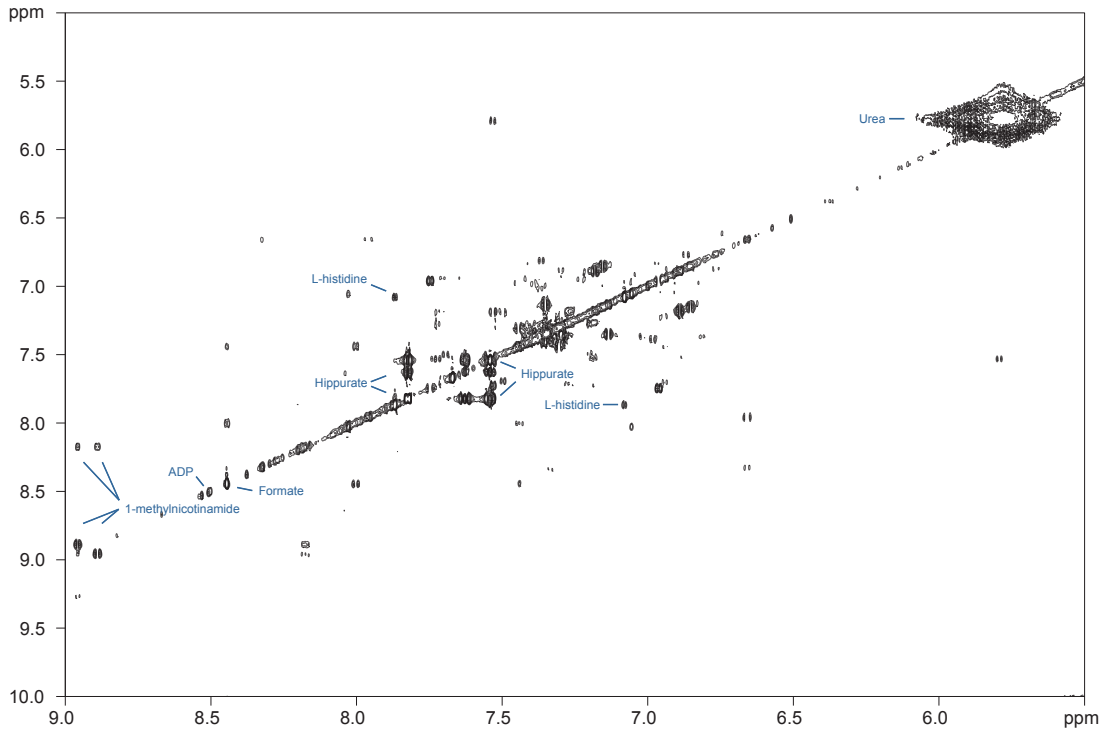


Figure 2.4.2: **Overview of the ^{1}H-^{1}H TOCSY NMR Spectrum (600 MHz) for a QC sample, from 5 to 9 ppm.**

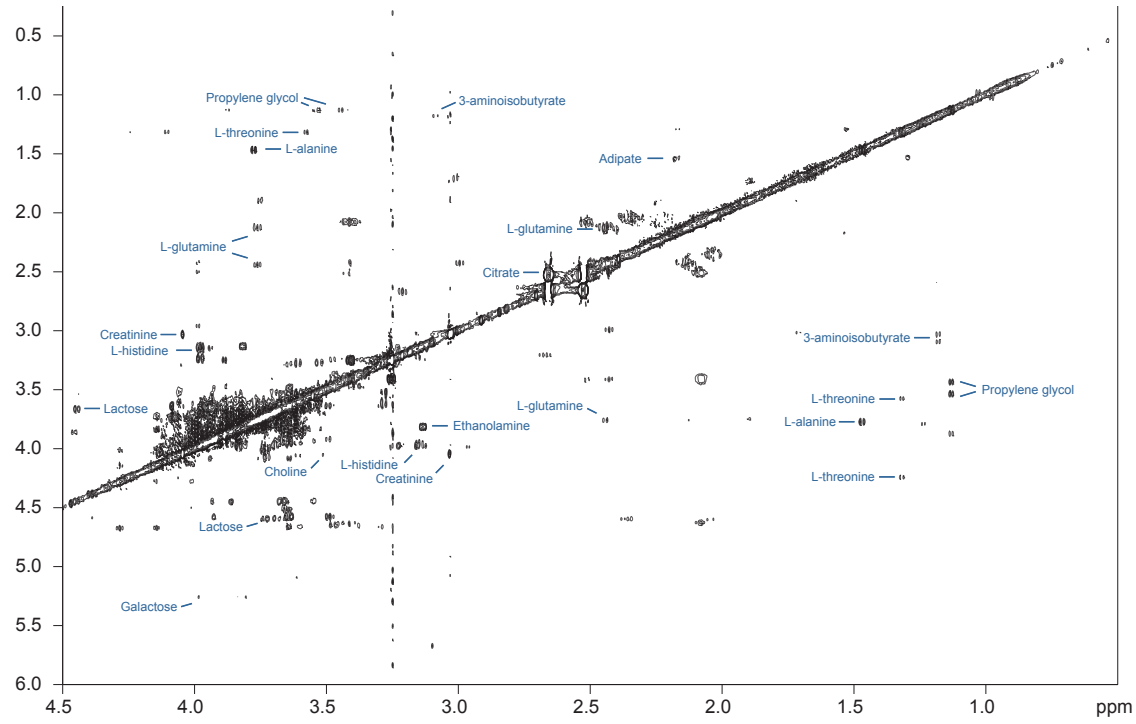


Figure 2.4.3: **Overview of the ^{1}H-^{1}H TOCSY NMR Spectrum (600 MHz) for a QC sample, from 0 to 4.5 ppm.**

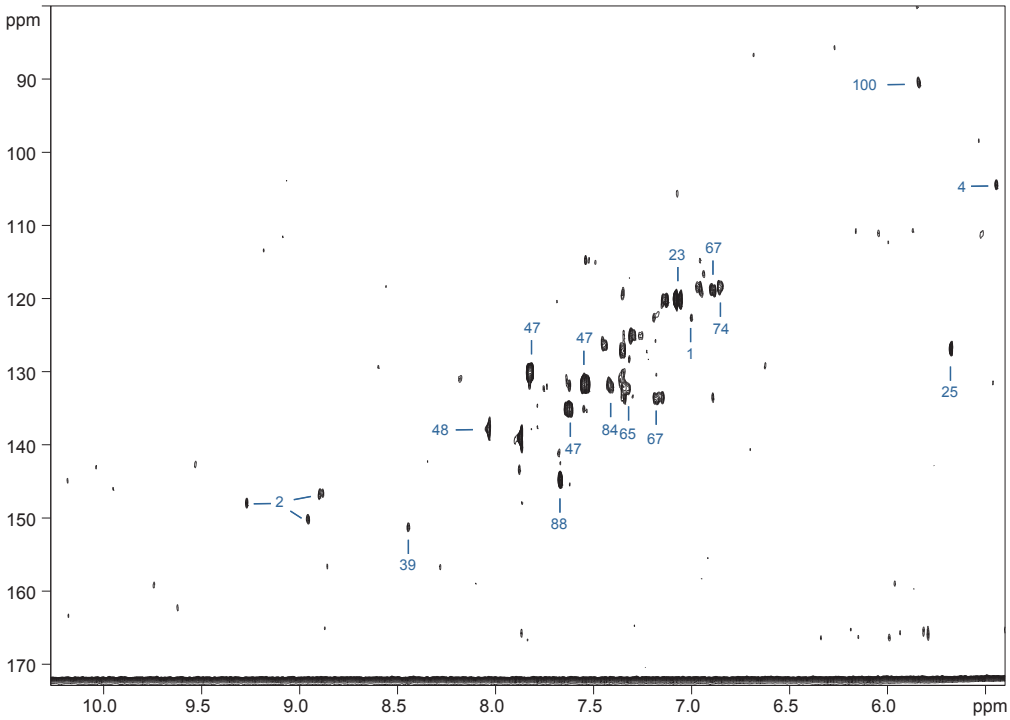


Figure 2.4.4: **Overview of the ^{1}H-^{13}C HSQC NMR Spectrum (600 MHz) for a QC sample from 5 to 10 ppm.** *Numbers correspond to metabolites in Table 2.5.2*

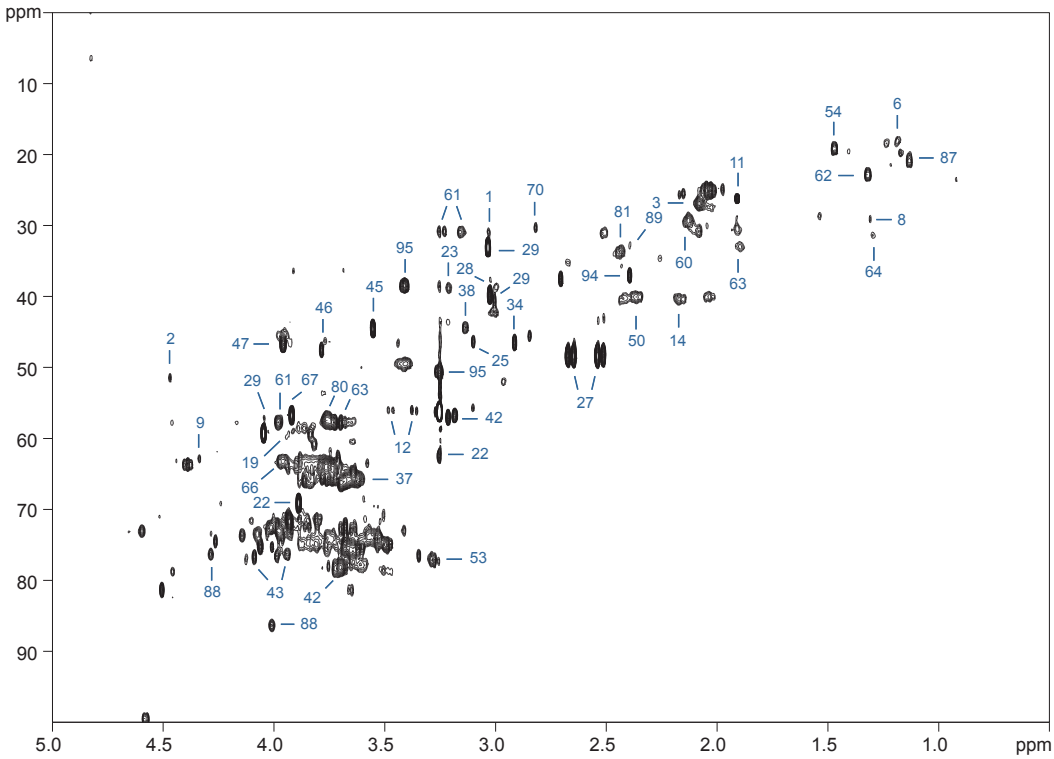


Figure 2.4.5: **Overview of the ^{1}H-^{13}C HSQC NMR Spectrum (600 MHz) for a QC sample from 0 to 5 ppm.** *Numbers correspond to metabolites in Table 2.5.2.*

Identified metabolites are listed in *Table 2.4.2.* The ^{1}H and ^{1}C chemical shifts of each metabolite are displayed in *Appendix G.*

Table 2.4.2: **List of metabolites identified on ^{1}H-NMR spectra of newborns urine**

1. 1-Methylhistidine (HMDB00001)	2. 1-Methylnicotinamide (HMDB00699)
3. 1,3-Diaminopropane (HMDB00002)	4. 1,6-anhydro-beta-D-Glucose (HMDB00640)
5. 2-Hydroxyisobutyrate (HMDB00729)	6. 3-Aminoisobutyrate (HMDB03911)
7. 3-Hydroxyisovalerate (HMDB00754)	8. 3-Hydroxymethylglutarate (HMDB00355)
9. 4-Hydroxyproline (HMDB00725)	10. 5-Aminopentanoate (HMDB03355)
11. Acetate(HMDB00042)	12. Acetoacetate (HMDB00060)
13. Acetone (HMDB01659)	14. Adipate (HMDB00448)
15. ADP (HMDB01341)	16. 16. Agmatine (HMDB01432)
17. Allantoin (HMDB00462)	18. Alpha-ketoisovalerate (HMDB04260)
19. Aminoadipate (HMDB00510)	20. Ascorbate (HMDB00044)
21. Beta-alanine (HMDB00056)	22. Betaine (HMDB00043)
23. Carnosine (HMDB00033)	24. Choline (HMDB00097)
25. cis-Aconitate(HMDB00072)	26. Citramalate (HMDB00426)
27. Citrate (HMDB00094)	28. Creatine (HMDB00064)
29. Creatinine (HMDB00562)	30. Cyclohexanone (HMDB03315)
31. D-Glucuronate (HMDB00127)	32. D-Glucose (HMDB00122)
33. Dimethylamine (HMDB00087)	34. Dimethylglycine (HMDB00092)
35. Dimethyl sulfone (HMDB04983)	36. D-threitol (HMDB04136)
37. Erythritol (HMDB02994)	38. Ethanolamine (HMDB00149)
39. Formate (HMDB00142)	40. Fumarate (HMDB00134)
41. Galactitol (HMDB00107)	42. Galactose (HMDB00143)
43. Glucarate (HMDB00663)	44. Gluconate (HMDB00625)
45. Glycine (HMDB00123)	46. Guanidoacetate (HMDB00128)
47. Hippurate (HMDB00714)	48. Homocarnosine (HMDB00745)
49. Hypoxanthine (HMDB00157)	50. Isobutyrate (HMDB01873)
51. Isocitrate (HMDB00193)	52. L-Acetylcarnitine (HMDB00201)
53. Lactose (HMDB00186)	54. L-Alanine (HMDB00161)
55. L-Alpha-aminobutyrate (HMDB00452)	56. L-Arabinose (HMDB00646)
57. L-Arginine (HMDB00517)	58. L-Carnitine (HMDB00062)
59. L-Fucose (HMDB00174)	60. L-Glutamine (HMDB00641)
61. L-Histidine (HMDB00177)	62. L-Lactate (HMDB00190)

63. L-Lysine (HMDB00182)	64. L-Octanoylcarnitine (HMDB00791)
65. L-Phenylalanine (HMDB00159)	66. L-Serine (HMDB00187)
67. L-Tyrosine (HMDB00158)	68. L-Valine (HMDB00883)
69. Mannitol (HMDB00765)	70. Methylguanidine (HMDB01522)
71. Myoinositol (HMDB00211)	72. N-Acetylglutamine (HMDB06029)
73. N-Acetyl-L-aspartate (HMDB00812)	74. N-Acetyl-L-tyrosine (HMDB00866)
75. N-Acetylornithine (HMDB03357)	76. N-Acetyl-D-glucosamine (HMDB00215)
77. N-Acetylputrescine (HMDB02064)	78. N,N dimethylaniline (HMDB01020)
79. O-phosphoethanolamine (HMDB00224)	80. Ornithine (HMDB00214)
81. Oxoglutarate (HMDB00208)	82. Pantothenate (HMDB00210)
83. Phenylacetate (HMDB00209)	84. Phenylacetylglycine (HMDB00821)
85. Pimelate (HMDB00857)	86. p-Hydroxyphenylacetate (HMDB00020)
87. Propylene glycol (HMDB01881)	88. Pseudouridine (HMDB00767)
89. Pyroglutamate (HMDB00267)	90. Pyruvate (HMDB00243)
91. Quinolinate (HMDB00232)	92. Sorbitol (HMDB00247)
93. Suberate (HMDB00893)	94. Succinate (HMDB00254)
95. Taurine (HMDB00251)	96. Threonate (HMDB00943)
97. trans-Aconitate (HMDB00958)	98. Trimethylamine N-oxide (HMDB00925)
99. Urea (HMDB00294)	100. Xanthosine (HMDB00299)

Quality of the ^{1}H-NMR data

An initial principal component analysis (PCA) was carried out to derive the main sources of variance from the ^{1}H-NMR data sets.[107] All samples were analyzed together, including 15 QC samples, the urine samples of 70 newborns with UTD and 90 controls collected within 120 days from birth. *(Figure 2.4.6)* The good stability of the NMR set-up and reproducibility of the experiments are attested by the grouped set of QC samples on the PCA score plot. Controls samples and patients' samples are not clearly separated by this unsupervised analysis. One patient sample was identified as a potential outlier. The quality of this spectrum was checked, and this sample was not removed for subsequent analyses as no specific clinical characteristics were identified for this patient.

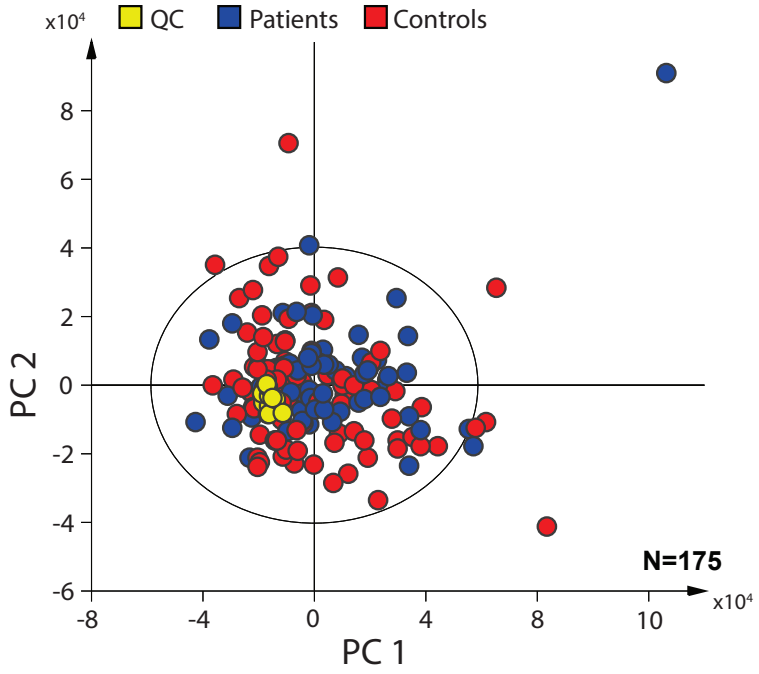


Figure 2.4.6: **Principal Component Analysis (score plot) based on ^{1}H-NMR spectra of 15 QC samples and urine samples of 70 newborns with UTD and 90 controls collected within 120 days from birth.** *The good stability of the NMR setup and reproducibility of the experiments is attested by the grouped set of QC samples on the PCA score plot.*

Comparison between ^{1}H-NMR spectra of patients and controls

A supervised analysis was carried out by calculating an O-PLS model to discriminate between metabolic profiles of patients and controls. No significant discrimination was obtained, as assessed by the low goodness of fit parameters (1+1 components; R^2Y = 0.186; Q^2 = -0.001). *(Figure 2.4.7)*

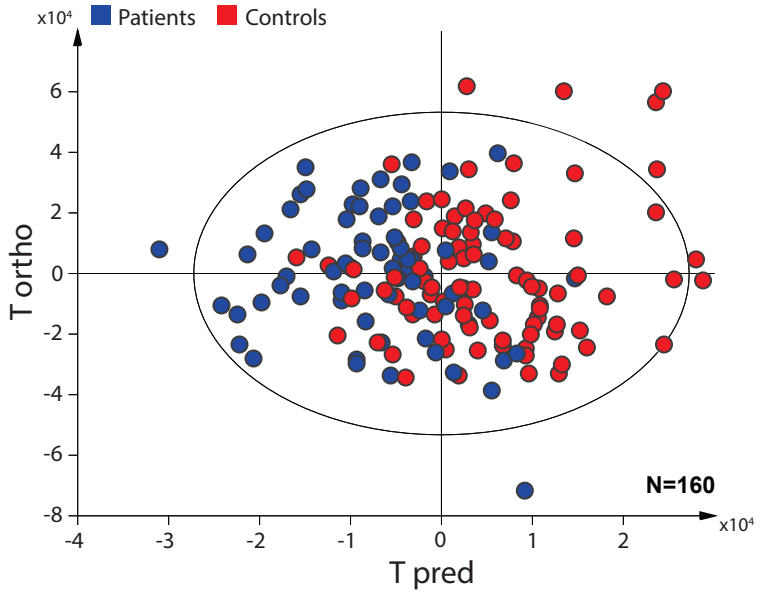


Figure 2.4.7: **Score plot of the O-PLS model based on ^{1}H-NMR spectra to discriminate between 70 patients urine samples and 90 controls urine samples.**

(1+1 components; $R^2Y = 0.186$; $Q^2 = -0.001$). There was no significant discrimination between patients and controls.

It could seem that there is a weak separation between the two groups, but this small separation is not statistically significant as the Q^2 value is negative. Thus, there is no evidence of significant difference between patients and controls metabolic profiles.

The same analysis was carried out using UV scaling instead of Pareto scaling, but this new model did not show any significant difference between the metabolic profiles either. (1 component, $R^2X = 0.128$, $R^2Y = 0.139$, $Q^2 = 0.00149$, p-value = 0.89)

Comparison between the surgical and the non-operative management groups

Another O-PLS analysis was carried out to discriminate between patients who required surgery during follow-up and patients who did not. No significant discrimination was obtained, as assessed by the low goodness of fit parameters.

(1+1 components; $R^2Y = 0.254$; $Q^2 = -0.32$). *(Figure 2.4.8)*

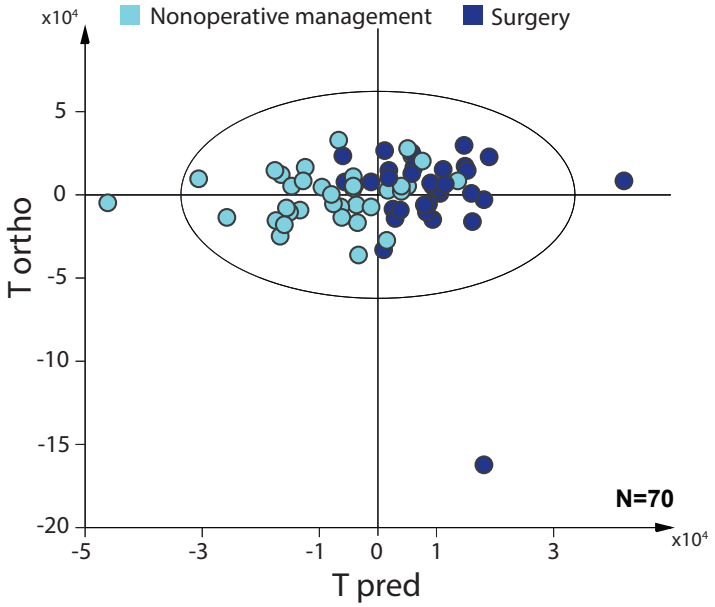


Figure 2.4.8: **Score plot of the O-PLS model based on ^{1}H-NMR spectra to discriminate patients who needed surgery and patients who did not within 2 years of follow-up.** *(1+1 components; $R^2Y = 0.254$; $Q^2 = -0.32$). There was no significant discrimination between these two groups.*

The results were not significant either using UV scaling. (1 component, $R^2X = 0.159$, $R^2Y = 0.15$, $Q^2 = -0.0626$, p-value = 1)

Other models were calculated to discriminate metabolic profiles according to groups 1 and 2, to the decrease of the relative renal function, and to UTD ultrasound grading system. The results of these models are summarized in *Table 2.4.3.* No clear discrimination was observed with these models.

