Investigations into Rare Kidney Disorders

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DECLARATION

‘I, Sergio Camilo Lopez-Garcia, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.’

ABSTRACT

An inherent feature of rare diseases is the scarcity of data to better define management and prognosis. International collaborations facilitate information sharing and can help overcome these challenges. With support from international scientific networks, this project has gathered and analysed data from large cohorts with very rare inherited conditions, that addressed relevant research questions to inform routine clinical practice.

The dRTA Cohort (N=340) analysed paediatric and adult clinical, genetic and demographic data from 29 countries. Long-term outcomes showed reduced stature in adults, increased prevalence of chronic kidney disease (CKD) from paediatric age (35%) to adults (82%), large prevalence of nephrocalcinosis (88%), urolithiasis (21-42%) and hearing loss. Adequate metabolic control was positively correlated with countries' wealth (GDP), but also with better kidney function and final height, highlighting the impact of health care access' inequalities on long term outcomes.

The NDI Cohort (N=315) reported on patients from a total of 22 countries. Long term outcome showed normal height but unexpectedly an increased prevalence of obesity in adults (41%). NDI patients also had an increased prevalence of CKD (children 32%; adults 48%) and urological complications (38%). While education and employment outcomes were satisfactory, there was a high proportion with mental health problems (36%).

A large paediatric urolithiasis cohort (N=141) was also analysed providing informative medical and surgical outcomes. This report showed a varied number of atypical features on presentation in children: including bilateral stone formation, high prevalence of metabolic abnormalities and a larger than expected prevalence of CKD. Finally, a large cohort of paediatric cystinuria patients (N=52) showed a high prevalence of comorbidities and a reassuring profile of thiol drugs regarding safety and efficacy.

IMPACT STATEMENT

While rare diseases are defined as affecting less than 1:2000 individuals, some of them are ultra rare with prevalences less than 1:100.000. Consequently, individual clinicians and also centres typically take care of very few, if any patients and treatment as well as prognostic counselling may be more based on anecdotes and individual experiences rather than solid evidence. Traditionally, such evidence is gathered through registries, but these usually need to exist for many years, before long-term outcomes can be assessed and require regular data entry from the participating centres.

I aimed to overcome these issues through an international collaboration of adult and paediatric nephrologist with the support of recognised scientific networks such as ERA/EDTA (European Renal Association/ European Dialysis and Transplant Association), ESPN (European Society of Paediatric Nephrology) and ERKNet (European Reference Network for Rare Kidney Diseases). Rather than gathering longitudinal data that would be extensive and thus could prevent clinicians from participating, I aimed to get a “snapshot” by asking only a few basic questions that focussed on the last clinical follow-up and thus would be easy to retrieve. Using innovative methodology that facilitated data recruitment via an anonymized online survey and a meticulous screening of submitted data, information from hundreds of patients from all over the world and across the whole age spectrum revealed novel insights into two very rare inherited kidney conditions such as distal renal tubular acidosis and nephrogenic diabetes insipidus.

A second part of the thesis was based on the investigations of two very large cohorts of paediatric patients with rare stone-forming kidney disorders. A comprehensive review of medical and surgical aspects was performed in a cohort of children with urinary stone disease attending a single quaternary centre during a period of a year from both a perspective. In addition, a detailed review of more than 50 children from the same centre diagnosed with cystinuria and followed up during more than 20 years offers a historic and detailed view of the management of this very rare disease.

Through this work relevant questions with regards to clinical practice, long-term outcomes and patient quality of life have been successfully answered and some new ones have been raised with the aim to guide future research.

Within the limitations of these observational and retrospective studies, the informative results here presented have contributed to current knowledge and will hopefully help to improve patients' care, as reflected in their citation by international treatment recommendations (cite dRTA, for NDI, you can write "manuscript in preparation"). This innovative data collection method through an online survey has since been followed by multiple other projects using this information gathering strategy.

Finally, this work has been selected for presentation at three international conferences (European Society of Paediatric Nephrology in 2018 and 2021, and International Paediatric Nephrology Association 2019), a national conference (BAPN 2018) and has been cited more than a hundred times in the scientific literature.

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ABBREVIATIONS:

17-AAG	17-Allylamin Geldanamycin
α-MPG	Alfa-Mercapto Propionyl Glycine
AC	Adenylate Cyclase
ACEI	Angiotensin-Converting Enzyme Inhibitor
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal Dominant
ADH	Antidiuretic Hormone
AE1	Anion Exchanger 1
AGT1	Aspartate/Glutamate Transporter 1
AKAP	PKA-Anchoring Protein
AKI	Acute Kidney Injury
AME	Apparent Mineralocorticoid Excess
APRT	Adenine phosphoribosyltransferase
ATP6V0A4	ATPase H^+ transporting V0 subunit a4
ATP6V1B1	ATPase H^+ transporting V1 subunit B1
AQP1/2/3/4	Aquaporin-1/2/3/4 channels
AR	Autosomal Recessive
ATP	Adenosine Triphosphate
AVP	Arginine Vasopressin
AVPR2	AVP receptor type 2
BBMV	Border Brush Membrane Vesicles
BC	Before Christ
BMI	Body Mass Index
CAII	Carbonic Anhydrase Type 2
CAG	Cytosine-Adenine-Guanine repeat
cAMP	cyclic Adenosine Monophosphate
CaSR	Calcium-Sensing Receptor
CBTDs	Cystine-Binding Thiol Drugs
CDC	Centre for Disease Control
cGMP	cyclic Guanine Monophosphate
CHO	Chinese Hamster Ovary
CIC	Clean Intermittent Catheterization

CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CNS	Central Nervous System
CO_2	Carbon Dioxide
COX	Cyclo-oxygenase
CREB-1	cAMP-Responsive Element-Binding Protein 1
CT	Computed Tomography
CXCL12	CXC-motif chemokine ligand 12
dDAVP	1-desamino-8-D-Arginine Vasopressin
DHA	2,8-dihydroxyadenine
DNA	Deoxyribonucleic Acid
dRTA	Distal Renal Tubular Acidosis
EAAC1	Excitatory Amino-Acid Carrier 1
EGF	Epidermal Growth Factor
eGFR	estimated Glomerular Filtration Rate
ENaC	Epithelial Sodium Channel
EP	E-Prostanoid receptor
Epac1	Exchange Protein Directly Activated by cAMP 1
EPO	Erythropoietin Hormone
ER	Endoplasmic Reticulum
ERA-EDTA	European Renal Association – European Dialysis and Transplant Association
ERKnet	European Rare Kidney Disease Network
ESPN	European Society for Paediatric Nephrology
ESWL	Extracorporeal Shock Wave Lithotripsy
FHHNC	Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis
FMP-API-1	3,3'-diamino-4,4'-dihydroxydiphenylmethane
FOXI1	Forkhead box I1
G3PD	Glyceraldehyde-3-Phosphate Dehydrogenase
GDP	Gross Domestic Product
GPCR	G Protein Coupled Receptors
GPCRK	G-protein-Coupled Receptor Kinase
GSK3	Glycogen Synthase Kinase type 3
GTP	Guanine Triphosphate

GWAS	Genome Wide Association Study
H_2S	Hydrogen Sulphide
HCO_3^-	Bicarbonate
HCS	Hypotonia Cystinuria Syndrome
HGMD	Human Gene Mutation Database
Hsp90	Heat Shock Protein 90
HU	Hounsfield Units
ICC	Internation Cystinuria Consortium
iPTH	intact Parathyroid Hormone
IQR	Interquartile Range
JAK-2	Janus Kinase 2
KCC4	Potassium Chloride Co-transporter 4
KCNJ1	Potassium Inwardly Rectifying Channel Subfamily J Member 1
kDa	Kilodalton
KDIGO	Kidney Disease Improving Global Outcomes
KDOQI	Kidney Disease Outcomes Quality Initiative
KUB	Kidneys Ureters and Bladder
LLN	Long Loop Nephrons
MDCK	Madin-Darby Canine Kidney
MDRD	Modification of Diet in Renal Disease (Study)
mRNA	messenger RNA
MRU	Magnetic Resonance Urography
MVBs	Multivesicular Bodies
NC	Nephrocalcinosis
NCC	Sodium Chloride Co-transporter
NCKX4	Sodium Calcium Potassium Exchanger 4
NDI	Nephrogenic Diabetes Insipidus
NH_3	Ammonia
NH_4^+	Ammonium
NH_4Cl	Ammonium chloride
NHANES-III	3rd National Health and Nutrition Survey
NHE3	Sodium Hydrogen Exchanger 3
NKCC	Sodium Potassium Chloride Co-transporter
NP-II	Neurophysin II