Table 2.4.3: **Goodness of fit parameters of O-PLS models calculated for discrimination based on relative renal function, Group 1 and 2 (ureteral dilatation) and UTD grading.**

O-PLS models	Effective	Components	R^2X	R^2Y	Q^2	*p-value*
Relative renal function < 40 % (yes/no)	70	1	0.25	0.08	0.03	0.13
Group 1 / Group 2	70	1	0.22	0.12	-0.09	1
UTD grading	70	1	0.23	0.117	-0.009	1

2.4.4. Discussion

This clinical study evaluating the difference in the urinary metabolome of newborns with UTD and controls failed to demonstrate a characteristic metabolic signature associated with UTD. We did not find any characteristic signature associated with the need for surgery or the decrease of relative renal function. Either the urinary metabolome of newborns is not modified in case of UTD, or a difference exists that the present study failed to demonstrate.

These negative results contrast with the work of Decramer et al. who observed a specific proteomic profiles in newborns with PUJ who needed surgery.[79] It is possible that the differences in urine composition between patients with transient UTD and patients with PUJ needing surgery only concern proteins. However no clear definition of urinary obstruction was provided in this proteomic study, and indications for surgery were not specified. Moreover, the specific proteomic profile associated with PUJ was not confirmed in children after one year.[80]

To our knowledge only one metabolomic study analyzed urinary metabolome of patients with obstructive uropathy and this study has several flaws.[147] Twenty-one patients affected by different nephrouropathies were included (5 renal dysplasia, 7 VUR, 4 urinary tract infections, 2 acute kidney injuries and 3 not specified). This group of patients was therefore very heterogeneous. Controls were 19 healthy subjects, but there was no precision regarding potential excluding criteria. The authors calculated an O-PLS model to discriminate between patients and controls and concluded that their urinary metabolic profiles were different, but no information was provided regarding the goodness of fit parameters of the model. It is therefore difficult to make conclusions based on the results of this study.

Despite greater effectives and rigorous methodology, we did not find such difference in metabolic profiles of UTD and controls. Consequently, we investigated potential confounding factors that could have influenced the results.

Small differences in the patient selection or in the collection and handling of samples can have dramatics effects in analytical reliability and reproducibility, in statistical analysis and interpretation of results.[148] The presence of these small differ-

ences, which are mainly related to the use of biological specimen, leads the presence of biases also known as confounding factors. They can be introduced at all levels of the NMR metabolomic study, from the patient selection to spectral acquisition. A number of studies have focused on these confounding factors and their impact on the metabolic profile of individuals in biological fluids. The biases can be classified into three main categories: the patients,[149-151] sample handling[152-156] and the spectral acquisition and analysis.[85,157] *(Table 2.4.4)*

Table 2.4.4 : **Possible confounding factors in NMR metabolomic studies**

Patients	**Sample handling**	**Spectral acquisition and analysis**
Physiologic characteristics	**Procedures of sample collection**	**Spectrometer**
Age	Biologist	Field strength
Gender	Type of tube	Field homogeneity
Genetics	Type of anticoagulant (EDTA, citrate…)	Randomization samples
Menopausal status	Time before centrifugation	Run order
	Multicentric	Solvent suppression
Hormonal effects	Biological replicates	Batches
		Technical replicates
Extrinsic factors	**Transport**	
Diet		**Analyze**
Physical activities	**Procedures of sample preparation**	Processing (phasing, Baseline correction…)
Medication	Manual or automated	Procedure of normalization and scaling
Fasting status	Material	
Lifestyle	Protocol (centrifugation, type of solution…)	
Gut microflora		
	Storage conditions	
	Material	
	Temperature	
	Freezing/thawing cycles	
	Length of time storage	

A recent study by Maitre et al. showed the influence of the sample collection time-point of the day on the metabolic phenotype of children aged 8-9 years old. Trimethylamine, N-acetyl neuraminic acid, 3-hydroxyisobutyrate, 3-hydroxybutyrate/3-aminoisobutyrate, tyrosine, valine and 3-hydroxyisovalerate exhibited the highest stability

over a period of 6 days. Ideally for a metabolite to be validated as a clinical or exposure biomarker, the analytical variability and the intra-individual variability must be smaller than the inter-individual variability. Pooled samples captured the best inter-individual variability and could be a way to improve biomarker discovery.[158] In the present study, the sample collection time-point of the day was variable among patients. The fasting status was not known, which could be another confounding factor.

Although initially we did not found significant difference between patients and controls mean age, we made another test considering age in days and not in months. The mean age was 59.4 days +/-23.8 in the patients group, and 67.2 days +/-23.8 in the control group. The result of the Student t test revealed that this small difference was significant with a *p*-value of 0.04.

This new result revealed a difference between groups we did not suspected.

For this reason, we decided to investigate the influence of age on the metabolic profiles in controls and patients.

3. Influence of growth parameters on newborns urinary metabolic profiles

3.1. Influence of age, weight and height on urinary metabolome in healthy newborns

3.1.1. Newborns metabolic maturation

At birth, the continuous transplacental supply of glucose is abruptly disrupted, and emerging neonates undergo a dramatic metabolic adaptation associated with alternating periods of feeding and fasting.[159] The first months of life are characterized by a significant increase of the body weight and height, as well as a complex maturation of the immune, endocrine, nephrologic and neurologic systems. An infant usually doubles birth weight by the age of 4 to 6 months and triples birth weight within the first year of life.[160,161] The pattern of subsequent changes in the metabolic and hormonal milieu is influenced by various genetic and environmental factors including gestational maturity, intrauterine growth characteristics and postnatal feeding practices.[159]

Current knowledge of the newborns metabolic maturation is partial. Epidemiological studies have led to the hypothesis that the risk of developing chronic diseases in adulthood is largely influenced by environmental factors acting during the periconceptual, fetal and infant phases of life.[19] A large body of research has focused on low birth weight and growth patterns as risk factors for metabolic diseases in adulthood,[20-23] and early nutrition is known to have long-term effects on health, disease and mortality risks in adults.[162] A better understanding of the complex metabolic processes during early infancy may not only improve the management of neonatal diseases, but also contribute to the prevention of chronic pathologies in adulthood.[163]

The influence of age on urinary metabolomic profiles was previously reported in children ranging from 6 months to 4 years of age[161] and from 1 to 12 years of age.[164] Age-related variations of urinary metabolomic profiles were also demonstrated in adults, using 40 years as a cutoff[165] and comparing subjects aged < 35 and > 50 years.[166] Marincola *et al.* also demonstrated the impact of early postnatal nutrition on the NMR urinary metabolic profile from birth to 4 months of life.[32]

In the present study, we investigate the influence of age, gender, and growth parameters, namely height and weight, on the newborns urine metabolome during the first 4 months of life using ^{1}H- NMR spectroscopy combined with multivariate statistical analyses.[167]

3.1.2. Experimental section

Study population

All ninety newborns (24 females and 66 males) from the control group were included in this part of the analysis. The mean age, weight and height were respectively 67 days (11-112), 5 kg (3-8) and 56 cm (47-67). The demographical characteristics of the study population and the motives of medical consultations are summarized in *Table 3.1.1 and 3.1.2.* Pearson correlation analysis conducted with SPSS (version 23.0; SPSS, Inc, Chicago, IL) software and a significance threshold of 0.05 confirmed the high correlation between weight and height (correlation coefficient 0.725, p-value = 0.0001) and lower correlations between age and weight (correlation coefficient 0.355, p-value = 0.001), and age and height (correlation coefficient 0.393, p-value = 0.0001).

Table 3.1.1: **Demographical characteristics of the population of healthy newborns**

	Total cohort	Males	Females
Number of individuals (%)	90 (100)	66 (73)	24 (27)
Mean age, days	67 (11-112)	68 (28-112)	64 (11-102)
Mean height, cm	56 (47-67)	56 (47-65)	56 (51-67)
Mean weight, kg	5 (3-8)	5 (3-7)	5 (4-8)

Table 3.1.2: **Motives of medical consultations**

Motives of medical consultation	n
Inguinal hernia	42
Abdominal discomfort	27
Suspicion of pyloric stenosis	11
Undescended testis	5
Umbilical granuloma	3
Umbilical hernia	2

Metabolomic analysis

The steps of the metabolomic analysis are described in chapter 2.5.2. The following analyses used the same dataset. When the calculated O-PLS models were found statistically robust, the Statistical Recoupling of Variables (SRV) algorithm was used implemented with MATLAB homemade routines. Consecutive and correlated variables were associated into a series of clusters. The significance of these new NMR variables for the effect under study (age, weight, height, gender) was calculated by ANOVA analysis and multiple testing correction of the p-values (Benjamini-Hochberg false discovery rate).[121] Correlation profile of the significant variables was represented on a loading plot to allow a visual and efficient identification of candidate biomarkers.[168]

Quality of the ^{1}H-NMR data

Good stability of the NMR setup and reproducibility of the experiments are attested by the grouped set of QC samples on the PCA score plot. *(Figure 3.1.1)* The PCA score plot was also used to check population homogeneity. No severe outlier was identified and all samples were included in the final analysis.

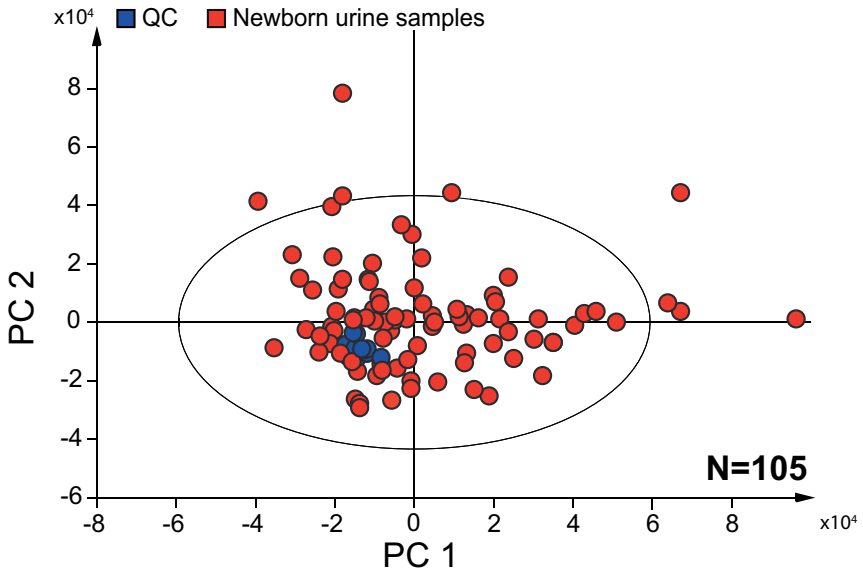


Figure 3.1.1: **PCA score plot of the 90 urine metabolic profiles and 15 quality control samples.** *(N = 105, 17 components; R^2 = 0.757, Q^2 = 0.367). The good stability of the NMR setup and reproducibility of the experiments are attested by the grouped set of QC samples.*

3.1.3. Metabolic signature associated with age

Figure 3.1.2 represents the results of supervised multivariate analysis (O-PLS model) carried out in order to identify the latent variable correlated with age. This correlation is illustrated along the score plot horizontal (predictive) axis. *(Figure 3.1.2a)* Statistical significance of the model is assessed by high values of goodness-of-fit parameters (R^2X = 0.235, R^2Y = 0.554, Q^2 = 0.376), a *p*-value of $1.3x10^{-8}$ by CV-ANOVA and model permutations. *(Figure 3.1.2b)* Validation by response permutation is an internal cross-validation, which creates a training set and a test set of samples. A model is fitted to explain the test set based on the training set and the values for R^2Y (explained variance) and Q^2 (predicted variance) are computed and plotted. By random permutation of the training and test sets, a number of R^2Y/Q^2 are obtained. The validate plot is then created by letting the Y-axis represent the R^2Y/Q^2-values of all models, including the "real" one, and by assigning the X-axis to the correlation coefficients between permuted and original response variables. A regression line is then fitted among the R^2Y points and another one through the Q^2 points. The intercepts of the regression lines are interpretable as measures of "background" R^2Y and Q^2 obtained to fit the data. R^2Y intercept value lower than the original point of R^2Y-value, and negative Q^2 intercept value indicate valid models. These criteria are met in this model, which confirm its validity.

The loadings plot depicting the metabolic signature associated with age is complemented by color-coded correlation and individual statistical significance information obtained from univariate analysis. *(Figure 3.1.2c)*

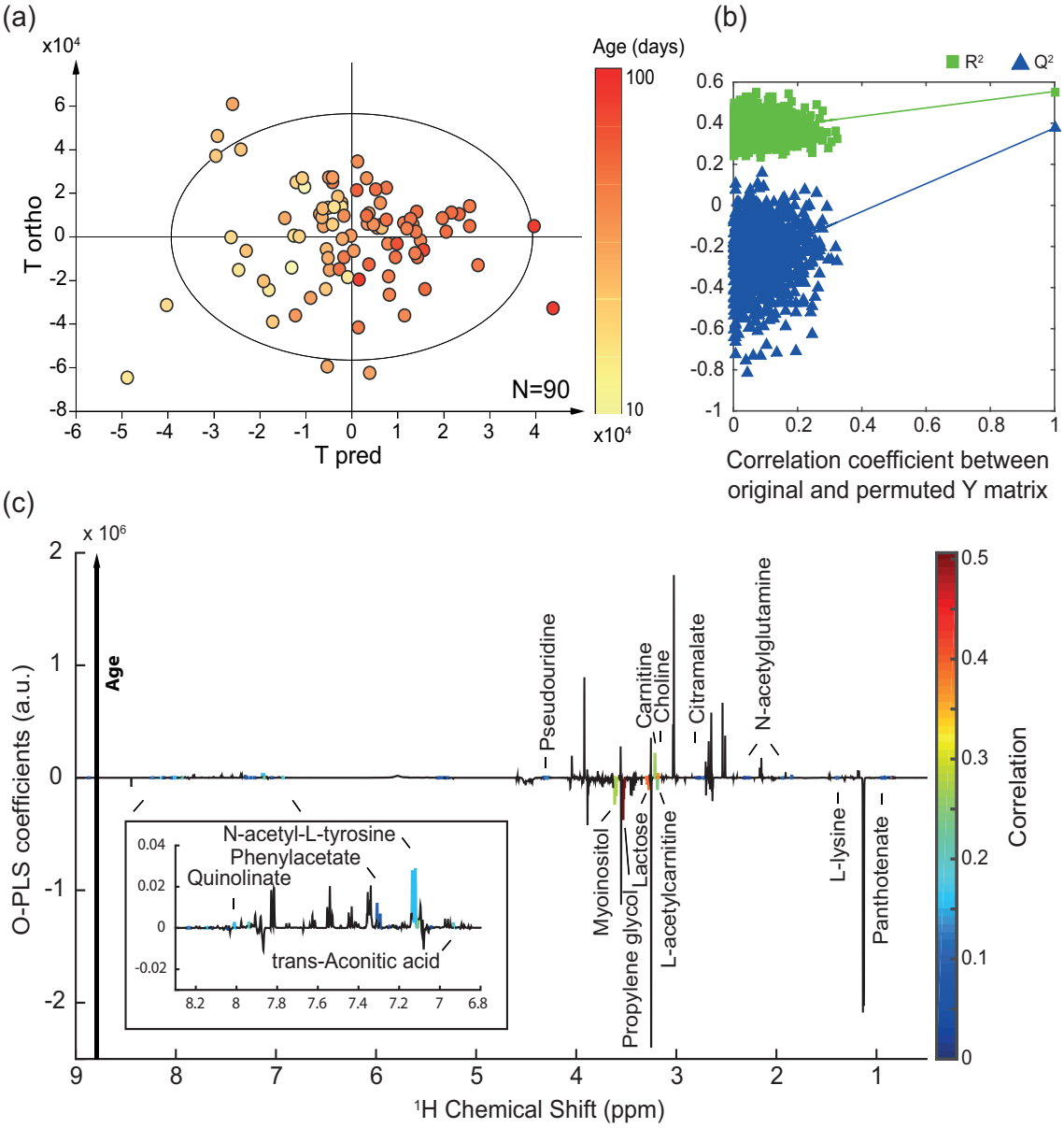


Figure 3.1.2: **O-PLS model constructed from the 90 ^{1}H NMR spectra of newborns urine for a regression based on age.** *(1+ 1 components; R^2X = 0,235; R^2Y = 0.554; Q^2 = 0.376; p-value = 1.3x10^{-8} by CV-ANOVA). (a) Score plot; (b) model validation by re-sampling 1000 times under the null hypothesis; (c) O-PLS loading plot after SRV analysis and Benjamini–Hochberg multiple testing correction. Statistically significant signals correspond to the colored spectral regions.*

Metabolites identified as key molecules with significant correlation with age are listed in *Table 3.1.3.*

Table 3.1.3: **Metabolites identified as key molecules significantly correlated with age**

Urine concentration increasing with age			**Urine concentration decreasing with age**		
Metabolite	***p*-value***	***q*-value****	**Metabolite**	***p*-value***	***q*-value****
2-Hydroxyisobutyrate	0.02	0.16	4-Hydroxyproline	0.049	0.27
Carnitine	0.0004	0.005	Citramalate	5.10^{-16}	9.10^{-14}
Choline	0.005	0.04	Galactose	0.008	0.07
Ethanolamine	0.04	0.24	Isobutyrate	0.04	0.24
Glucarate	0.01	0.12	Lactose	0.002	0.02
Histidine	0.04	0.24	L-Lactate	0.006	0.05
L-Acetylcarnitine	7.10^{-5}	0.0009	L-Lysine	3.10^{-6}	5.10^{-5}
L-Glutamine	0.007	0.06	Myoinositol	0.0003	0.004
N-Acetyglutamine	1.10^{-5}	0.0003	Pantothenate	0.0004	0.005
N-Acetyl-L-tyrosine	9.10^{-13}	4.10^{-11}	Propylene glycol	0.002	0.02
Phenylacetate	2.10^{-7}	5.10^{-6}			
Pseudouridine	3.10^{-7}	7.10^{-6}			
Quinolinate	5.10^{-13}	3.10^{-11}			
trans-Aconitate	7.10^{-5}	0.001			

**p-value without multiple testing correction*

***q-value after Benjamini-Hochberg false discovery rate multiple testing correction*

These results highlight modifications of the urine metabolome during the neonatal period. This clear evolution of metabolomic profiles is concomitant with a period of rapid growth and maturation of the renal system. This metabolic signature is specific to newborns, with different key metabolites than in adults, in particular those involved in carbohydrate metabolism.[32,161,165,166] Evolution of carbohydrate metabolism is particularly central during the studied neonatal period, as concentrations of lactose, galactose, myoinositol and lactate decrease over time. Lactose is the major sugar in breast and formula milk, and the source of glucose and galactose. Small amounts of lactose is absorbed intact across the intestine and excreted unchanged in urine. Lactose and galactose concentrations in urine decreased from 0 to 4 months, which can be related to lactose intestinal uptake and urinary excretion that are significantly higher in newborns compared to adult subjects.[169] Myoinositol can be synthesized from glucose-6-phospate,

and is naturally present in breast milk and added in formula milk. It plays an important role in glucose metabolism and transport. It is a precursor of a number of secondary messengers and an important component of the structural lipids phospatidylinositol.[170] Our results confirm its decrease in urine from 0 to 4 months, as reported earlier.[32]

The increase in choline levels observed in newborns from 0 to 4 months of age is concordant with data reported by Marincola *et al.*[32] Choline is an essential nutrient, mainly provided by food intake, precursor of acetylcholine and a component of phosphatidylcholine. The average choline consumption is frequently below the adequate intake and choline deficiency may play a role in liver disease and atherosclerosis.[171] Choline level is elevated in formula milk and its level in breast milk is correlated with respective level in maternal blood.[172] It is usually grouped with B vitamins, but can also be synthesized from glycine, which concentration is interestingly correlated with age in children.[161,164] Choline is also a precursor of betaine, another metabolite identified as age-dependant in newborns and adults.[32,161,164-166] Betaine urinary concentration has been reported to increase from 0 to 2 months and decreasing from 2 to 4 months.[32] This could explain why it is not correlated with age in the present study. Meanwhile, propylene glycol is an excipient in drug formulation and is contained in vitamins supplementation largely prescribed in newborns. We hypothesize that the observed decrease in propylene glycol concentration may be explained by a decreasing observance of vitamins supplementation with infant's age, though data is not accessible to confirm this information, as the study design did not include follow-up.

Some of the metabolites found to be age-related in newborns in the present studies are not specific to the neonatal period, as they have been reported earlier in children and adults. This is the case for carnitine,[165] for which the observed increased concentrations with age was also previously described for newborns fed by breast milk.[32] L-acetylcarnitine was also correlated with age in our study. Most of the carnitine comes from breast milk and formula milk in newborns, and meat consumption in adults.[173] Its role is to transport long-chain acyl groups from fatty acids into the mitochondria matrix, providing energy to muscle cells. Its increasing concentration in newborns urine throughout the first four months of life could be correlated to the significant growth of skeletal muscle mass during infancy.[164]

We note that evolution of the urine metabolome during the first four months of life seems progressive. O-PLS discriminant analysis performed to investigate variations between groups of subjects classified by age showed a significant discrimination between groups 0 (newborns younger than 1 month) and 3 (3 months old newborns) and between group 1 (1 month old newborns) and 3, *(Figure 3.1.3)* with a lack of significant discrimination between the other groups. This analysis shows the strength of the correlation between metabolomic profiles and age, which is still seen despite small effectives. However, there does not seem to be any rapid switch in urinary metabolomic profiles, which suggests a progressive maturation of newborns metabolism throughout this period of life.