NRF2	Nuclear factor erythroid 2-related factor 2
NSAIDs	Non-steroidal Anti-Inflammatory Drugs
NSF	N-ethylmaleimide-Sensitive Factor
PCCL	Percutaneous Cystolithotomy
PCNL	Percutaneous Nephrolithotomy
PCO2	Partial Pressure of Carbon Dioxide
PDE4	Phosphodiesterase-4
PGE_2	Prostaglandin E_2
PH1/2/3	Primary Hyperoxaluria Type 1/2/3
PKA/C/G	Protein Kinase A/C/G
PO_4^{3-}	Orthophosphate
PUJ	Pyelo-Ureteral Junction
PVN	Paraventricular Nuclei
pRTA	Proximal Renal Tubular Acidosis
RBC	Red Blood Cell
RFS	Renal Fanconi Syndrome
RhAG	Rhesus Glycoprotein A
RhBG	Rhesus Glycoprotein B
RhCG	Rhesus GlycoproteinC
RNA	Ribonucleic Acid
ROMK	Renal Outer Medullary Potassium channel
RR	Relative Risk
RTA	Renal Tubular Acidosis
SAO	Southeast Asian Ovalocytosis
SDS	Standard Deviation Score
SG	Sediment Gravity
SLN	Short Loop Nephrons
SON	Supraoptic Nuclei
SNHL	Sensorineural Hearing Loss
sp.	Single unnamed bacterial species
sPRR	Soluble Protein Receptor
TAL	Thick Ascending Limb
TDL	Thin Descending Limb
TCO2	Total Carbon Dioxide

t-SNARE	target membrane-Soluble NSF Attachment Protein Receptor
UAG	Urine Anion Gap
UCa	Urinary Calcium
UCr	Urinary Creatinine
UL	Urolithiasis
UOx	Urinary Oxalate
URS	Ureteroscopy
USA	United States of America
UT-A1/A2/A3	Urea Transporters A1/A2/A3
UTI	Urinary Tract Infection
v-SNARE	vesicle-Soluble NSF Attachment Protein Receptor
WHO	World Health Organization

CHAPTER 1: PRIMARY DISTAL RENAL TUBULAR ACIDOSIS

1.1. INTRODUCTION

1.1.1. Syndromes of renal tubular acidosis. Historical context to classification.

The study and understanding of the renal tubular acidosis began in the early 20th century and has continued to evolve in the 21st century with the expansion of molecular biology.

In a British Paediatric Association meeting of 1935 Lightwood first reported a group of 6 young children (from a large autopsy series) with what he described as “calcium infarction” of the kidneys (1). This was later recognised as the first report of infants with nephrocalcinosis from renal tubular acidosis. Only a year later, Butler and colleagues described four infants with similar features and hyperchloremic acidosis, although the connection between acidosis and nephrocalcinosis was at that time unclear (2).

About a decade later Albright brilliantly provided what is considered the first pathophysiological description of RTA (3). Key insights provided by Albright in his extensive essay on “osteomalacia and late rickets” included: a) an understanding of the disease as “renal acidosis resulting from tubular insufficiency without glomerular insufficiency”, b) the understanding that acidosis causes hypercalciuria, c) the consequent link to nephrocalcinosis/urolithiasis, d) a correlation between this “renal tubular insufficiency” and the previously described familial periodic paralysis and e) the treatment recommendation of these patients with a solution of sodium citrate previously designed by Alfred Shohl. The term “renal tubular acidosis” was coined in 1951 (4).

During the following years, further research on the physiology of renal tubular acidification led to the development of different techniques to measure the capacity of the distal tubules for acid secretion. This identified “distal tubular insufficiency” as the main mechanism for the disease. In particular the work of Wrong and Davies not only outlined what later on became the gold standard diagnostic protocol (ammonium

chloride test), but also provided an extended phenotype with the description of the so called “incomplete renal tubular acidosis”, defined as a failure to acidify urine in response to an oral acid load but with an absence of overt systemic acidosis in routine conditions (5).