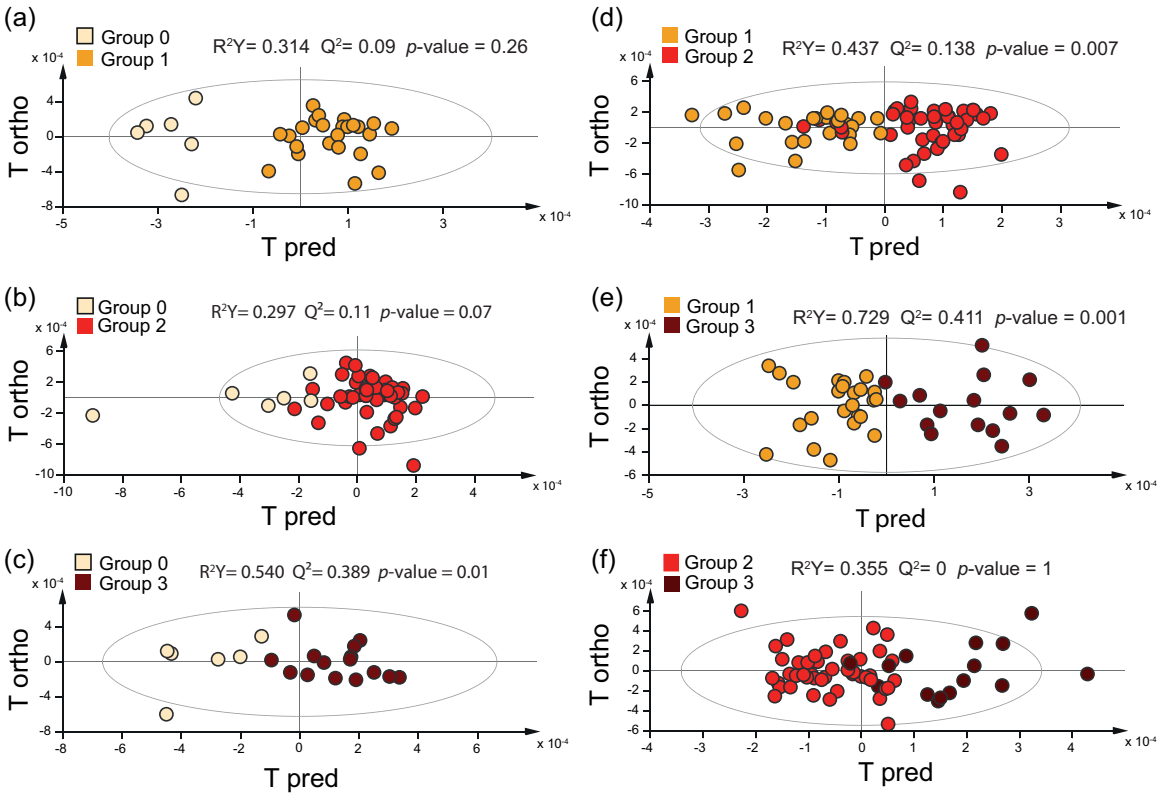


Figure 3.1.3: **Score plots of the O-PLS models based on ^{1}H-NMR spectra of newborns urine for classification based on age groups.** *A significant discrimination was found between groups 0 and 3, between groups 1 and 2 and between groups 1 and 3.*

3.1.4. Metabolic signatures associated with growth parameters.

Supervised analysis by O-PLS revealed significant variations in the dataset related to height and weight. *(Figure 3.1.4)* High goodness-of-fit parameters and model resampling under the null hypothesis *(Figure 3.1.5)* assessed the robustness of the calculated models according to height ($R^2X = 0.196$, $R^2Y = 0.551$, $Q^2 = 0.341$, p-value $= 5.1 \times 10^{-7}$) and weight ($R^2X = 0.11$, $R^2Y = 0.588$, $Q^2 = 0.118$, p-value $= 0.03$).

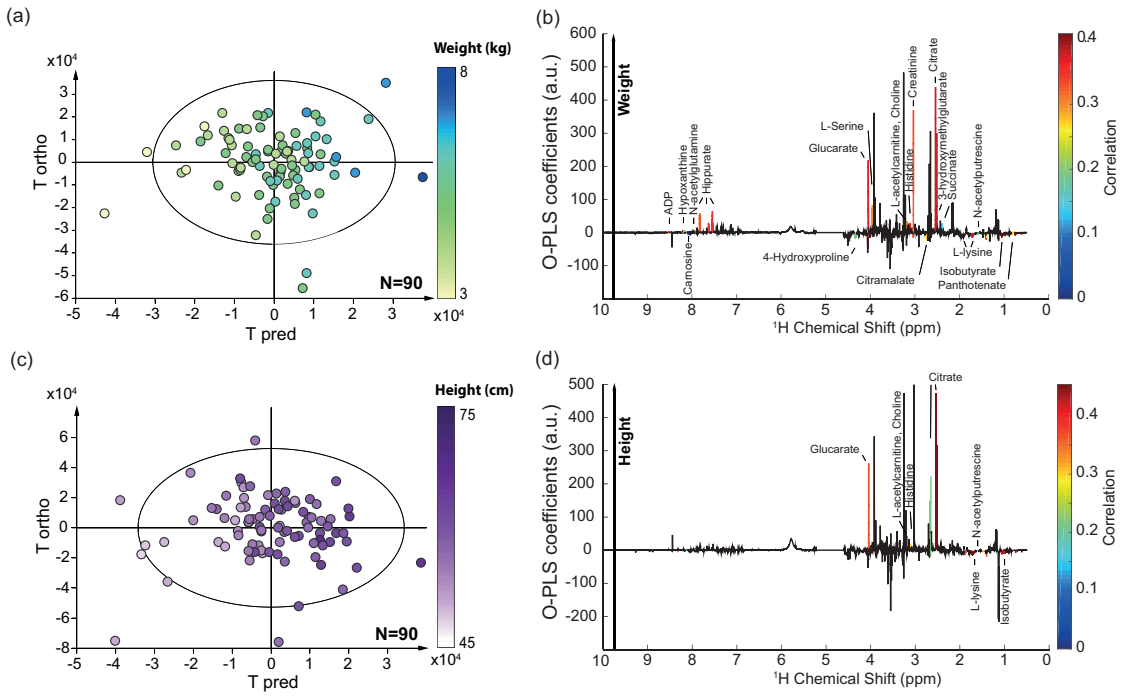


Figure 3.1.4: **O-PLS regression model against newborns weight, constructed from the set of 90 ^{1}H NMR spectra** *(1+ 1 components; $R^2X = 0.11$, $R^2Y = 0.588$, $Q^2 = 0.118$, p-value = 0.03 by CV-ANOVA): (a) score plot and (b) corresponding loadings plot after univariate analysis and Benjamini–Hochberg multiple testing correction. Statistically significant signals correspond to the colored spectral regions. Significant concentrations variations correlated with weight are summarized in Table 3.1.4.* **O-PLS regression model against newborns height** *(1+ 1 components; $R^2X = 0.196$, $R^2Y = 0.551$, $Q^2 = 0.341$, p-value = $5.1x10^{-7}$ by CV-ANOVA): (c) score plot and (d) corresponding loadings plot after univariate analysis and Benjamini–Hochberg multiple testing correction. Statistically significant signals correspond to the colored spectral regions.*

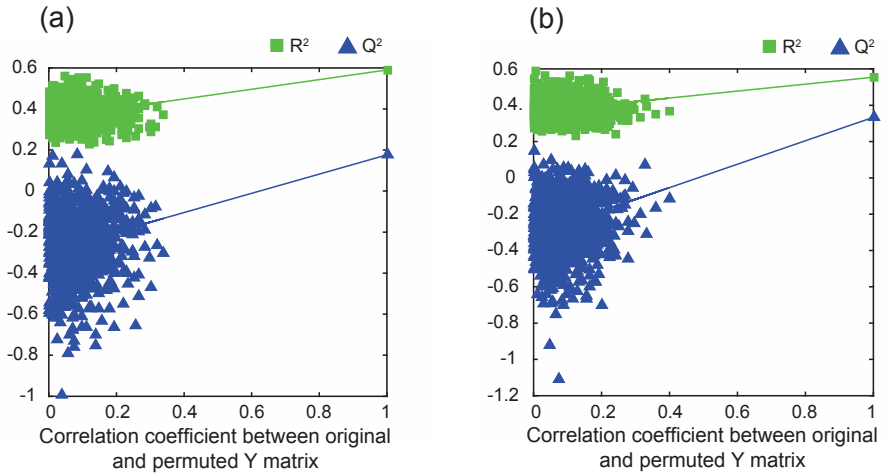


Figure 3.1.5: O-PLS models for regression against weight (a) and height (b) were validated by re-sampling 1000 times under the null hypothesis.

Metabolic signatures associated with height and weight are supplemented with univariate analysis of the NMR metabolomic profiles. Metabolites significantly correlated with weight and height are listed in *Table 3.1.4.*

Table 3.1.4: **Evolution of urine concentration of metabolites significantly associated with age, weight and height in 90 newborns under 4 months of age.**

	Age			Weight			Height		
	Evolution	*p*-value*	*q*-value**	Evolution	*p*-value*	*q*-value**	Evolution	*p*-value*	*q*-value**
1-Methylnicotinamide	-			↓	0.04	0.2	↓	0.03	0.3
2-Hydroxyisobutyrate	↑	0.02	0.16	-			-		
3-Hydroxyisovalerate	-			-			↑	0.02	0.2
3-Hydroxy-methylglutarate	-				0.004	0.03			
4-Hydroxyproline	↓	0.049	0.27	↓	0.0009	0.009	↓	0.04	0.3
ADP	-			↑	8.10^{-15}	5.10^{-13}	-		
Carnitine	↑	0.0004	0.005	↑	0.02	0.09	↑	0.04	0.3
Carnosine	-			↓	0.002	0.02	-		
Choline	↑	0.005	0.04	↑	1.10^{-6}	2.10^{-5}	↑	0.0001	0.004
***cis*-Aconitate**	-				0.04	0.2		0.01	0.1
Citramalate	↓	5.10^{-16}	9.10^{-14}	↓	0.0009	0.009	-		
Citrate	-			↑	7.10^{-5}	0.003	↑	6.10^{-7}	2.10^{-5}
Creatinine	-			↑	4.10^{-5}	0.0007	↑	0.008	0.09
Dimethylamine	-			↑	0.01	0.09	-		
Ethanolamine	↑	0.04	0.24	-			-		
Galactose	↓	0.008	0.07	-			-		
Glucarate	↑	0.01	0.12	↑	1.10^{-5}	0.0003	↑	0.003	0.04
Hippurate				↑	0.003	0.02	-		
Histidine	↑	0.04	0.24	↑	0.003	0.02	↑	0.003	0.04
Hypoxanthine	-			↑	8.10^{-10}	3.10^{-8}	-		
Isobutyrate	↓	0.04	0.24	↓	4.10^{-11}	2.10^{-9}	↓	3.10^{-7}	1.10^{-5}
L-Acetylcarnitine	↑	7.10^{-5}	0.0009	↑	0.0004	0.005	↑	0.0007	0.01
Lactose	↓	0.002	0.02	-			-		
L-Glutamine		0.007	0.06	-			-		
L-Lactate	↓	0.006	0.05	-			-		
L-Lysine	↓	3.10^{-6}	5.10^{-5}	↓	7.10^{-7}	2.10^{-5}	↓	1.10^{-8}	8.10^{-7}
L-Serine	-			↑	0.05	0.2	-		
Myoinositol	↓	0.0003	0.004	-			-		
N-Acetyglutamine	↑	1.10^{-5}	0.0003	↑	0.0002	0.003	-		
N-Acetyl-L-tyrosine	↑	9.10^{-13}	4.10^{-11}	↑	0.01	0.07		0.04	0.3
N-Acetylputrescine	-			↓	0.006	0.04	↓	0.003	0.04
Oxoglutarate	-			↑	0.002	0.02	↑	0.02	0.2
Pantothenate	↓	0.0004	0.005	↓	0.0004	0.005	-		
Phenylacetate	↑	2.10^{-7}	5.10^{-6}	-			-		
Propylene glycol	↓	0.002	0.02	-			-		
Pseudouridine	↑	3.10^{-7}	7.10^{-6}	-			-		
Quinolinate	↑	5.10^{-13}	3.10^{-11}	-			-		
Succinate	-			↑	2.10^{-3}	0.02	-		
***trans*-Aconitate**	↑	7.10^{-5}	0.001	-			-		

**p-value without multiple testing correction*

***q-value after Benjamini-Hochberg false discovery rate multiple testing correction*

We investigated here for the first time concomitantly the influence of age and growth parameters on urine metabolomic profiles in newborns. The tremendous growth occurring during the neonatal period is associated with important modifications of newborns metabolism. Despite the existing correlations between age, height and weight parameters, several metabolites appear to only correlate to one of these factors, *(Figure 3.1.6)* and our study highlight a number of specific associations with newborns height and weight that were so far described as associations with age, in previous reports neglecting the former factors.

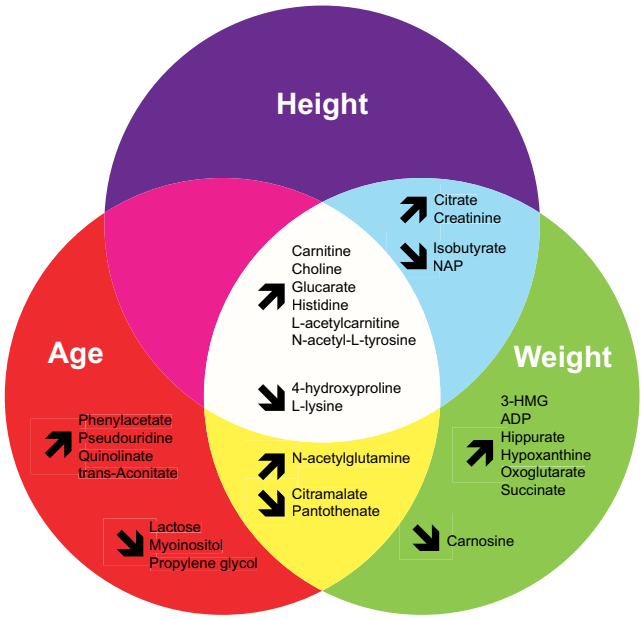


Figure 3.1.6: **Venn diagram summarizing key metabolites significantly associated with age, weight and/or height.** *Metabolites with at least one q-value < 0.05 are represented. NAP: N-Acetylputrescine; 3 HMG: 3-Hydroxymethylglutarate.*

We found that concentrations of phenylacetate, pseudouridine, quinolate and trans-aconitate are specifically increasing with age, which to our knowledge was not reported before. Dimethylamine, 1-methylnicotinamide, hippurate and succinate have been reported as increasing with age in children from 6 months to 1 year,[161] while no such correlation was evidenced in adults.[165,166] Our results suggest that concentrations of these metabolites in newborns urine are in fact more specifically correlated to the individual's weight, with a significant association confirmed for hippurate and succinate after multiple testing correction. We also identified 3-hydroxymethylglutarate, ADP, hypoxanthine and oxoglutarate as specifically increasing with weight. ADP and hypoxan-

thine are both results of the breakdown of intracellular ATP, which suggest an increase of purine metabolism. The increase in citrate, succinate and oxoglutarate urinary concentrations is also consistent with an enhanced activity of the tricarboxylic acid cycle as newborns gain weight. In the case of citrate that we found positively associated with height and weight, age-dependant concentrations have been reported in several studies investigating the urine metabolome[32,161,164-166,174] though inconsistent increase or decrease in concentration with age were so far described for newborns.[32,174] Factors regulating citrate excretion in urine are not fully understood. It is an important intermediate in the tricarboxylic acid cycle, a central pathway regulating metabolism carbohydrates, fats, and proteins metabolism. Urinary concentration of cis-aconitate, an intermediate in the tricarboxylic acid cycle produced by the dehydration of citrate also increased with weight in our observations. Creatinine is the breakdown product of creatine phosphate in muscle. Its blood concentration is known to be correlated with age and weight and is routinely used to evaluate renal function.[175] Creatinine concentration in blood and urine is a marker of glomerular filtration influenced by numerous factors including age, weight, gender, meat consumption, exercise or drugs intake. Creatinine urinary concentration was correlated with age in previous metabolomic studies,[32,161,164-166] yet we found here weight to be the most important factor modifying its urinary concentration in newborns. Our metabolic signature also detects an increase of hippurate with weight, while it was reported as increasing with age in children from 6 months to 1 year in a previous study.[161] Hippurate is an acyl glycine formed by the conjugation of benzoic acid with glycine, and is a normal component of urine increasing with consumption of phenolic compounds.

We found pantothenate urinary concentration to decrease with weight although it was reported to increase over time in newborns fed with formula milk.[32] This essential nutrient from the B vitamin group (vitamin B5) is present in breast and formula milk. Panthotenate is needed for the synthesis of coenzyme-A and plays a critical role in the metabolism and synthesis of carbohydrates, proteins, and fats.

We also observed an evolution in histidine and carnosine urinary concentrations related to weight. Carnosine is a dipeptide of the amino acids beta-alanine and histidine. It is highly concentrated in muscle and brain tissues and could have suppressive effects on the biochemical changes that accompany aging.[176] This evolution of histidine and carnosine concentrations could be related to the growth of muscle mass in newborns.

Considering these results, we emphasize the necessity to systematically and precisely report children age, height and weight in metabolomic studies, as they have differential impact on urinary metabolic profiles. This is particularly important in metabolomic studies investigating urine metabolome of premature children or intrauterine growth retardation,[14,28] where newborns at the same age may have very different weight and height. Inclusion criteria must be precise and rigorous considering the fast evolution of newborns metabolism. Consequently, we recommend patients to be matched by age, weight and height whenever possible before any group comparison.

3.1.5. Influence of gender on the urine metabolomic profiles

Supervised analysis was also applied to find variations in the dataset related to gender, but the O-PLS model did not show significant separation between males and females. ($R2X = 0.154$, $R2Y = 0.117$, $Q2 = -0.02$).

These results contrast with one previous study reporting significant differences between urinary profiles of newborns one or two days old according to their gender.[177] In adults, 3-hydroxybutyrate, creatinine and taurine and TMAO urinary concentrations were reported as higher in male subjects, whereas citrate, creatine, phenylalanine, hippurate, lactate, glycine and succinate were higher in women subjects.[166] Interestingly, the differences observed between adult male and female urinary metabolomes were not identified in children.[161,164,178] At birth, higher levels of testosterone in males have been early documented.[179] Testosterone levels decrease very rapidly after birth to prepubertal values. This phenomenon is commonly referred as “mini-puberty” and could explain differences in metabolic profiles associated with gender observed at birth and in adults, but not in prepubertal children.

3.1.6. Limitations of the study

The main limitation in our study is the lack of information regarding the intake of breast milk or formula milk. Yet, Marincola *et al.* recently reported the effects of three different milk supplies on the urinary metabolic profiles of newborns during the first 4 months of life.[32] Concentrations of fucosylate oligosaccharides, N-acetyl compounds, formate and citrate were elevated in the breast milk group, whereas the levels of pantothenate, choline, threonate, tartrate, cis-aconitate and lactate were higher in the formula milk group. Dessi *et al.* compared urine metabolomic profiles of 7 days neonates fed

with breast milk or formula milk. Urine of neonates fed with formula milk contained higher levels of glucose, galactose, glycine and myoinositol, while up-regulated aconitate, aminomalonate and adipate were found in breast milk fed neonates.[180] Lactose, L acetylcarnitine, myoinositol, carnitine and pantothenate are detected in breast milk collected at day 90 postpartum.[173] A switch in newborns nutrition from breastfeeding to formula milk in the first months of life could have modified the observed metabolite urinary concentrations. However, Chiu *et al.* did not find significant differences regarding different patterns of breastfeeding in newborns less than one year of age,[161] and Marincola *et al.* reported the same age-dependent metabolites in the breast milk group and in the formula milk group.[32]

Although the number of patients included is higher than in previous studies investing age-related modifications of urine metabolome, it is still modest, which is a recurrent issue in neonatal metabolomic studies. A cohort study analyzing the dynamic modifications of urine metabolomic profiles throughout the first months of life with more samples per newborns will be required to further generalize our results.

3.1.7. Conclusions

The present study is the first one to highlight the simultaneous influence of newborns age, weight and height on the urine metabolome during the first four months of life. The tremendous growth occurring during the neonatal period is associated with specific modifications of newborns metabolism. We identified key-metabolites responsible for this evolution. Conversely, gender appears to have no impact on the urine metabolome in early infancy. These results allow a deeper understanding of newborn metabolic maturation and contribute to identify potential confounding factors for metabolomics investigations in newborns. Considering these results, we emphasize the necessity to systematically and precisely report children height and weight in metabolomic studies, as they have a critical impact on urinary metabolic profiles.

3.2. Influence of age, weight and height on the urinary metabolome of newborns with urinary tract dilatation

3.2.1. Introduction

The comparison of urinary metabolic profiles of healthy newborns and newborns with prenatally diagnosed UTD did not reveal significant differences between the two groups. However, there is a strong influence of age and growth parameters on the urinary metabolome of healthy newborns. The objective of the following chapter is to evaluate the influence of age, weight, height and gender on the urinary metabolite profiles of newborns with prenatally diagnosed UTD.

3.2.2. Methods

The following analyses use the same dataset as previous chapters, plus 49 urine samples from patients collected between 7 and 18 months of age. The original study design included these additional urine samples with the objective of studying a potential normalization of urinary metabolome of patients over time. The ^{1}H NMR spectra were acquired in the same batch as other samples. The steps of metabolomic analysis and multivariate statistical analysis were described in chapter 2.4.2.

3.2.3. Metabolic signature associated with age in patients with UTD before 4 months

An initial PCA was carried out to derive the main sources of variance from the ^{1}H-NMR data sets. All samples were analyzed together, including 15 QC samples, the urine samples of 70 newborns with UTD and 90 controls collected within 120 days from birth and the 49 additional samples from patients collected between 7 and 18 months of age. *(Figure 3.2.1)* The good stability of the NMR setup and reproducibility of the experiments were attested by the set of QC samples grouped on the PCA score plot. This unsupervised analysis showed a clear discrimination between spectra of samples collected before 4 months (controls and patients first sample) and between 7 and 18 months (patients second sample), suggesting a strong influence of age on the urinary metabolome of patients. No outsider was excluded after this initial analysis.

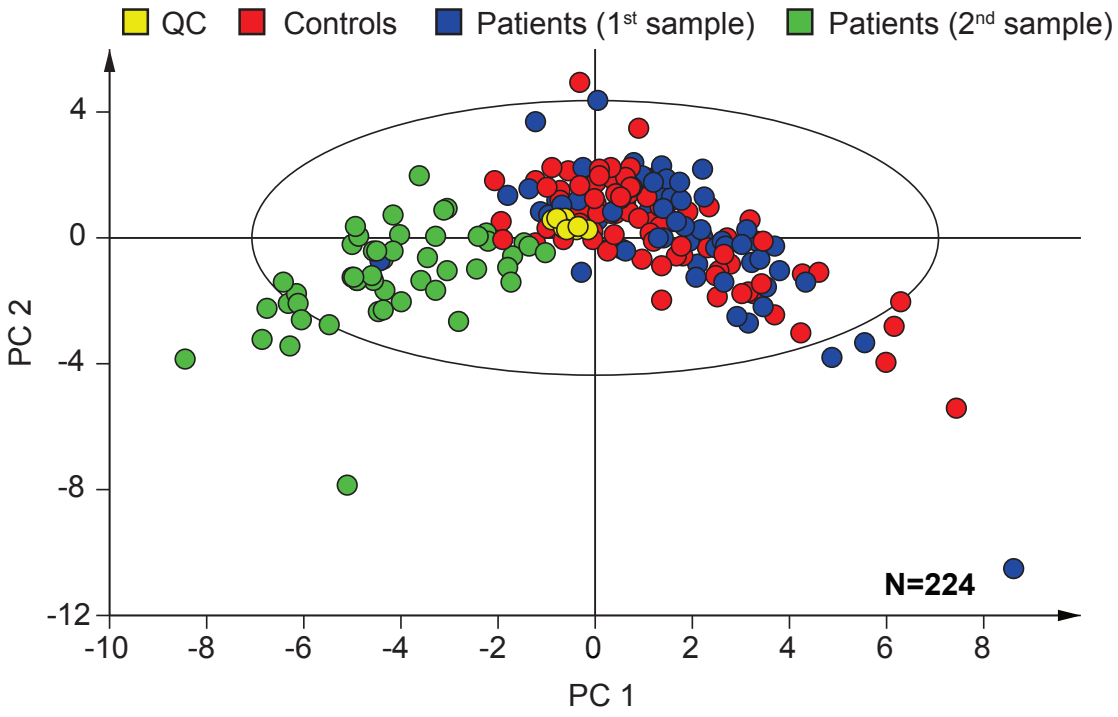


Figure 3.2.1: **Principal Component Analysis (score plot) based on ^{1}H-NMR spectra of 15 QC samples, urine samples of 70 newborns with UTD (1st sample) and 90 controls collected within 120 days from birth, and the 49 urine samples of newborns with UTD collected between 7 and 18 months of age (2nd sample).** *The good stability of the NMR setup and reproducibility of the experiments were attested by the grouped set of QC samples on the center of the plot. This unsupervised analysis showed a strong influence of age on the urinary metabolome of patients.*

To check if the influence of age on the urinary metabolome observed on controls in chapter 2 also applied to patients, an O-PLS model was constructed from the 70 ^{1}H NMR spectra from patients first sample (within 4 months of age) for a regression based on age. *(Figure 3.2.2)* The statistical significance of the model was assessed by high values of goodness-of-fit parameters (1+3 components; $R^2X = 0.369$; $R^2Y = 0.849$; $Q^2 = 0.31$) and a p-value of 0.002 by CV-ANOVA and model resampling. The good performance of this model indicated a strong influence of age in the urine metabolite profiles of newborns with UTD. The metabolic signature associated with age was identified with univariate analysis of the NMR metabolomic profiles and represented on a loading plot. Metabolites identified as key molecules with high correlation to age are listed in *Table 3.2.1.*

Table 3.2.1: **Metabolites identified as key molecules with high correlation to age from 10 to 115 days in 70 newborns with congenital urinary tract dilatation.**

Increasing with age until 4 months			Decreasing with age until 4 months		
Name	***p*-value***	***q*-value****	**Name**	***p*-value***	***q*-value****
3-Hydroxyisovalerate	4.10^{-6}	4.10^{-5}	1-Methylhistidine	1.10^{-8}	1.10^{-7}
Ascorbate	3.10^{-5}	2.10^{-4}	1-Methylnicotinamide	0.04	0.13
Acetate	0.009	0.04	Dimethylglycine	0.004	0.02
Citrate	1.10^{-7}	1.10^{-6}	Homocarnosine	0.001	0.008
Creatine	7.10^{-5}	6.10^{-4}	Isobutyrate	2.10^{-6}	2.10^{-5}
Glucarate	1.10^{-4}	1.10^{-3}	L-Lysine	3.10^{-13}	2.10^{-13}
Hippurate	0.005	0.02	L-Lactate	5.10^{-11}	7.10^{-10}
Histidine	0.006	0.03	Lactose	9.10^{-4}	0.005
L-glutamine	6.10^{-15}	0.06	Myoinositol	0.001	0.007
L-Serine	0.007	0.03	N-acetylputrescine	0.001	0.007
N-Acetyglutamine	1.10^{-4}	9.10^{-4}	Pantothenate	9.10^{-15}	3.10^{-13}
N-Acetyl-L-tyrosine	4.10^{-6}	4.10^{-5}	Propylene glycol	8.10^{-4}	0.005
trans-Aconitate	0.02	0.09	Xanthosine	0.03	0.1

**p*-value without multiple testing correction

***q*-value after Benjamini-Hochberg false discovery rate multiple testing correction

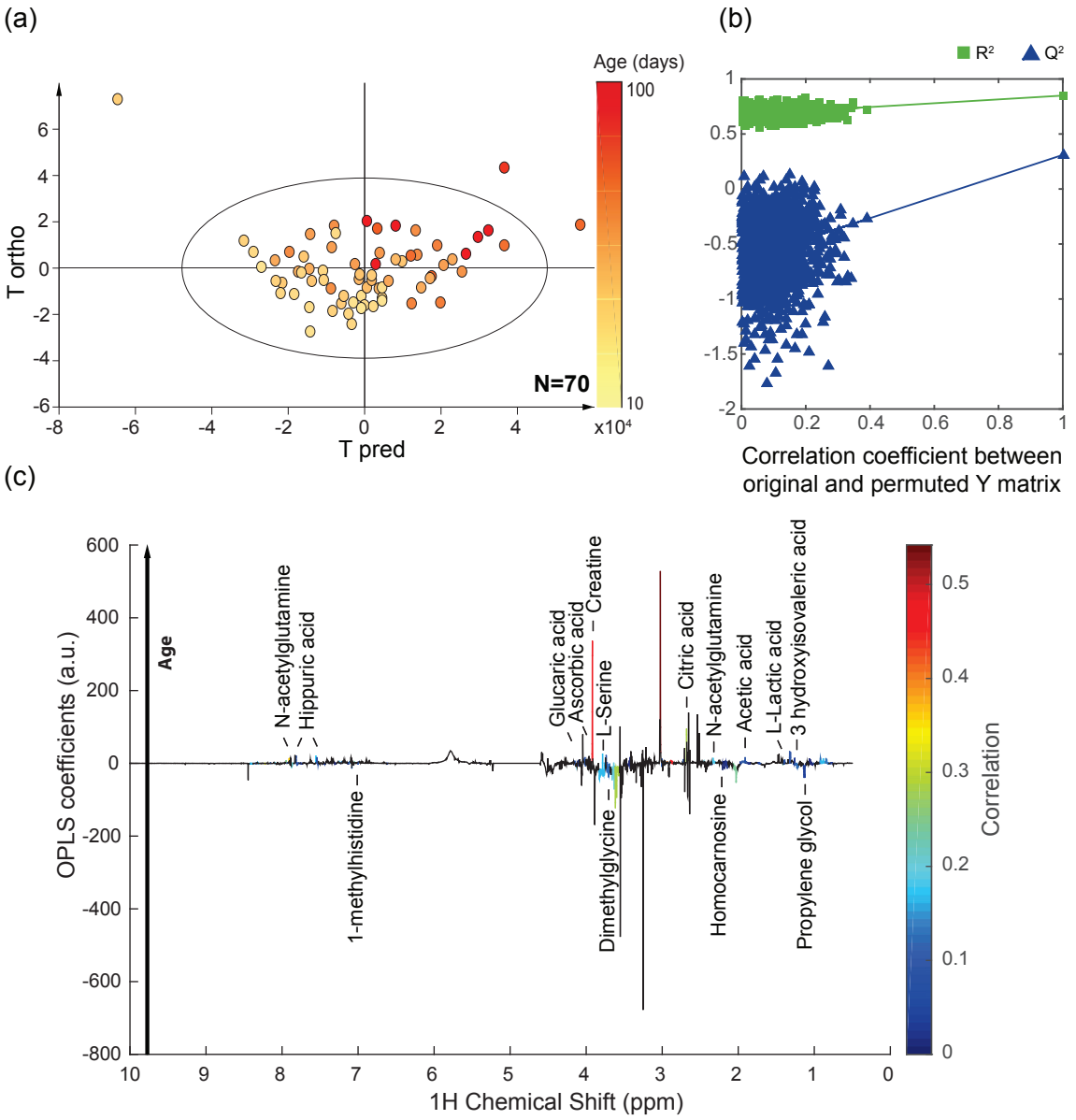


Figure 3.2.2: **Score plot of the O-PLS model based on 70 urine sample of newborns with congenital urinary tract dilatation for classification based on age from 10 to 115 days** *(A = 1+3, R^2X = 0.369, R^2Y = 0.849, Q^2 = 0.31, p-value = 0.002 by CV-ANOVA) (a) Score plot; (b) model validation by re-sampling 1000 times under the null hypothesis; and (c) O-PLS loading plot after SRV analysis and Benjamini–Hochberg multiple testing correction. Statistically significant signals correspond to the colored spectral regions.*

This analysis revealed similarities between metabolic signatures associated with age in patients and controls. Glucarate, Histidine, L-Glutamine, N-acetylglutamine, N-Acetyl-L-tyrosine and *trans*-Aconitate were significantly increasing with age in both groups, while Isobutyrate, L-Lactate, L-Lysine, Lactose, Myoinositol, Panthotenate and Propylene glycol were significantly decreasing with age in both groups.

The urinary concentrations of 1-Methylnicotinamide, 3-Hydroxyhydrovalerate, Citrate, Hippurate, L-Serine and N-Acetylputrescine were influenced by age in the UTD group whereas they were associated with weight or height in the control group.

Despite these similarities, the evolution of urinary metabolic profiles in newborns with UTD was different than the evolution observed in healthy newborns. The increase in concentrations of 2-Hydroxyisobutyrate, Carnitine, Choline, Ethanolamine, L-Acetylcarnitine, Pseudouridine and Quinolinate, and the decrease in concentrations of 4-Hydroxyproline, Citramalate and Galactose highlighted in healthy newborns were not found in newborns with UTD.

On the other hand the evolution of Ascorbate, Creatine, 1-Methylhistidine, Dimethylglycine, Homocarnosine and Xanthosine concentrations with age were specific to the patients group.

Although the evolution of urinary metabolic profiles with age seems to be different in the two groups, these results should be considered with caution, as multivariate analysis did not reveal significant differences between metabolic profiles of patients with UTD and controls. Consequently, the above metabolites should not be associated to the physiopathology of UTD based on these results.

A new O-PLS model was calculated for classification of all samples (patients and controls) for classification based on age. (Figure 3.2.3) With a greater effective, the

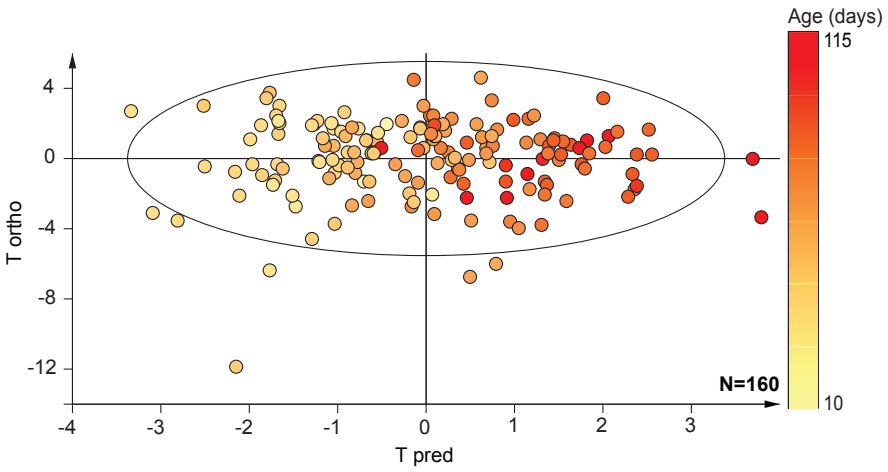


Figure 3.2.3: **Score plot of the O-PLS model based on 70 urine sample of newborns with congenital UTD and 90 healthy newborns for classification based on age.** *N = 160, 1+2 components, $R^2X = 0.262$, $R^2Y = 0.649$, $Q^2 = 0.464$, p-value = 1.10^{-18}*

goodness of fit parameters of this new model were higher: 1+2 components, R^2X = 0.262, R^2Y = 0.649, Q^2 = 0.464, p-value = 1.10^{-18}

This later model was based on a heterogeneous population and therefore its interest is limited, but these results only confirm the strong influence of age on the urinary metabolic profile, whether UTD is present or not.

3.2.4. Influence of age on the urinary profile of patients with UTD until 18 months

To investigate the evolution of urinary metabolic profiles in newborns with UTD over a longer period, a new O-PLS model was calculated for classification based on age, including all patients samples: 70 patients first samples collected before 4 months and 49 patients second samples collected between 7 and 18 months. *(Figure 3.2.4)* This model revealed a strong influence of age and a clear discrimination between patients first and second samples. The goodness of fit parameters confirm the robustness of the model. 1+2 components, R^2X = 0.393, R^2Y = 0.93, Q^2 = 0.861, p-value = 1.10^{-10} by CV-ANOVA.

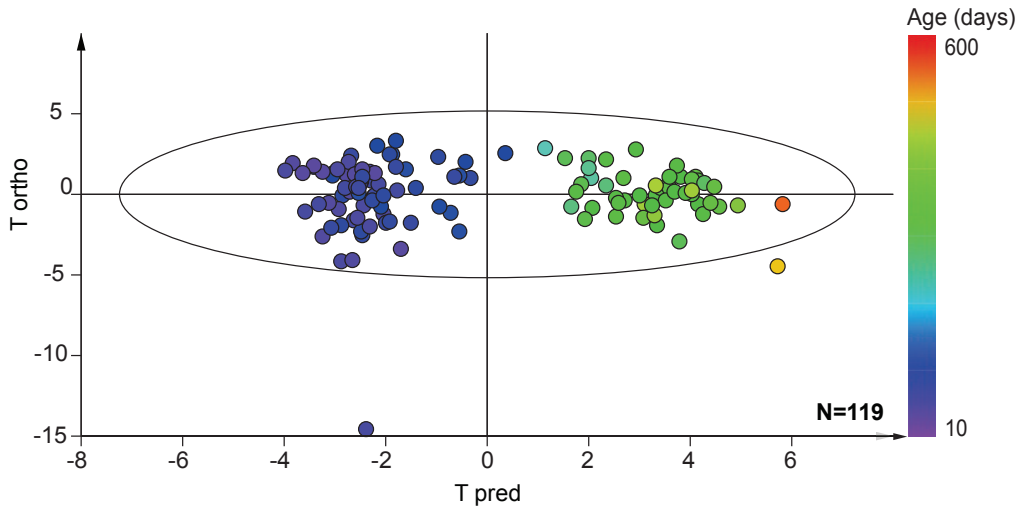


Figure 3.2.4: **Score plot of the O-PLS model based on 70 patients with UTD samples collected before 4 months and 49 patients second samples collected between 7 and 18 months, for discrimination based on age.** *N = 119, 1+2 components, R^2X = 0.393, R^2Y = 0.93, Q^2 = 0.861, p-value = 1.10^{-10} by CV-ANOVA.*

The metabolic signature associated with age was identified with univariate analysis of the NMR metabolomic profiles and represented on a loading plot. *(Figure 3.2.5)* Metabolites identified as key molecules with high correlation to age are listed in *Table 3.2.2.*

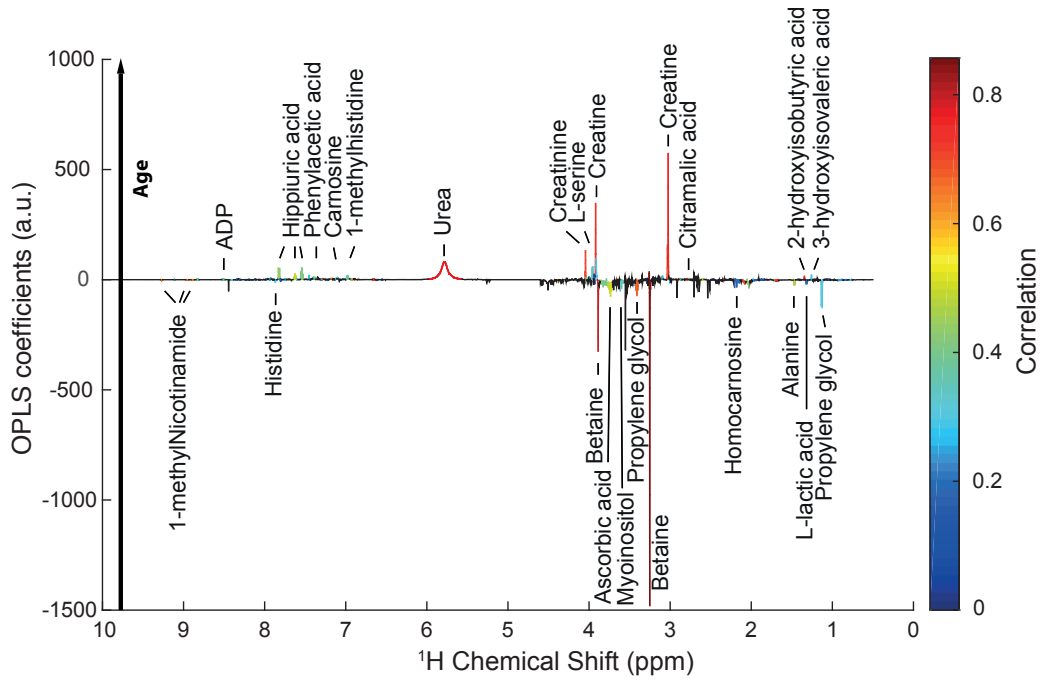


Figure 3.2.5: **O-PLS loading plot after SRV analysis and Benjamini–Hochberg multiple testing correction.** *Statistically significant signals correspond to the colored spectral regions.*

Table 3.2.2: **Metabolites identified as key molecules with high correlation to age from 10 to 600 days in newborns with congenital urinary tract dilatation.**

Increasing with age until 18 months			Decreasing with age until 18 months		
Name	***p*-value***	***q*-value****	**Name**	***p*-value***	***q*-value****
3-Hydroxyisovalerate	2.10^{-4}	0.001	1,3-Diaminopropane	7.10^{-5}	3.10^{-4}
3-Hydroxymethylglutarate	1.10^{-12}	2.10^{-11}	1-Methylnicotinamide	0.002	0.006
2-Hydroxyisobutyrate	6.10^{-4}	0.002	Alanine	0.007	0.02
ADP	9.10^{-6}	5.10^{-5}	Ascorbate	2.10^{-4}	0.001
Creatine	9.10^{-8}	8.10^{-7}	Betaine	1.10^{-4}	4.10^{-4}
Creatinine	0.001	0.004	Carnosine	2.10^{-7}	1.10^{-6}
Hippurate	6.10^{-5}	3.10^{-4}	Citramalate	0.001	0.004
Histidine	0.009	0.02	Homocarnosine	2.10^{-12}	3.10^{-11}
L-Serine	3.10^{-5}	4.10^{-4}	Gluconate	0.004	0.01
L-Tyrosine	0.001	0.004	Guanidoacetate	0.01	0.03
N-Acetylglutamine	1.10^{-4}	5.10^{-4}	Isobutyrate	3.10^{-6}	2.10^{-5}
O-phosphoethanolamine	4.10^{-4}	0.001	L-Lactate	5.10^{-10}	6.10^{-9}
Phenylacetate	9.10^{-13}	2.10^{-11}	L-Lysine	0.001	0.003
Pseudouridine	0.01	0.03	Myoinositol	0.02	0.04
trans-Aconitate	4.10^{-6}	2.10^{-5}	N-acetylputrescine	0.002	0.005
Urea	1.10^{-6}	7.10^{-6}	Pantothenate	2.10^{-9}	2.10^{-8}
			Propylene glycol	0.003	0.008
			Taurine	2.10^{-4}	9.10^{-4}
			Xanthosine	0.005	0.01

**p*-value without multiple testing correction

***q*-value after Benjamini-Hochberg false discovery rate multiple testing correction

Table 3.2.3. summarizes the evolution of metabolites associated with age in healthy newborns before 4 months, in patients with UTD before 4 months, and in patients with UTD until 18 months**.**

Table 3.2.3: **Key metabolites evolution with age in healthy newborns before 4 months, in patients with UTD before 4 months, and in patients with UTD until 18 months.**

Metabolites	**Evolution in healthy newborns under 4 months**	**Evolution in newborns with UTD before 4 months**	**Evolution in newborns with UTD before 18 months**
Histidine, N-Acetylglutamine, *trans*-Aconitate	↗	↗	↗
3-Hydroxyisovalerate, Creatine, Hippurate, L-serine	-	↗	↗
Glucarate, L-Glutamine, N-Acetyl-L-Tyrosine	↗	↗	-
2-Hydroxyisobutyrate, Phenylacetate, Pseudouridine	↗	-	↗
Carnitine, Choline, Ethanolamine, L-Acetylcarnitine, Quinolinate	↗	-	-
Ascorbate, Acetate, Citrate	-	↗	-
3-Hydroxymethylglutarate, ADP, Creatinine, L-tyrosine, O-phosphoethanolamine, Urea	-	-	↗
Isobutyrate, L-Lactate, L-Lysine, Myoinositol, Pantothenate, Propylene glycol	↘	↘	↘
1-methylnicotinamide, Homocarnosine, N-Acetylputrescine, Xanthosine	-	↘	↘
Lactose	↘	↘	-
Citramalate	↘	-	↘
4-Hydroxyproline, Galactose	↘	-	-
1-Methylhistidine, Dimethylglycine	-	↘	-
1,3-Diaminopropane, Alanine, Ascorbate, Betaine, Carnosine, Citramalate, Gluconate, Guanidoacetate, Taurine	-	-	↘

The influence of age we previously highlighted on some of this metabolites before 4 months continues during the first 18 months. For example, the urinary concentrations of Histidine, N-Acetylglutamine and *trans*-Aconitate increased with age in healthy newborns and in patients. In the same way, Isobutyrate, L-Lactate, L-Lysine, Myoinositol, Pantothenate and Propylene glycol decreased with age in all groups. In consequences, processes influencing these metabolites excression are persistent during the first 18 months of life and are not modified in newborns with UTD.