Stapleton and colleagues in 1949 described patients with significant bicarbonaturia in a context of low serum bicarbonate (6). This suggested that in some patients the disease mechanism was not impaired distal acidification but rather proximal bicarbonate reabsorption. Rodriguez-Soriano and Edelman subsequently proposed a new classification, based on the threshold for bicarbonate excretion and thereby distinguishing between proximal and distal RTA (7).

Later on, Morris and colleagues described another subtype, denominated type III and characterised by a combination of proximal and distal RTA (8). This was based on observations in 2 infants and most likely reflected the transient proximal tubular dysfunction commonly seen in patients with distal RTA at presentation, which resolves with treatment (9). However, in the 1980s, a rare autosomal recessive syndrome presenting with osteopetrosis, combined proximal and distal renal tubular acidosis, cerebral calcification, and mental impairment was described and linked to pathogenic variants in *CA2*, encoding carbonic anhydrase type II, expressed both in proximal and distal segments and involved in bicarbonate reabsorption, as well as proton secretion (10).

The discovery of aldosterone did not only have a significant impact on our understanding of sodium and potassium physiology but also subsequently led to the recognition of a fourth type of renal tubular acidosis. This form was seen in patients with aldosterone insufficiency or resistance (also known as pseudo-hypoaldosteronism) and is characterised by hyperkalaemia rather than hypokalaemia (11). This form of RTA was then referred to as type IV RTA. It is important to recognize, however, that the primary defect here is a failure to reabsorb sodium in the collecting duct with the resultant “voltage defect” impairing acid secretion (12). Thus, type IV RTA is primarily a salt-wasting disorder with a secondary defect in distal acidification.

Over the past century, advances in renal physiology, clinical investigations and molecular genetics have greatly improved our understanding of the various forms of renal tubular acidosis. Currently, the diagnosis and classification of the various types of renal tubular acidosis continue to rely on biochemical measurements of blood and urine but is increasingly complemented by genetic testing.

More than 50 disease genes for kidney tubulopathies are recognised at present and the list keeps on expanding. Because of the specific phenotype associated with mutations in most genes, an accurate clinical diagnosis can usually be established. However, even in expert centres, genetic testing can sometimes further specify or even correct the clinical diagnosis, so that genetic confirmation is usually recommended because of potentially important implications not only for genetic counselling, but also for treatment (13, 14).