The concentrations of some other metabolites only seem to change during the first 4 months of life. During this period, the concentrations of Glucarate, L-Glutamine, N-Acetyl-L-Tyrosine increase while Lactose urinary concentration decrease. This decrease in Lactose urinary concentration is only seen during the first 4 months of life, which corresponds to the maturation of intestinal permeability and mucosal function.[169]

The urinary concentrations of Carnitine, Choline, Ethanolamine, L-Acetylcarnitine, Quinolinate, 4-Hydroxyproline and Galactose were influenced by age only in the control group, while 3-Hydroxyisovalerate, Creatine, Hippurate, L-Serine, 1-Methylnicotinamide, Homocarnosine, N-Acetylputrescine, Xanthosine were only influenced by age in patients with UTD. Although these evolutions should be considered cautiously, they represent potential leads for further investigations on the metabolic profiles of newborns with UTD.

Urinary concentrations of 3-Hydroxymethylglutarate, ADP, Creatinine, L-Tyrosine, O-phosphoethanolamine, Urea, 1,3-Diaminopropane, Alanine, Ascorbate, Betaine, Carnosine, Citramalate, Gluconate, Guanidoacetate and Taurine were only influenced by age in the latter analysis including all samples from patients with UTD. It is not possible to determine if these evolutions are specific to UTD. The same evolutions could be seen in healthy newborns, but our study design does not allow confirming this hypothesis. However, increasing Creatinine urinary concentrations with age and decreasing betaine urinary concentration has been reported before in healthy children.[161,164] Creatinine and Betaine increase latter in adults,[166] and Alanine urinary concentrations are also related to age after 40 years.[165]

Ascorbate, also called vitamin C was the only metabolite found as increasing with age before 4 months in newborns with UTD, but decreasing with age when including the patients second samples in the analysis. Although this could be related to the intake

of vitamin C provided by breast and formula milks, these results should again be taken with caution, as no such evolution was found in the control group.

We reported the influence of growth parameters on the urinary metabolic profiles of healthy newborns in chapter 3.1.4. Other models were calculated to investigate the influence of height and weight on the urinary metabolic profiles of patients with UTD.

3.2.5. Influence of growth parameters and gender in patients

New O-PLS models were calculated to investigate variations related to height, weight and gender in patients first samples (before 4 months).

Considering weight, goodness of fit parameters of the O-PLS model were (N = 70, 1 component) $R^2X = 0.148$, $R^2Y = 0.315$ and $Q^2 = 0.099$ with a p-value = 0.02 by CV-ANOVA. Despite a p-value < 0.05, the predictive value of the model is poor as assessed by the low Q^2 value.

The O-PLS model constructed from the same samples for discrimination based on height was not predictive either: (N = 70, 1 component) $R^2X = 0.176$, $R^2Y = 0.26$, $Q^2 = 0.08$, p-value = 0.06.

The influence of growth parameters on metabolic profiles of controls was not confirmed in the population of patients with UTD, with a smaller effective than controls.

Surprisingly, some metabolites are influenced by age in patients, although they were only influenced by growth parameters in the control group. For example, 3-hydroxyhydrovalerate and L-serine increased with control height but were increasing with age in patients.

Gender had no significant influence on the metabolic profiles of patients with UTD, as assessed by the parameters of the corresponding O-PLS model: 1 component, $R^2X = 0.08$, $R^2Y = 0.35$, $Q^2 = -0.38$, p-value = 1.

The last analysis performed on the 49 patients second samples collected between 7 and 18 months found no significant difference between metabolic profiles of patients that needed surgery and patients managed conservatively: N = 49, 1 component, $R^2X = 0.06$, $R^2Y = 0.44$, $Q^2 = -0.59$, p-value = 1.

These results showed that age was the main factor influencing the metabolic profiles of newborns with UTD.

3.2.6. Toward improvement of metabolomics investigations in newborns

Although the number of metabolomic studies is constantly rising, methodology is not always standardized. Based on the results of the present study, we propose ways to improve metabolomic studies in neonatology, following best practices and taking account of the influence of age and growth parameters.

Metabolomic studies aiming to identify potential biomarkers are often case-control studies. Inclusion criteria must be clearly and precisely defined for patients and control groups. Ideally, the only difference between groups should be the effect under study, like the disease. As we highlighted the important influence of age, weight and height in newborns, these factors should be correctly controlled by inclusion criteria.[167] Patients and controls should be matched according to age whenever possible, with age calculated in days for more precision. In the same way, matching patients and controls by weight and height would improve the precision and power of the subsequent analysis. Another possible solution to avoid bias generated by differences in age, weight and height between newborns would be to only collect samples from patients with the same age, and to match them on weight and height. This could be applied to neonatology studies including only patients at birth. Matching patients on weight and height is challenging and could limit the effective, which is often modest in neonatology and in studies of rare diseases. A new method was developed recently, aiming to perform sample size determination and power analysis in metabolic phenotyping studies.[181] This method helps to determine the number of patients to include based on the data of a pilot study. Such methodology could be applied to metabolomic studies to calculate the effective needed to identify specific metabolic profiles.

Nutrition is another factor influencing the urinary metabolic profile of newborns, which should be taken into account.[182] In the present study, information regarding the exact alimentation of newborns was lacking. We recommend to always record the type of feeding of each newborns, and to match patients and controls accordingly whenever possible. This influence of nutrition is modest in newborns, as they are all alimented with milk, whether breast milk, formula or both, before the age of 4 months. Moreover, a common age-dependent modification of the urine metabolome was observed for newborns fed by breast milk, formula and mixed alimentation.[182] The influence of diet on the metabolomic profile is higher after dietary diversification, usually recommended

from 4 to 6 months of age. For this reason, samples should be collected after a period of fasting in older children. Moreover, daily variations of the urinary metabolome of children, specific to the individual, were reported.[158] Pooled sample including first morning void and night-time urinary samples was shown to capture the most inter-individual variance in the metabolic phenotype.

Although resource-consuming, a metabolomic study comparing urine metabolic profiles of newborns with UTD to age-matched healthy newborns, with successive samples throughout the first years of life, could improve our comprehension of metabolic maturation in newborns and impact of UTD on metabolism.

Conclusion

By detecting without a priori metabolites present in a biological sample, metabolomic analysis is a useful tool to identify metabolic profiles correlated with a specific disease or status. This approach represents a huge potential for biomarker discovery, and the number of metabolomic studies is rapidly rising. As genomics and proteomics, metabolomics enables analysis at a system level. It captures a global picture of metabolism at a given time, which is influenced by numerous factors such as genetics, disease, diet or lifestyle.

Metabolomics uses robust analysis technologies like NMR spectroscopy or Mass spectrometry to determine the metabolic composition of biological samples. Various biological fluids can be analyzed via these methods, like blood, plasma, or urine. The later can be collected non-invasively and is particularly suited for metabolomics investigations in pediatrics and neonatology. Moreover, evidences are pointing toward the influence of environmental factors acting during the early phases of life on the risk of developing chronic disease in adulthood. For these reasons, physicians working in the fields of neonatology and pediatrics express a growing interest for metabolomics as tool for translational research.

Congenital UTD is an important diagnosis and prognosis challenge for pediatric urologists. It can either be transient or represent the first sign of urinary obstruction needing surgical management to prevent urinary infections and the degradation of renal function. The diagnosis can only be assessed by repetitive US and additional imaging, including renal isotope studies and possibly micturating cystogram, with significant consequences on childhood radiation, parental anxiety and healthcare resources. Inves-

tigating urinary biomarkers with diagnosis and prognosis value is therefore a dynamic field of research.

In this thesis, we evaluated the prognostic value of three ultrasound grading systems in newborns with prenatally diagnosed unilateral urinary tract dilatation using a prospective cohort study including 70 newborns. The simplest one, the Antero-Posterior Diameter of the renal pelvis was compared to the Society for Fetal Urology grading system, which is the most commonly used, and to the most recent Urinary Tract Dilatation grading system. We confirmed the validity of this new grading system, and showed that the Antero-Posterior Diameter of the renal pelvis and abnormal parenchymal thickness are the most important criteria for identification of children at risk for needing surgery.

In a large case-control study using ^{1}H-NMR spectroscopy and metabolomic analysis, we compared the urinary metabolic profiles of these 70 newborns with prenatally diagnosed unilateral urinary tract dilatation and the urinary metabolic profiles of 90 controls. Contrary to expectations, the evolution of urinary metabolomic profiles through the first 4 months of life was comparable in patients and controls. There was neither a significant difference between urinary profiles of patients who needed surgery during follow-up and newborns managed conservatively.

However, we demonstrated the influence of age, weight and height on the urinary metabolome of newborns during the first four months of life. We reported specific modifications of newborns metabolism associated with the tremendous growth occurring during the neonatal period and key metabolites responsible for this evolution. Conversely, gender appeared to have no impact on the urine metabolome in early infancy.

Moreover, we analyzed the evolution of urinary metabolome in children with urinary tract dilatation from 15 to 600 days of life, and precisely described the evolution of numerous metabolites during this period. These results are a step toward a better comprehension of the physiopathology of UTD.

This thesis allows a deeper understanding of newborn metabolic maturation and contributes to identify potential confounding factors for metabolomics investigations in newborns. It highlights the necessity to systematically and precisely report children age, height and weight in metabolomic studies, as they have a critical impact on urinary metabolic profiles. Such methodology improvements give hope for new applications of metabolomics in personalized medicine.

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Appendices

Appendix A

Embryology of the renal system

Images by Pr Pierre-Yves Mure.

Understanding the normal embryology of the urinary tract is vital when considering congenital uropathies. The kidneys progress through three developmental stages: the pronephros, the mesonephros, and the metanephros that persists as the definitive adult kidney.

The pronephros

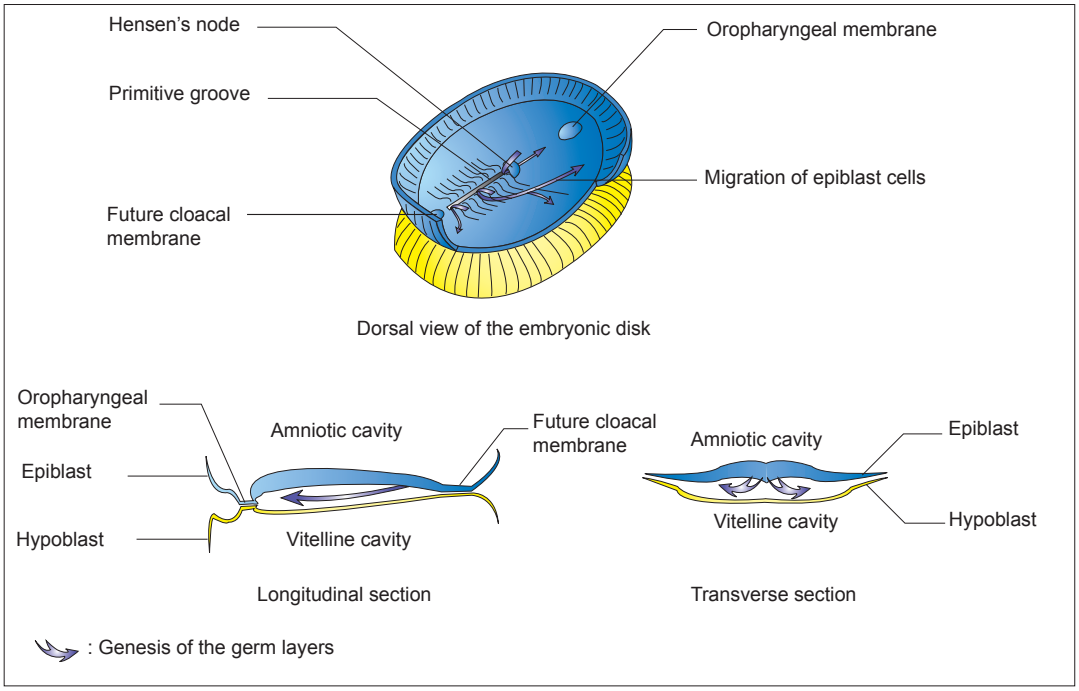
The pronephros *(Figure 1 and 2)* develops in the cervical region of the embryo during gestational week 3 as a condensation of intermediate mesoderm, and almost entirely regresses in gestational week 4.

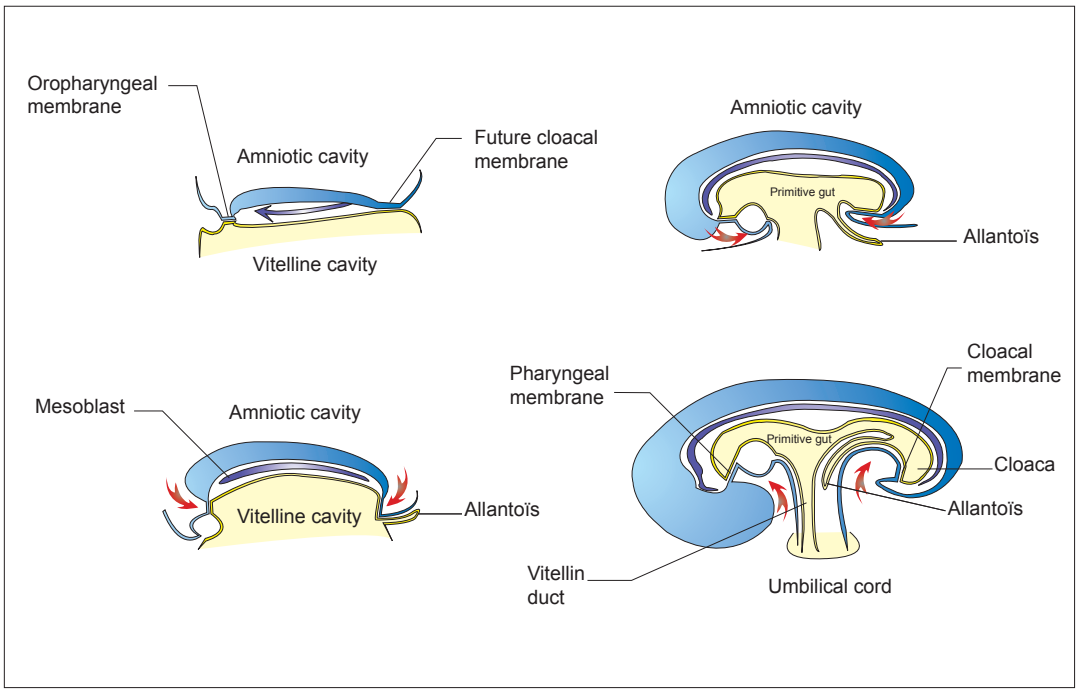
The pronephric duct arises from dorsal and caudal evaginations of the pronephros and ultimately give rise to the mesonephric duct.

The mesonephros

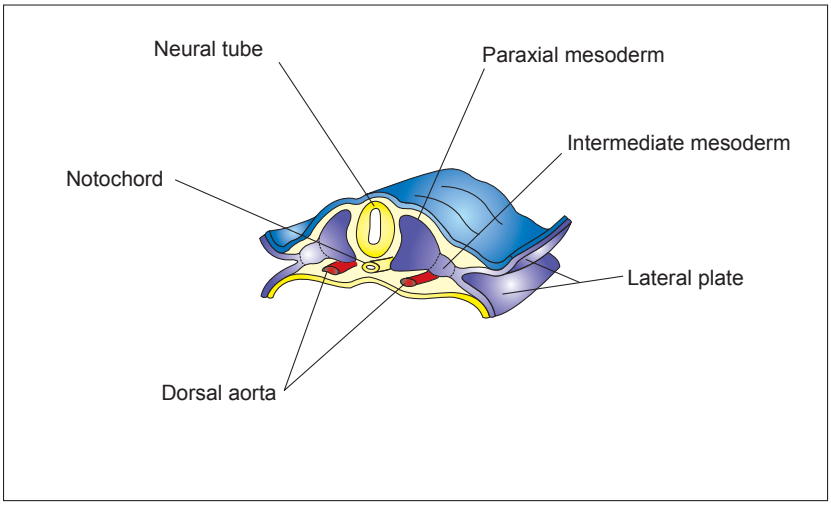
More caudally, the mesonephros develops into mesonephric tubules and the mesonephric duct (Wolffian duct) during gestational week 4. Although the majority of these tubules degenerate, the mesonephric duct persists bilaterally and opens to the cloaca at the tail of the embryo.

Appendix A *Figure 1*

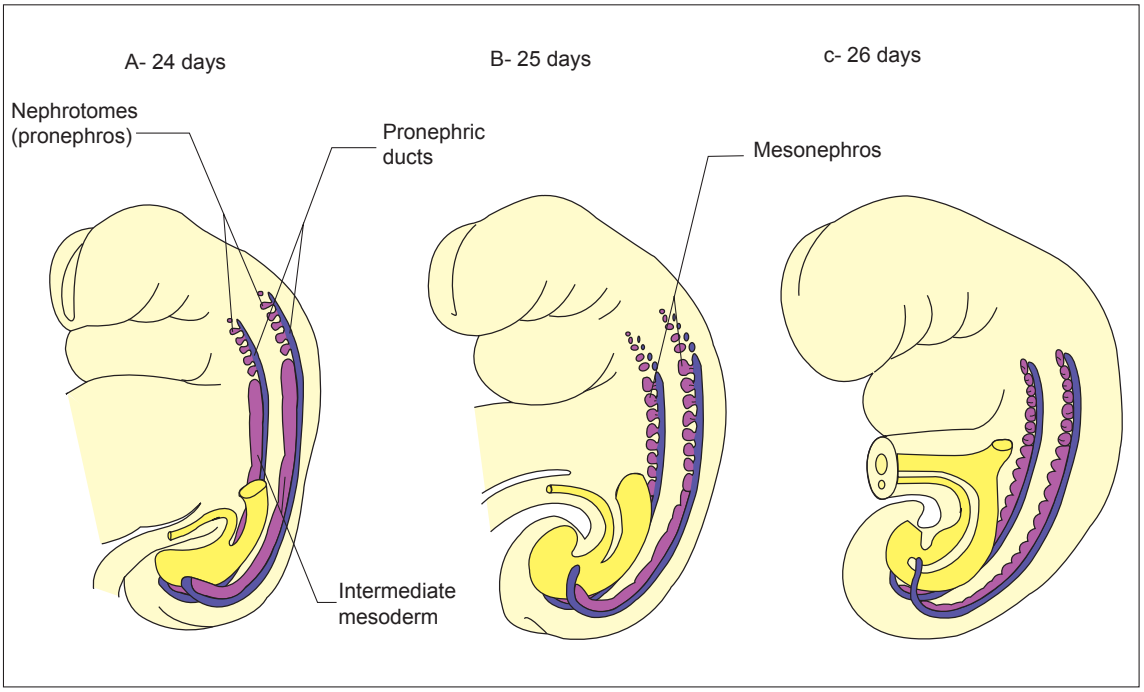




Appendix A *Figure 2*



Intermediate mesoderm at 23 days



Pronephros and mesonephros development

The metanephros

The ureteric bud is a protrusion from the mesonephric duct appearing after its opening to the cloaca. *(Figure 3)* The ureteric bud comes in contact with the metanephric blastema, forming the metanephros. The ureteric bud and metanephric blastema reciprocally induce growth, forming the kidney. The ureteric bud progressively enlarges and divides to form the renal pelvis, infundibula, collecting ducts, and 8-12 major and minor calyces. *(Figure 4)*

The collecting tubules invaginate metanephric mesoderm to form metanephric vesicles, which subsequently elongate to form metanephric tubules. As the metanephric tubules are invaginated by capillaries (glomeruli), nephrons are formed. This process continues until the 32nd gestational week. At birth, approximately 750,000 to 1 million nephrons are present in each kidney;

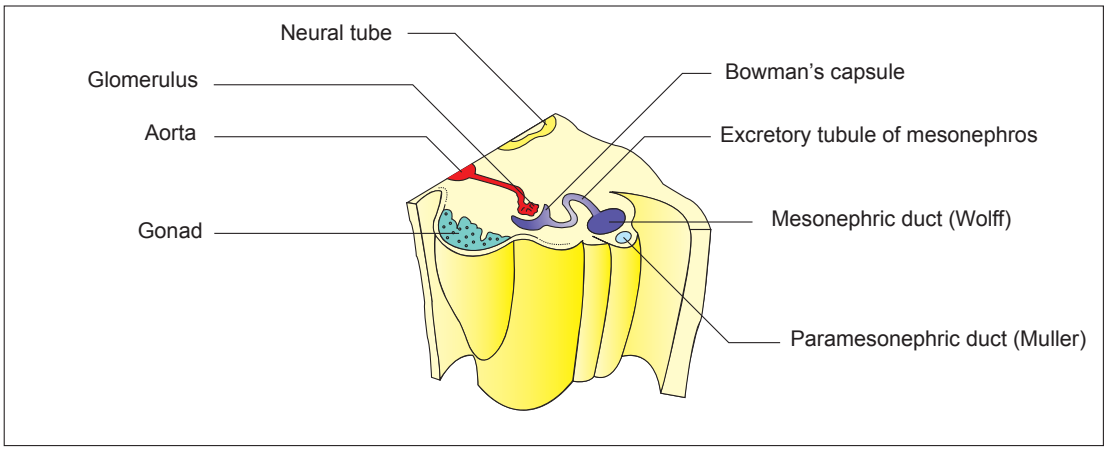
With differential longitudinal growth of the embryo, the kidney "ascends" from its initial location in the pelvis to its final location in the upper retroperitoneum. *(Figure 5)*

Development of the bladder and ureters

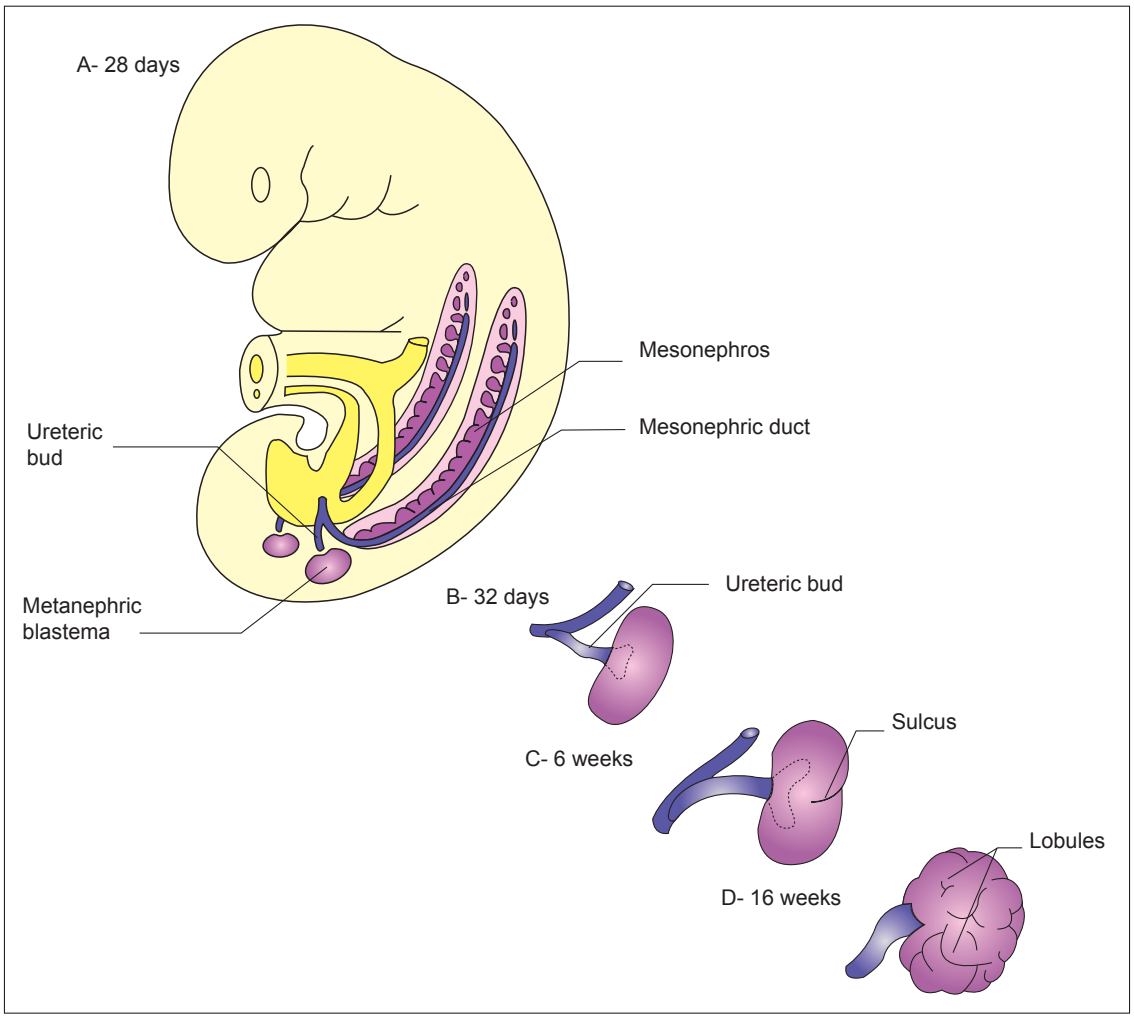
Until gestational week 7, the embryo has a cloaca, a single orifice at the caudal aspect. *(Figure 6)* During gestational week 7, the urogenital membrane grows caudally, dividing the cloaca into ventral (urogenital sinus) and dorsal (rectum) components. The urogenital sinus can be further subdivided into cranial (future bladder) and caudal (future prostate, urethra, and external genitalia) portions. The vesical epithelium is entirely derived from the endodermal layer of the urogenital sinus.

In both sexes, the ureters, renal pelvis, and bladder trigone are derived from the mesonephric duct; in the male, the mesonephric duct also gives rise to the vasa deferentia, epididymides, and seminal vesicles.

Appendix A *Figure 3*

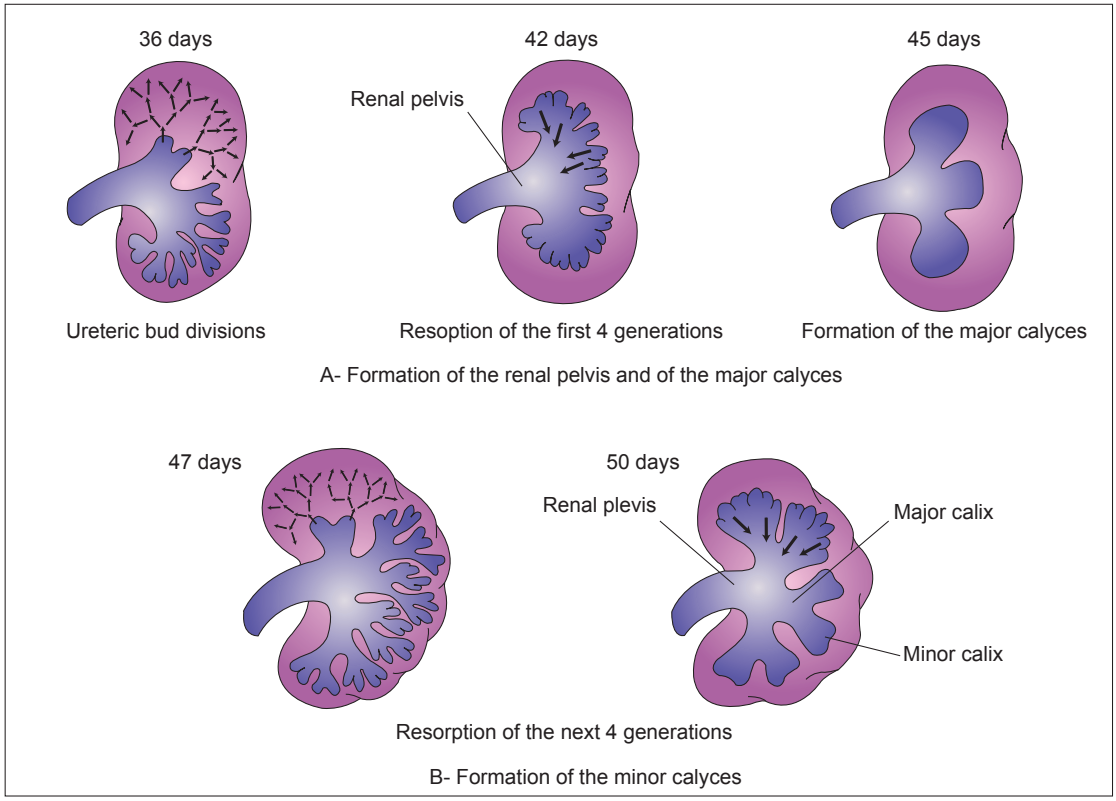


Mesonephric excretory unit

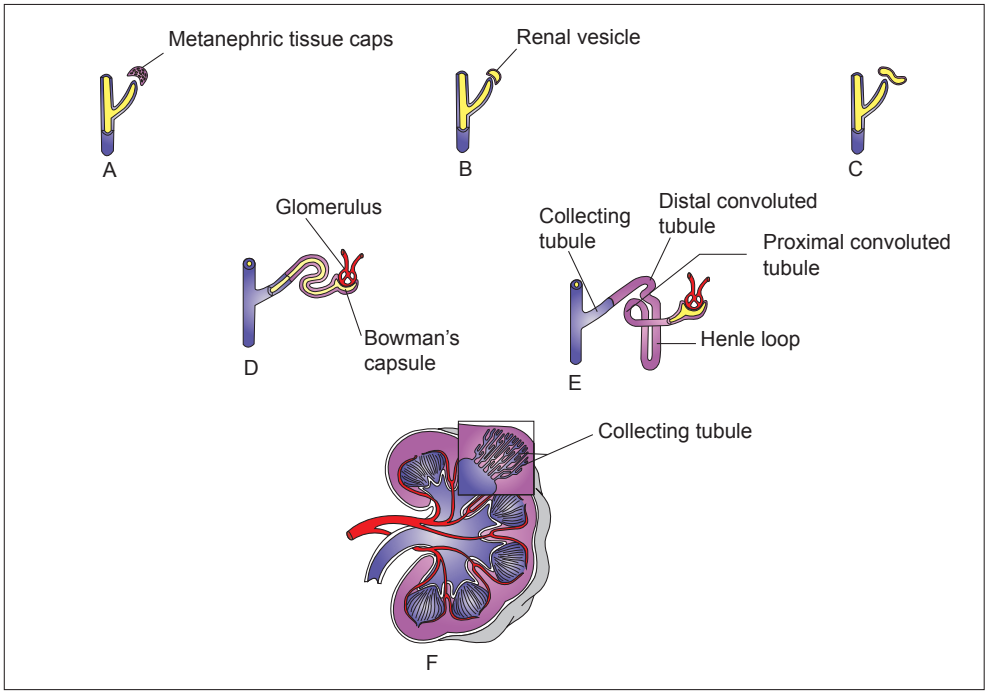


Origin and development of metanephros

Appendix A *Figure 4*

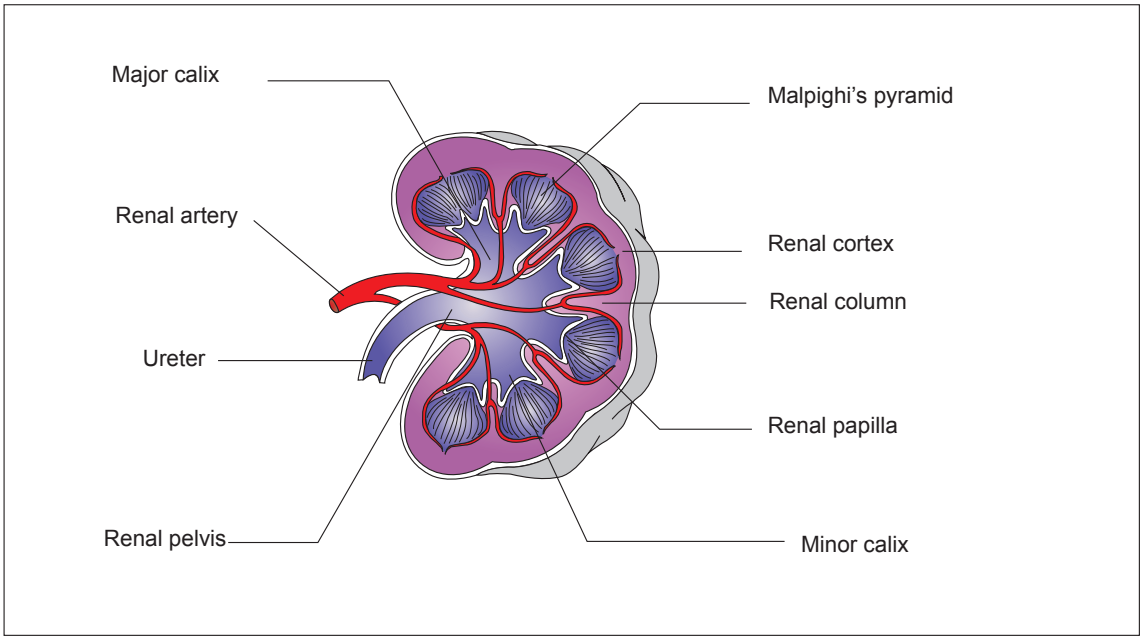


Formation of the renal pelvis and calyces

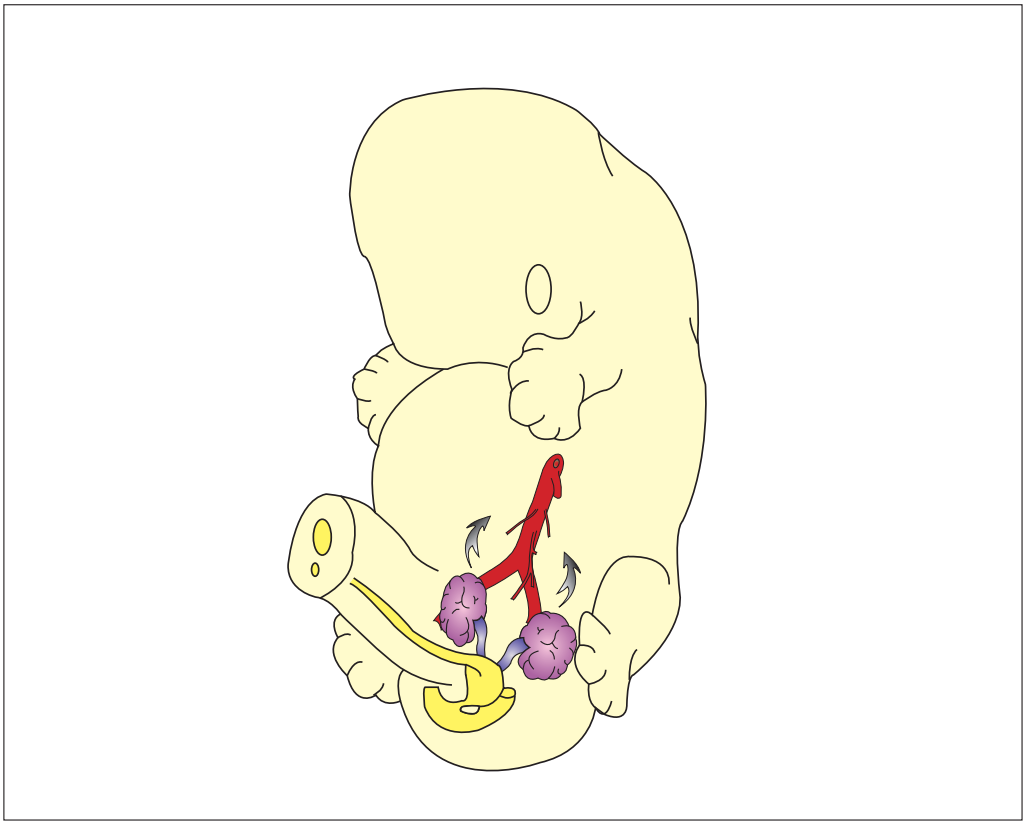


Stages in the development of a metabephric excretory unit (nephron)

Appendix A *Figure 5*

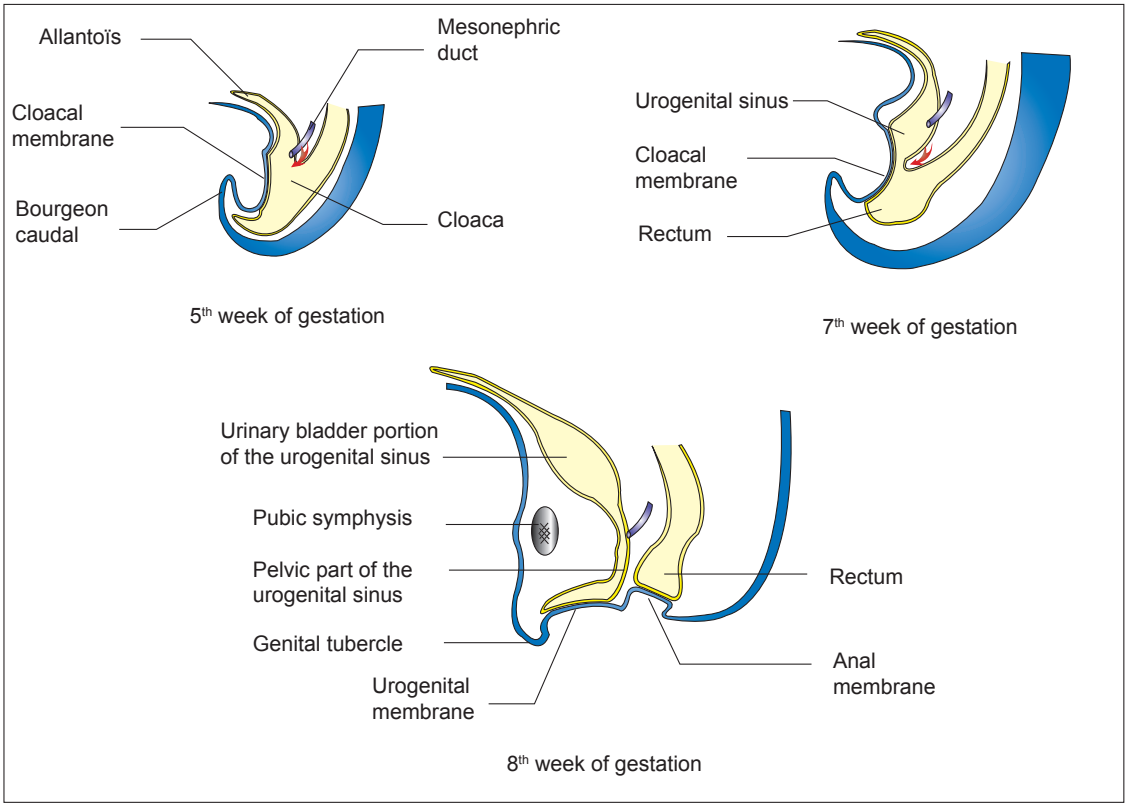


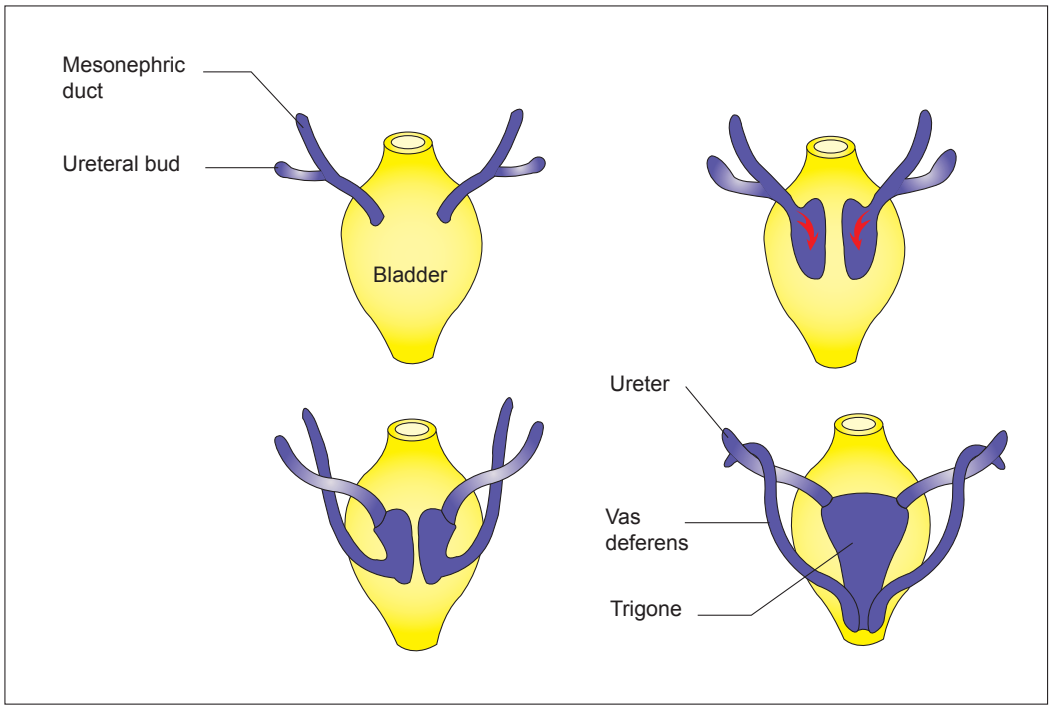
Definitive structure of the metanephros



Ascent of the kidneys from its location in the pelvis
to the upper retroperitoneum between weeks 6 and 9

Appendix A *Figure 6*





The development of the bladder trigone

Appendix B

Diagnosis and treatment of uropaties associated with prenatally diagnosed urinary tract dilatation

Postnatal ultrasonography

US should provide information regarding the side of UTD (unilateral or bilateral), the aspect of the renal parenchyma, the presence of ureteral dilatation, bladder anomalies and associated malformations. *(Figure 1)* The downside of US for evaluating UTD is that the distension of the renal pelvis and calyces may vary depending on factors such as hydration status, degree of bladder filling, and patient position. Different classifications can be used to evaluate the severity of UTD. The measure of the APD and the Society for Fetal Urology (SFU)[126] grading systems were the most commonly used before the creation of the Urinary Tract Dilatation (UTD) grading system introduced in 2014 by a multidisciplinary consensus.[46] For unilateral UTD and unless posterior urethral valves are suspected, it is recommended that this first postnatal US be delayed for at least 48 h after birth to avoid underestimating the severity of dilatation.[48] Postnatal US findings are reported as normal in 21–28% of patients referred for prenatal US.[55] If the first US is normal, it is advisable to repeat it on two or three occasions during the first year of life as some uropathies may only become patent during the months following birth.[56] When posterior urethral valves are suspected (bilateral UTD with bladder anomalies), US should be performed on the first day of life.