1.1.2. Distal Renal Tubular Acidosis (Type 1 RTA or dRTA)

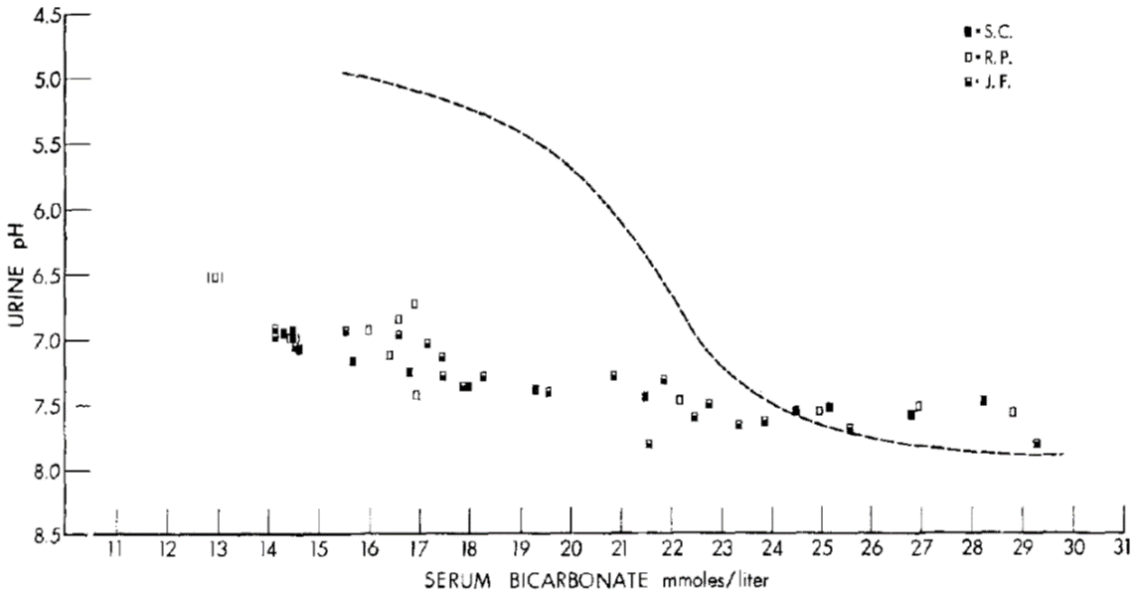
1.1.2.1. Pathophysiology

As initially described in 1967(15), dRTA is defined as the impaired ability of the kidney to maximally decrease the urine pH under conditions of systemic acidosis. This particular project presented by Soriano and collaborators represented the first study describing patients (N=3) with a defect in distal tubular acidification (Figure 1.1). Those patients underwent a routine acidification test performed via enteral administration of ammonium chloride at a standard dose of 0.1g/kg (details on the various urinary acidification tests are provided in Section Diagnosis: urinary acidification tests).

Although dRTA was already described as an entity in 1967 it took about 20 years more to better understand the molecular mechanisms of acid secretion and why acid retention occurs in these patients. Indeed, the main role of the distal tubule in acid-base balance is based on the luminal excretion of protons which mainly derive from the metabolism of our diet. A common western diet is usually generating a daily acid load of 1 mmol/kg of body weight (16) and on top of this amount children typically produce an extra 1-2mmol/kg of body weight as a result of bone mineralization (generation of hydroxyapatite), which generates an acid load of approximately 1-3mmol/kg/day that the kidneys need to excrete (17, 18). If the distal nephron is not

capable of performing this function, the patient will use the existing buffers in the body to neutralize this excess of acid (mainly bicarbonate, but also phosphate, plasma proteins such as haemoglobin and ultimately the skeleton) (19, 20).

Figure 1.1. Graph representation of first urinary acidification test



While individuals with normal urinary acidification are expected to follow a sigmoidal decrease in urine pH following a drop in serum bicarbonate (mean represented by broken line) with values as low as <5.0 during severe acidotic conditions, the presented patients (S.C., R.P. and J.F.) followed a linear distribution of urinary pH with a small slope and no values under a pH of 6.5. Extracted from Soriano et al. 1967.

1.1.2.2. Importance of acid-base homeostasis

Extracellular pH is normally regulated in an extremely tight range (7.37-7.43), reflecting changes in proton concentration only in the nanomolar range. As protons can bind to amino acids like histidine, a change in pH will lead to a change in the charge of a given protein, which in turn can affect protein folding and function (21). This highlights the critical importance of acid-base homeostasis for cellular function in general, and many of the non-specific clinical symptoms encountered in untreated patients with dRTA such as fatigue and lack of appetite may reflect this.

1.1.2.3. Pathophysiological consequences

Most of the pathophysiologic consequences of distal RTA are due to the accumulation of acid (nausea, vomiting, fatigue, tachypnoea, mineral bone disease, hypercalciuria, salt wasting, hypocitraturia, nephrocalcinosis/urolithiasis and in up to 2/3 of paediatric cases also proximal tubular dysfunction on presentation) (9, 22-25). Although a normal functioning proximal tubule will continue reabsorbing the filtered bicarbonate, the distal

inability to excrete the excess body acid will cause a progressive increase of the base deficit.

1.1.2.3.1. Bone disease

At the extracellular level, bicarbonate is consumed due to its buffering action and once depleted, other buffers start to take over, such as phosphate, which has the skeleton as its main storage, in the form of hydroxyapatite [$Ca_5(PO4)_3(OH)$]. Hydroxyapatite dissolves in an acidic environment and one of the consequences is the release of hydroxyl ions (OH^-) involved in acid neutralization, but also of calcium which subsequently must be excreted through the urinary tract, leading to hypercalciuria and calcification (nephrocalcinosis and/or urolithiasis). Indeed, a negative calcium balance in individuals with dRTA has been shown (7). In children the clinical consequence of this chronic acidosis disturbing the growing bone is the development of rickets, while in adults it is osteomalacia (3).