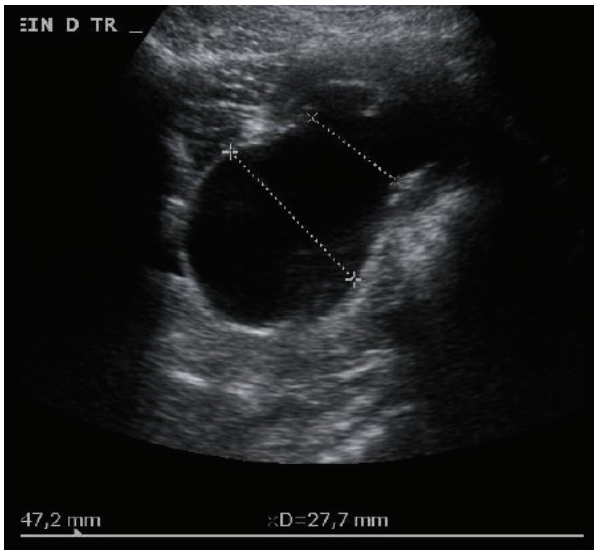


Figure 1 : **Renal ultrasonography showing intrasinusal and extrasinusal dilatation of the renal pelvis.**

Radioisotope renography

Radioisotope renography using ^{99m}Tc dimercaptosuccinic acid (DMSA), a tracer that binds to the proximal convoluted tubules after intravenous injection, is considered as the best investigation for detecting renal parenchymal anomalies. *(Figure 2)* It also provides quite accurately the relative function of each kidney. Guidelines have been edited by the European Association of Nuclear Medicine.[185] The normal range of differential renal function that is accepted is 45-55%. Dynamic isotope studies using Mercaptoacetyltriglycine (MAG3), Ethylene dicysteine (EDICIS) or ^{99m}Tc di-ethylene-tertra-amine-penta-acetic acid (DTPA), may also aid assessment of a congenital UFI, although interpretation of the drainage curve can be difficult.

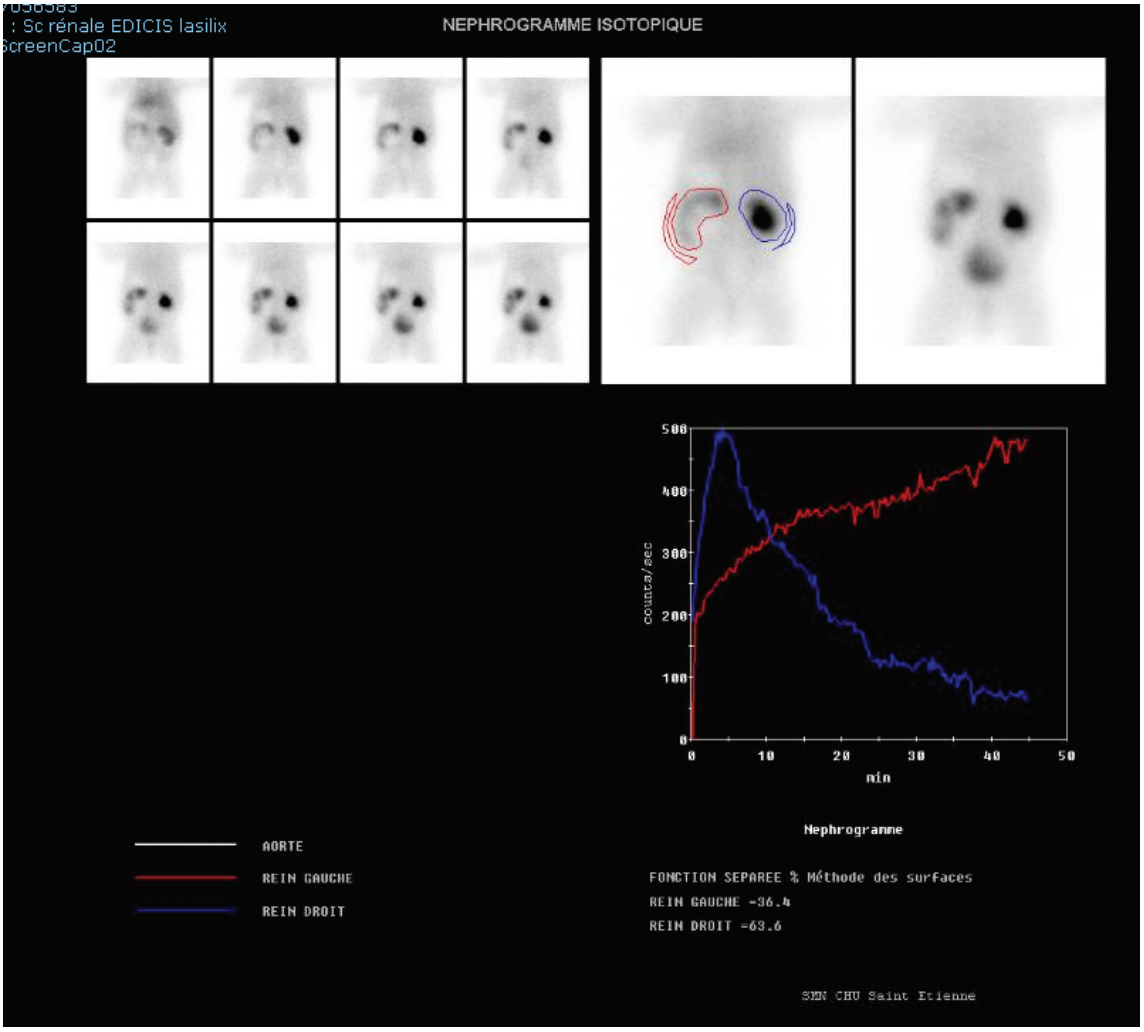


Figure 2: **Radioisotope renography using ethylene dicysteine showing a decrease in relative renal function on the left (36% vs 64% on the right kidney) and an abnormal homolateral drainage curve**

Micturating cystography

Micturating cystography (MCUG) is a radiography procedure with instillation of radiocontrast in the bladder by urethral or suprapubic catheterization. *(Figure 3)* Its indications are not consensual, given the risk of subsequent urinary infection. It is although recommended in children with a dilated ureter on US (> 6 mm) and an abnormal isotope study, and in case of posterior urethral valves suspicion (bilateral UTD and bladder anomalies).[51] MCUG enables detection of posterior urethral valves, evaluation of the bladder aspect and size, detection of bladder diverticulum and vesico-ureteral reflux.

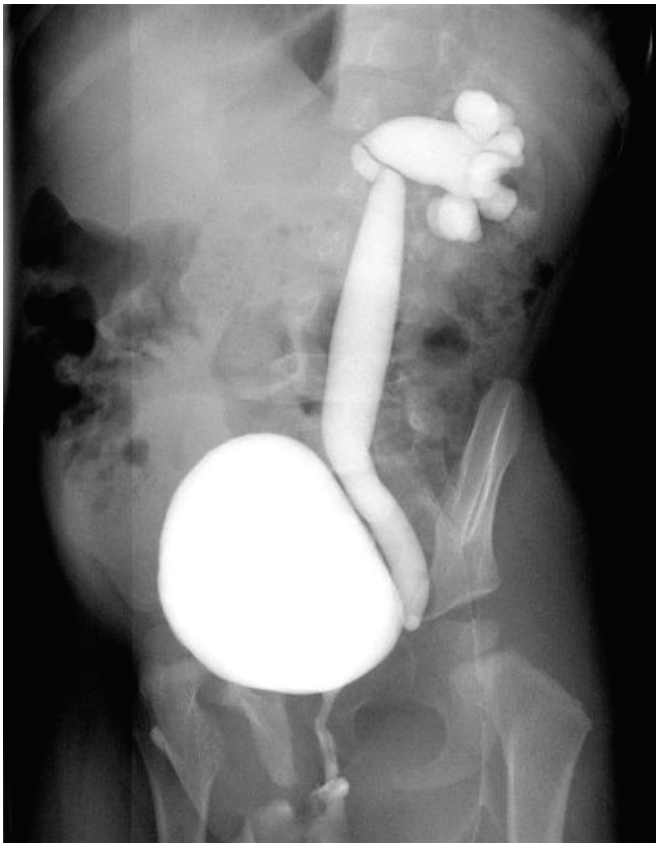


Figure 3: **Micturating cystography showing a high-grade left vesico-ureteral reflux**

Magnetic resonance urography

Dynamic contrast-enhanced magnetic resonance urography using gadolinium-DT-PA allows anatomical and functional assessment of the kidneys and urinary tract.[186, 187] However, US performed by an experienced radiologist already provides anatomical description of the urinary tract, and magnetic resonance urography may imply sedation or general anesthesia in children under 6 years old, and is not easily available. Magnetic resonance urography also has potential in the evaluation of kidney function, but is not currently used in clinical application.[188]

Etiologies of prenatally diagnosed UTD are summarized in *Table 1.*

Table 1. **Etiologies of prenatally diagnosed UTD (adapted from Nguyen et al. 2010)[61]**

Etiology	Incidence (%)
Transient/physiologic UTD	41 - 88
Pyelo-ureteral Junction anomalies	10 – 30
Vesico-ureteral reflux	10 – 20
Uretero-vesical junction obstruction/megaureter	5 – 10
Multicystic dysplastic kidney disease	4 – 6
Posterior urethral valves	1 – 2
Ureterocele, ectopic ureter, duplex system,	5 – 7
Urethral atresia, Prune belly syndrome, polycystic kidney diseases…	< 1

Pyelo-ureteral junction anomalies

Pyelo-ureteral Junction anomalies (PUJ), also called pelvi-ureteric junction anomalies are the commonest detected uropathies in newborns with UTD with an incidence of 1/1000 to 1/2000 live births.[46] PUJ anomalies are unilateral in 90% of cases, and more frequent in males than females.[48, 190] PUJ can be caused by extraluminal, parietal or intraluminal anomalies of the ureter. Extraluminal anomalies are mainly caused by aberrant vessels or more rarely by kinks, bands, adhesions and vascular malformations spanning the PUJ and intermittently reducing the urine flow.[48,191] Parietal anomalies are due to an abnormal distribution of the muscular and collagen fibers at the level of the PUJ with failure of peristalsis producing an incomplete, functional obstruction. They are the most common etiology (75% of cases). Intraluminal anomalies are rare, often associated to parietal anomalies, and are essentially benign fibroepithelial polyps.[192]

PUJ anomalies are suspected when US shows a dilatation of the renal pelvis, while the homolateral ureter is not dilated and the appearance of the bladder is normal. *(Figures 4 and 5)* In case of severe obstruction, the renal parenchyma appears thinner and the differentiation between the medulla and the renal cortex disappears. Other urological anomalies (renal agenesis, multicystic renal dysplasia, vesico-ureteral reflux, horseshoe kidney) are associated in about 25% of cases.[51]

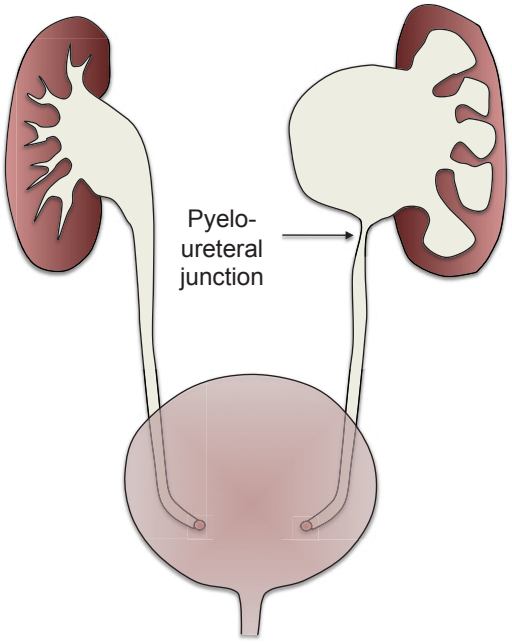


Figure 4: **Scheme of a pyelo-ureteral junction anomaly.**
The renal pelvis is dilated but the ureter is not.

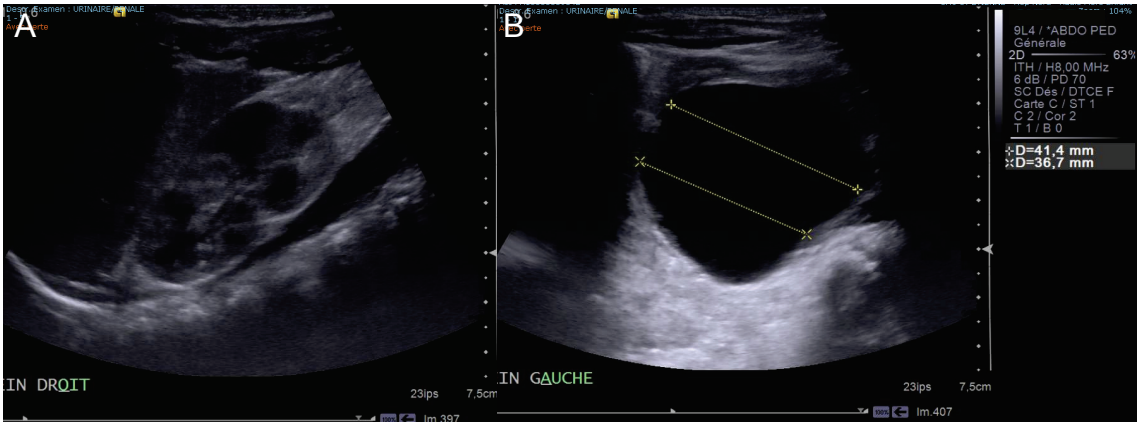


Figure 5: **Renal ultrasonography:** *A) Normal right kidney B) Dilatation of the left renal pelvis of 41mm with abnormal parenchymal thickness. The ureter being not dilated, the diagnosis of PUJ anomaly is the most likely.*

Children with a stable UTD, asymptomatic, with no history of urinary tract infection or decrease of the homolateral renal function on isotope studies can be managed conservatively.[51,146,193] Consensual indications for surgery of the PUJ anomaly are declining function of the homolateral kidney, important increase of the UTD over time, recurrent pyelonephritis and homolateral flank pain. Surgery aims to improve urine flow by resection of the PUJ and anastomosis of the ureter to the renal pelvis (Anderson-Hynes technique). *(Figure 6)*

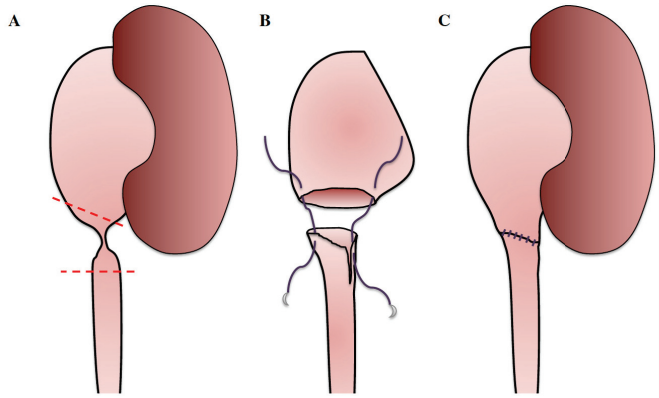


Figure 6: **Pyeloplasty.**
A: Resection of the pyelo-ureteral junction.
B: Anastomosis of the ureter to the renal pelvis.
C: Surgery completed.

Vesico-ureteral junction anomalies

Vesico-ureteral reflux (VUR) is another common uropathy defined as a permanent or intermittent retrograde flow of urine from the bladder into the upper urinary tract.[194,195] It is diagnosed in 10 to 20% of UTD detected by prenatal ultrasonography. Its incidence in the general pediatric population is estimated at about 1-2%,[48,196,197] and it is usually diagnosed in infants being investigated for an urinary tract infection. VUR facilitates the ascension of bacteria from the bladder to the upper urinary tract, which can result in pyelonephritis and alteration of the renal function. MCUG is the gold standard imaging for the diagnosis of VUR. VUR can be classified according to the result of the MCUG. *(Figure 7)* Management of VUR is mostly conservative, as spontaneous resolution occurs in more than 50% of cases. The indications for surgery are high-grade reflux ≥III with deterioration of renal function on radioisotope studies or recurrence of acute pyelonephritis despite antibiotic prophylaxis.[198] Surgery aims to restore anti-refluxing mechanism of the vesico-ureteral junction.[194] Open procedures such as Cohen technique are the current gold standard with a success rate over 98%.[199] Minimally invasive techniques such as endoscopic, laparoscopic or robotic approaches are promising alternatives.[200,201]

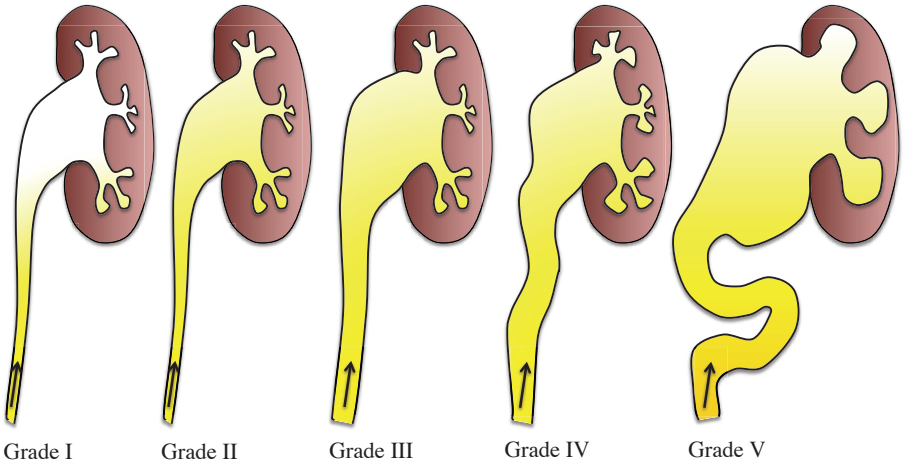


Figure 7: **International Study Classification of vesico-ureteral reflux**

Primary non-refluxing megaureters

Primary non-refluxing megaureters account for 5 to 15% of prenatally detected UTD. They are caused by a narrowing of the vesico-ureteral junction. The male-to-female ratio is approximately 2:1, with up to 25% of cases bilateral.[202] The diagnosis of primary megaureters is suspected whenever US reveals a dilatation of the ureter (> 6 mm diameter) by US, the bladder appearance and volume being normal. *(Figure 8)*

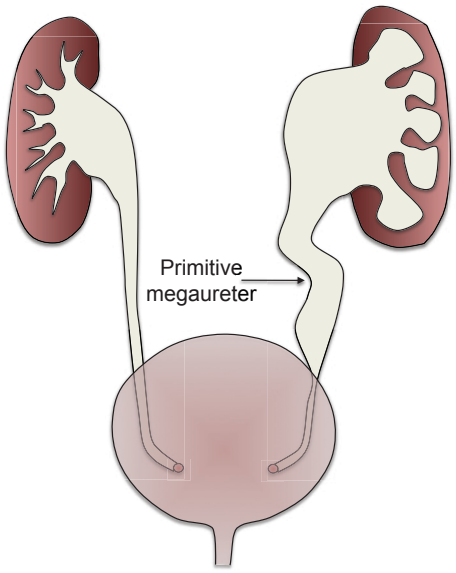


Figure 8: **Scheme of a primitive megaureter.** *The ureter and the homolateral renal pelvis are dilated.*

Postnatal investigations include US, MCUG and diuresis renography. The diagnosis of primary obstructive megaureters is confirmed if MCUG proves that the dilatation is not due to VUR. Asymptomatic children with a stable homolateral renal function on isotope studies are managed conservatively. Indications for surgery are recurrent urinary tract infections despite adequate antibiotic cover, decrease of the relative renal function on repeated isotope studies, or progressive increase of the ureteral dilatation on serial US. The operation consists in excising the distal obstructive segment of the ureter, which is then

reimplanted with an antireflux mechanism, with or without ureteric modeling. However, when renal function is poor (< 10%), a nephroureterectomy may be indicated.

Other uropathies

UTD can also be the sign of less common uropathies, like multicystic dysplastic kidneys, duplex systems or posterior urethral valves.

Multicystic dysplastic kidney is a renal dysplasia characterized by the presence of multiple, non-communicating cysts of varying size separated by dysplastic parenchyma. The affected kidney is non functional, which can be assessed by renal isotope study.[203] Multicystic dysplastic kidney is usually managed conservatively.

A duplex system describes a kidney that is divided into two separate pelvi-calyceal systems, with either complete or partial duplication of the ureters. Most duplex systems are asymptomatic and can be managed conservatively. Surgical treatment can be required in case of recurrent urinary tract infections or alteration of the renal function. The treatment of duplex systems with ureterocele remains controversial and varies from endoscopic incision to upper pole hemi-nephrectomy. Ureteral reimplantation with antireflux mechanism can also be required.[48]

A posterior urethral valve is an obstructive membrane in the posterior male urethra that cause an urinary obstruction that results in the dilatation of the posterior urethra, a bilateral UTD with a thickened bladder wall, thickened smooth muscle trabeculations and bladder diverticula. *(Figure 9)* The obstruction ultimately leads to renal failure. Posterior urethral valves are suspected prenatally when bilateral UTD is associated with bladder anomalies or oligoamnios. Renal US is performed rapidly after birth and a catheter should immediately be placed for bladder drainage. MCUG is mandatory to confirm diagnosis. The definitive treatment of choice is cystoscopic valve ablation. [204]

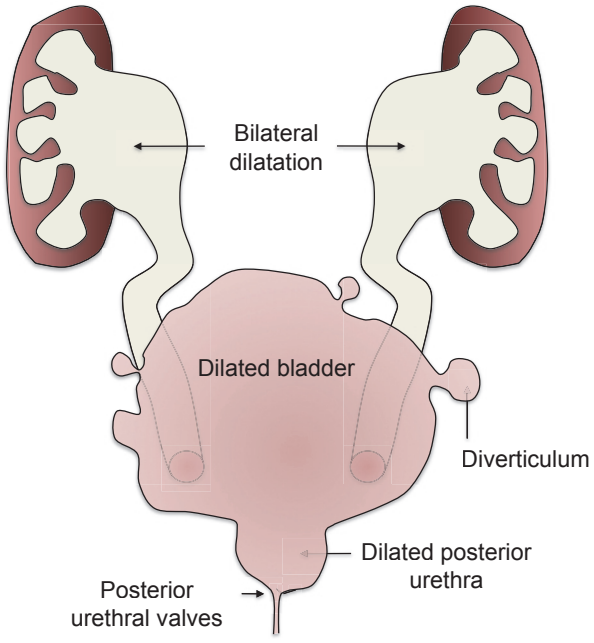


Figure 9: **Scheme of posterior urethral valves showing dilatation of the posterior urethra, a dilated bladder with diverticula and bilateral UTD.**

Appendix C

Authorization from the *Commission Nationale de l'informatique et des libertés*

CNIL

Hospices Civils de Lyon
21 FEV. 2012
Délégation à la Recherche Clinique et à l'Innovation

Le Vice-Président délégué

Madame Odile GELPI
DÉLÉGATION À LA RECHERCHE CLINIQUE
ET À L'INNOVATION
BP 2251 – 3 QUAI DES CÉLESTINS
69229 LYON CEDEX 02

N/Réf. : EGY/FLR/AR121865

Paris, le 13 FEV. 2012

Objet : NOTIFICATION D'AUTORISATION

Décision DR-2012-078 autorisant les HOSPICES CIVILS DE LYON à mettre en œuvre un traitement de données ayant pour finalité une étude clinique des modifications du profil métabolique urinaire secondaire à une anomalie congénitale de l'écoulement des urines (ACEU) par Résonance Magnétique Nucléaire (RMN) (Demande d'autorisation n° 911402)

Madame,

Vous avez saisi notre Commission d'une demande d'autorisation relative à un traitement de données à caractère personnel ayant pour finalité :

ÉTUDE CLINIQUE DES MODIFICATIONS DU PROFIL MÉTABOLIQUE URINAIRE SECONDAIRE À UNE ANOMALIE CONGÉNITALE DE L'ÉCOULEMENT DES URINES (ACEU) PAR RÉSONANCE MAGNÉTIQUE NUCLÉAIRE (RMN)

Ce traitement relève de la procédure des articles 54 et suivants de la loi du 6 janvier 1978 modifiée.

Les services de notre Commission ont étudié les conditions définies par le dossier de formalités préalables déposé à l'appui de cette demande et notamment celles de l'exercice effectif des droits des participants à l'étude.

Après avoir examiné les catégories de données traitées et les destinataires, je vous rappelle que conformément au 3ème alinéa de l'article 55, la présentation des résultats du traitement de données ne peut, en aucun cas, permettre l'identification directe ou indirecte les personnes concernées.

En application des articles 15 et 69 de la loi précitée et de la délibération n° 2009-674 du 26 novembre 2009 portant délégation d'attributions de la Commission nationale de l'informatique et des libertés à son président et à son vice-président délégué, j'autorise la mise en œuvre de ce traitement.

Je vous prie, Madame, d'agréer l'expression de mes salutations distinguées.

B. de Givry

Emmanuel de GIVRY

Commission Nationale de l'Informatique et des Libertés
8 rue Vivienne CS 30223 75083 PARIS Cedex 02 - Tél: 01 53 73 22 22 - Fax: 01 53 73 22 00 - www.cnil.fr

Appendix D

Authorization of the

Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé

Liberté • Égalité • Fraternité
RÉPUBLIQUE FRANÇAISE

MINISTÈRE
DE L'ENSEIGNEMENT SUPÉRIEUR
ET DE LA RECHERCHE

DIRECTION GENERALE POUR LA RECHERCHE ET L'INNOVATION

Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé

Dossier n° 10.515

Intitulé de la demande : **Etude clinique des modifications du profil métabolique urinaire secondaires à une anomalie congénitale de l'écoulement des urines (ACEU) chez le nouveau-né par Résonance Magnétique Nucléaire (RMN) et analyse métabolique.**

Responsable scientifique : **Pierre-Yves MURE**
Hôpital Femme-Mère-Enfant
Groupement Hospitalier Est
59 boulevard Pinel
69677 BRON Cedex

Demandeur : **Odile GELPI**
Hospices Civils de Lyon
Délégation Clinique et à l'Innovation - Siège administratif
BP 2251 - 3 quai des Célestins
69229 LYON Cedex 02

Dossier reçu le : 30.08.10

Dossier examiné le : **23 septembre 2010**

Avis du Comité consultatif :

Avis favorable

Toutefois, il faudrait fournir une lettre d'information et un formulaire de non-opposition aux parents des enfants témoins.

Fait à Paris, le 30 septembre 2010

Le Président du Comité consultatif
Docteur Mahmoud ZUREIK

1, rue Descartes – 75231 Paris Cedex 05
http://ww.recherche.gouv.fr

Appendix E

Authorization of the *Comité de protection des personnes*

COMITE DE PROTECTION
DES PERSONNES SUD-EST II
HOPITAL EDOUARD HERRIOT
Bâtiment 12 - 1er étage
5, Place d'Arsonval
69437 LYON CEDEX
Tél : 04.78.42.94.48
Fax : 04.78.42.94.69
Email : contact@cppsuest2.com
Site internet : http://www.cppsudest2.com

Lyon, le 7 octobre 2010

Monsieur le Docteur KASSAI-KOUPAI
CENTRE D'INVESTIGATION
CLINIQUE DE LYON
Site Pédiatrie
Bâtiment les Tilleuls
GROUPEMENT HOSPITALIER EST

Nos réf : C.A.L.23/2010
Copie à
Mme GAILLARD
CIC - Bâtiment Les Tilleuls
GRUPEMENT HOSPITALIER EST

Monsieur,

Le Comité de Protection des Personnes SUD-EST II, lors de la séance 6 octobre 2010, a étudié le projet d'étude que vous envisagez de réaliser intitulé : **« Etude clinique des modifications du profil métabolique urinaire secondaire à une anomalie congénitale de l'écoulement des urines (ACEU) par résonance magnétique nucléaire (RMN) et analyse métabolomique »**

A l'issue de la délibération et au regard des éléments que vous apportez dans votre courrier, le Comité a considéré que **cette étude n'entre pas dans le champ d'application de la Loi du 9 août 2004**, dans la mesure où il s'agit d'un recueil de données issues d'examens radiologiques réalisés dans le cadre du suivi habituel des patients et où aucun prélèvement invasif n'est effectué.

Pourriez-vous définir le mot « métabolomique » ?

Recevez, Monsieur, nos salutations les meilleures.

Pour le CPP SUD-EST II
Le Président – Professeur Michel DAVID

Membres ayant participé à la séance :

AMIET Nicole (Domaine Juridique) - BEAUMONT Laurent (infirmier) - BIENVENU Jacques (Recherche Biomédicale) BOISRIVEAUD Christine (Psychologue) - CHAMBOST Véronique (Pharmacienne) - CROZIER Etienne (Médecin Généraliste) - DAVID Michel (Recherche Biomédicale) - GIMENEZ-GEAY Isabelle (Infirmière) - KASSAï KOUPAï Behrouz (Recherche Biomédicale - méthodologie) - NAGEOTTE Alain (Pharmacien) PAULIGNAN Yves (Recherche Biomédicale) - PELEGRIN Serge (Représentant Association Malades et Usagers) - PHILIPPE-JANON Chantal (Domaine Social) - SI-AHMED Si-Nafa (Recherche Biomédicale) - SANN Léon (Recherche Biomédicale).

Appendix F

The standard for reporting diagnostic accuracy checklist (STARD)

The Standards for Reporting Diagnostic Accuracy (STARD) checklist (2015 version) *Page numbers in the table correspond to page numbers of the following publication:*

Prognostic Value of Ultrasound Grading Systems in Prenatally Diagnosed Unilateral Urinary Tract Dilatation. **Scalabre A**, Demède D, Gaillard S, Pracros JP, Mouriquand P, Mure PY. J Urol. 2017 Apr;197(4):1144-1149. doi: 10.1016/j.juro.2016.11.103.

Section and Topic	No	Item	Page
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	3
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	3
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	4
	4	Study objectives and hypotheses	5
METHODS			
Study design	5	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	5
Participants	6	Eligibility criteria	5
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	5
	8	Where and when potentially eligible participants were identified (setting, location, and dates)	5
	9	Whether participants formed a consecutive, random, or convenience series	5
Test methods	10a	Index test, in sufficient detail to allow replication	6 and table 1
	10b	Reference standard, in sufficient detail to allow replication	6
	11	Rationale for choosing the reference standard (if alternatives exist)	Reference standard: surgery

	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	6
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	-
	13a	Whether clinical information and reference standard results were available to the performers or readers of the index test	6
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	6
Analysis	14	Methods for estimating or comparing measures of diagnostic accuracy	6
	15	How indeterminate index test or reference standard results were handled	6
	16	How missing data on the index test and reference standard were handled	6
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	Not done
	18	Intended sample size and how it was determined	Not done
RESULTS			
Participants	19	Flow of Participants using a diagram	Not done
	20	Baseline demographic and clinical characteristics of participants	Table 2
	21a	Distribution of severity of disease in those with the target condition	Table 2
	21b	Distribution of alternative diagnoses in those without the target condition	Table 2
	22	Time interval and any clinical interventions between index test and reference standard	7
Test results	23	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	Table 3
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	Table 3
	25	Any adverse events from performing the index test or the reference standard	-
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	Not done
	27	Implications for practice, including the intended use and clinical role of the index test	10
OTHER INFORMATION			
	28	Registration number and name of registry	-
	29	Where the full study protocol can be accessed	-
	30	Sources of funding and other support; role of funders	-

Appendix G

List of metabolites identified on ^{1}H-NMR spectra of newborns urine

ID	Metabolite	HMDB number	^{1}H chemical shifts (ppm)	^{13}C chemical shifts (ppm)
1	1-Methylhistidine	00001	7.68	141.01
			7.00	122.51
			3.94	56.61
			3.69	36.17
			3.18	30.91
			3.03	30.85
2	1-Methylnicotinamide	00699	9.27	147.85
			8.96	150.10
			8.89	146.51
			8.18	130.80
			4.47	51.28
3	1.3-Diaminopropane	00002	3.12	28.11
			3.10	39.27
			2.08	26.78
4	1.6-Anhydro-beta-D-Glucose	00640	5.44	104.26
			4.63	80.49
			4.08	67.99
			3.75	68.08
			3.68	75.24
			3.68	72.57
			3.53	74.11
5	2-Hydroxyisobutyrate	00729	1.34	29.35
6	3-Aminoisobutyrate	03911	2.61	42.20
			1.18	17.92
7	3-Hydroxyisovalerate	00754	2.35	52.20
			1.26	30.93
8	3-Hydroxymethylglutarate	00355	2.42	51.34
9	4-Hydroxyprolyne	00725	4.65	72.95
			4.34	62.69
			3.48	55.88
			3.36	56.10
			2.43	40.29
			2.15	40.24
10	5-Aminopentanoate	03355	3.01	42.14
			2.21	39.84
			1.62	24.01

11	Acetate	00042	1.91	26.07
12	Acetoacetate	00060	3.43	56.13
			2.28	32.03
13	Acetone	01659	2.22	32.92
14	Adipate	00448	2.17	40.25
			1.54	28.58
15	ADP	01341	8.51	140.20
			4.55	73.05
			4.23	70.71
			4.14	64.88
			4.07	65.87
16	Agmatine	01432	3.23	43.47
			3.02	42.00
			1.73	28.65
			1.66	26.97
17	Allantoin	00462	5.38	66.22
18	Alpha-ketoisovalerate	04260	3.02	37.42
			1.09	18.36
19	Aminoadipate	00510	3.94	59.56
			3.72	57.65
			2.33	31.12
			2.23	39.62
			2.08	30.67
			1.86	32.96
			1.76	21.16
			1.76	27.85
			1.62	24.13
20	Ascorbate	00044	4.51	-
			4.01	-
			3.75	-
21	Beta-alanine	00056	3.17	40.00
			2.53	36.43
22	Betaine	00043	3.89	69.04
			3.25	55.94
23	Carnosine	00033	8.04	137.43
			7.07	119.71
			4.46	57.66
			3.21	38.67
			3.17	30.68
			3.01	30.89
			2.67	34.81
24	Choline	00097	4.05	58.68
			3.50	70.57
25	*cis*-Aconitate	00072	5.68	126.70
			3.10	46.24

26	Citramalate	00426	2.73	46.29
			2.42	49.15
			1.32	27.97
27	Citrate	00094	2.66	48.22
			2.53	48.22
28	Creatine	00064	3.92	56.65
			3.02	39.81
29	Creatinine	00562	4.05	59.22
			3.03	33.01
30	Cyclohexanone	03315	2.37	43.78
			1.84	30.32
			1.71	26.90
31	D-Glucuronate	00127	5.19	95.31
			4.64	99.09
			4.06	74.62
			3.71	78.31
			3.70	75.08
			3.50	78.53
			3.50	74.84
			3.29	76.80
32	D-Glucose	00122	5.23	94.77
			4.63	98.92
			3.88	63.40
			3.83	63.87
			3.81	74.47
			3.73	63.72
			3.70	75.24
			3.52	74.13
			3.47	78.43
			3.39	72.12
			3.22	76.90
33	Dimethylamine	00087	2.70	37.38
34	Dimethylglycine	00092	3.71	62.95
			2.91	46.40
35	Dimethyl sulfone	04983	3.14	44.37
36	D-threitol	04136	3.74	65.19
			3.72	65.06
			3.65	72.72
37	Erythritol	02994	3.77	64.26
			3.68	74.90
			3.64	65.38
38	Ethanolamine	00149	3.82	60.71
			3.14	44.26
39	Formate	00142	8.45	151.28

40	Fumarate	00134	6.50	139.65
41	Galactitol	00107	3.97	72.95
			3.69	66.24
			3.67	72.54
42	Galactose	00143	5.26	95.17
			4.57	99.31
			4.07	73.29
			3.98	72.52
			3.93	71.55
			3.84	72.07
			3.79	71.20
			3.74	63.97
			3.70	77.99
			3.65	75.55
			3.47	74.77
			3.18	56.65
43	Glucarate	00663	4.12	76.87
			4.09	76.57
			4.06	74.79
			3.94	76.30
44	Gluconate	00625	4.12	76.75
			4.01	72.46
			3.82	65.38
			3.76	75.09
			3.76	73.71
			3.65	65.29
45	Glycine	00123	3.55	44.53
46	Guanidoacetate	00128	3.78	47.39
47	Hippurate	00714	7.82	129.98
			7.63	134.99
			7.54	131.76
			3.96	46.63
48	Homocarnosine	00745	8.03	137.20
			7.06	119.96
			4.45	73.47
			3.18	30.86
			2.99	31.01
			2.92	42.31
			2.36	35.42
			1.87	24.44
49	Hypoxanthine	00157	8.20	144.52
			8.18	140.10

50	Isobutyrate	01873	2.37	39.86
			1.05	21.10
51	Isocitrate	00193	3.99	76.50
			2.96	51.85
			2.49	40.41
			2.44	40.35
52	L-Acetylcarnitine	00201	5.58	69.42
			3.85	71.86
			3.59	68.53
			3.19	56.64
			2.64	41.29
			2.51	40.80
			2.13	23.40
53	Lactose	00186	5.23	94.94
			4.66	99.19
			4.63	99.26
			4.45	105.79
			4.44	105.76
			3.93	71.60
			3.88	63.26
			3.83	74.47
			3.77	63.91
			3.72	78.20
			3.68	75.01
			3.64	75.56
			3.62	75.37
			3.57	73.63
			3.54	74.01
			3.29	77.04
54	L-Alanine	00161	3.78	53.43
			1.47	19.06
55	L-Alpha-aminobutyrate	00452	3.68	57.72
			1.89	25.82
			0.97	11.42
56	L-Arabinose	00646	7.08	129.53
			5.23	95.16
			4.51	99.48
			4.02	65.37
			3.98	72.40
			3.86	71.76
			3.79	71.33
			3.68	69.39
			3.64	65.21
			3.49	74.89

57	L-Arginine	00517	3.76	57.10
			3.23	43.34
			1.91	30.39
			1.66	26.96
58	L-Carnitine	00062	4.55	68.67
			3.41	72.85
			2.44	45.66
			2.43	45.71
			2.42	45.71
59	L-Fucose	00174	5.19	95.08
			4.54	99.33
			3.84	72.04
			3.81	74.63
			3.75	71.47
			3.74	74.00
			3.63	75.61
			3.44	75.40
			1.26	18.09
			1.20	18.23
			4.18	67.80
			3.84	67.95
			1.19	17.95
60	L-Glutamine	00641	3.75	57.31
			2.44	33.60
			2.13	29.21
61	L-Histidine	00177	7.08	119.98
			3.98	57.60
			3.25	30.76
			3.23	30.68
			3.16	30.79
62	L-Lactate	00190	4.10	71.43
			1.32	22.72
63	L-Lysine	00182	3.70	57.69
			3.00	42.17
			1.90	32.87
			1.72	29.43
			1.48	24.50
			1.44	24.50

64	L-Octanoylcarnitine	00791	5.61	69.67
			3.86	71.72
			3.61	71.53
			3.20	56.78
			2.62	42.53
			2.51	43.01
			2.43	35.56
			1.62	27.36
			1.30	30.81
			1.28	24.81
			1.27	32.81
			0.86	16.13
65	L-Phenylalanine	00159	7.41	131.83
			7.36	131.02
			7.32	132.14
			3.98	57.79
			3.28	39.11
			3.10	39.19
66	L-Serine	00187	3.97	63.27
			3.83	59.19
67	L-Tyrosine	00158	7.19	133.57
			6.89	118.61
			3.93	56.80
			3.17	38.75
			3.06	38.53
68	L-Valine	00883	3.58	63.42
			2.26	30.32
			1.04	20.84
			0.97	19.56
69	Mannitol	00765	3.86	65.75
			3.79	71.73
			3.76	74.01
			3.68	65.83
70	Methylguanidine	01522	2.82	30.15
71	Myoinositol	00211	4.06	74.62
			3.60	75.08
			3.52	74.09
			3.27	77.06
72	N-Acetylglutamine	06029	4.16	57.74
			2.31	34.39
			2.10	30.59
			2.03	24.99
			1.91	30.44

73	N-Acetyl-L-aspartate	00812	4.34	56.51
			2.59	42.25
			2.03	24.80
74	N-Acetyl-L-tyrosine	00866	7.13	132.98
			6.83	118.11
			4.37	59.57
			3.10	38.98
			2.83	37.80
			1.91	23.42
75	N-Acetylornithine	03357	4.17	57.62
			3.02	42.20
			2.05	24.96
			1.71	26.86
76	N-Acetyl-D-glucosamine	00215	5.23	94.61
			3.88	63.49
			3.87	75.05
			3.83	63.65
			3.77	63.64
			3.59	77.55
			3.54	78.31
			3.49	72.44
			2.01	24.96
77	N-Acetylputrescine	02064	3.21	41.39
			3.06	41.71
			1.97	24.74
			1.59	27.72
78	N.N dimethylaniline	01020	7.37	131.38
			7.07	117.09
			2.84	45.24
79	O-phosphoethanolamine	00224	3.99	63.28
			3.23	43.57
80	Ornithine	00214	3.76	57.25
			3.04	41.74
			1.93	30.39
			1.80	26.42
81	Oxoglutarate	00208	3.00	38.33
			2.43	33.53
82	Pantothenate	00210	3.98	78.52
			3.50	71.09
			3.44	38.66
			0.92	23.37
			0.88	21.81

83	Phenylacetate	00209	7.37	131.17
			7.30	131.82
			7.29	128.91
			3.53	47.14
84	Phenylacetylglycine	00821	7.42	131.79
			7.35	130.18
			7.35	131.79
			3.75	46.29
			3.66	44.99
85	Pimelate	00857	2.17	40.36
			1.54	28.49
			1.28	31.26
86	p-Hydroxyphenylacetate	00020	7.15	133.39
			6.86	118.09
			3.44	46.36
87	Propylene glycol	01881	3.81	71.48
			3.44	69.47
			3.37	69.51
			1.13	20.90
88	Pseudouridine	00767	7.67	144.61
			4.67	81.48
			4.28	76.16
			4.14	73.62
			4.01	86.21
			3.73	63.99
89	Pyroglutamate	00267	4.16	61.31
			2.48	28.08
			2.39	32.48
			2.02	27.30
90	Pyruvate	00243	2.36	29.19
91	Quinolinate	00232	8.44	150.67
			8.00	140.01
92	Sorbitol	00247	3.83	74.84
			3.64	60.31
			3.63	72.73
93	Suberate	00893	1.53	28.61
			1.30	31.25
			2.17	40.29
94	Succinate	00254	2.39	36.94
95	Taurine	00251	3.41	38.27
			3.25	50.53

96	Threonate	00943	4.06	75.04
			3.98	76.53
			3.70	66.05
			3.64	65.19
97	*trans*-Aconitate	00958	6.58	133.85
			3.44	38.54
98	Trimethylamine N-oxide	00925	3.25	62.33
99	Urea	00294	5.78	-
100	Xanthosine	00299	7.87	138.92
			5.84	90.25
			4.73	75.42
			4.40	73.69
			4.24	86.64
			3.90	63.72
			3.83	64.25

Communications

Publications

Evolution of Newborns' Urinary Metabolomic Profiles According to Age and Growth. **Scalabre A**, Jobard E, Demède D, Gaillard S, Pontoizeau C, Mouriquand P, Elena-Herrmann B, Mure PY. J Proteome Res. 2017 Aug 25. J Proteom Res. 2017, 16 (10), 3732-3740. doi: 10.1021/acs.jproteome.7b00421

PMID: 28791867

Prognostic Value of Ultrasound Grading Systems in Prenatally Diagnosed Unilateral Urinary Tract Dilatation. **Scalabre A**, Demède D, Gaillard S, Pracros JP, Mouriquand P, Mure PY. J Urol. 2017 Apr;197(4):1144-1149. doi: 10.1016/j.juro.2016.11.103. Epub 2016 Dec 1.

PMID: 27916713

Oral presentations

Prognostic value of ultrasound grading systems in prenatally diagnosed unilateral Urinary tract dilatation. **Scalabre A**, Demède D, Gaillard S, Pracros JP, Mouriquand P, Mure PY. 28th congress of the European Society for Paediatric Urology, Barcelona, Spain, 19-22th April 2017

Evolution of urine metabolic profile in newborns. **Scalabre A**, Jobard E, Demède D, Gaillard S, Pontoizeau C, Mouriquand P, Elena-Herrmann B, Mure PY. 10èmes journées du Réseau Français de Métabolomique et Fluxomique, Montpellier, le 2 juin 2016

Comparaison des valeurs pronostiques du diamètre antéro-posterieur du bassinet, du score Urinary Tract Dilation (UTD) et du score de la Society for Fetal Urology (SFU) dans les dilatations unilatérales des voies urinaires de diagnostic anténatal. **Scalabre A**, Demède D, Benchaib M, Gaillard S, Mouriquand P, Mure PY. 73ème congrès de la Société Française de Chirurgie Pédiatrique, Toulouse le 21 septembre 2016