The exact physio-pathological mechanisms linking mineral bone disease with systemic acidosis are yet to be elucidated. *In vitro* studies using rat cells suggest that acidosis does not directly affect the composition of the mineral osteoid but stimulates the resorptive activity of the osteoclasts by directly activating the cells (26) and also inhibiting the synthetic function of osteoblasts (27). However, some other authors have argued that *in vitro* environments only reflect responses to acutely induced acidosis while perhaps the influence of chronic acidosis may be different and in that respect data extracted from bone biopsies in human patients with dRTA have shown a supressed activity of both osteoblast and osteoclasts, as well as low turn-over bone disease and a reduced mineral density in both densitometry and histological studies (28).

1.1.2.3.2. Hypercalciuria and nephrocalcinosis

Not only hypercalciuria contributes to the development of nephrocalcinosis, but also the increased proximal reabsorption of citrate with subsequent hypocitraturia. Citrate is the most important and abundant organic urinary base equivalent, and it represents also the most important chelator for calcium in the urine. It is thereby working as an anti-lithogenic agent by preventing calcium binding to oxalate/phosphate and also their crystallisation.

The reabsorption of citrate happens exclusively in the proximal tubule through a specific cotransporter (sodium-dicarboxylic acids) denominated NaDC-1 (*SLC13A2*) which shows increased expression in the apical membrane during systemic acidosis (29, 30). Once citrate is taken up by the proximal tubular epithelial cell, it will be metabolized by the mitochondria through the citric acid cycle, generating CO_2 and H_2O while consuming 3 protons (H^+). This suggests that a normally functioning proximal tubule is needed to develop hypocitraturia and may explain why in clinical practice it is more likely to find nephrocalcinosis in patients with distal than proximal RTA. However, this is not always the case and patients with Dent's disease constitute a clear exception (31).

Nephrocalcinosis (NC) in itself can have a negative impact on the natural history of the disease with studies suggesting that it accelerates loss of kidney function by triggering a local inflammatory response (32, 33) and also impairs the urinary concentration ability, presumably due to interstitial calcification. Nephrocalcinosis has also been associated with increased production of red blood cells or erythrocytosis. Although the mechanisms have not been fully elucidated, it is believed to be in relation with renal hypoxia and increased synthesis of erythropoietin hormone (EPO) (34, 35). Urolithiasis shares the same pathophysiological mechanisms with nephrocalcinosis and as such is particularly common in patients with primary forms of dRTA with selected series from tertiary centres showing a prevalence as high as 77% and this seems to be also true for secondary forms, as for example dRTA associated with autoimmune disorders like Sjogren syndrome (36, 37).

The combination of hypocitraturia, hypercalciuria and a high urinary pH creates a favourable environment for calcium-phosphate precipitation and indeed brushite ($CaHPO_4 \cdot 2H_2O$) is the predominant mineral found in the urinary stones from patients with dRTA. However, although this composition is highly suggestive, in clinical practice it is common to find a broad spectrum of stone compositions, including struvite, calcium oxalate, purines and mixed stones perhaps reflecting the particularities of each individual and their respective stone formation risk: recurrent urinary tract infections, the degree of urinary acidification impairment, diet, medications and other comorbidities (36).

1.1.2.3.3. Hypokalaemia

Hypokalaemia, although not necessarily present in all individuals, is another classical finding in patients with dRTA (38, 39). The exact mechanisms involved in potassium wasting have perhaps not been fully elucidated by now, but most likely include a combination of different factors. Notably, hyperaldosteronism (39) has been documented and it does seem to be related to volume contraction and sodium wasting. Renal potassium wasting through abnormal activity of the H^+/K^+ ATPase in the collecting tubule cells has also been proposed as an alternative mechanism (38).

In addition, the impaired urinary concentrating ability and consequent increased urinary flow rate activate K-secretion via BKCa channels (40).

Hypokalaemia can be very severe and life threatening, causing rhabdomyolysis (41) and muscle paralysis, which can be one of the presenting signs of dRTA (42). In a report describing 14 patients with hypokalaemic rhabdomyolysis, 7 patients carried the diagnosis of distal RTA (41).

1.1.2.3.4. Salt wasting

Not only potassium wasting is a feature of dRTA, sodium reabsorption can be also impaired. However, the exact mechanisms why this is the case remain to be fully elucidated yet. In the first instance, sodium reabsorption in the collecting duct may be decreased, due to impaired exchange with protons. But also, acidosis per se may cause decreased sodium reabsorption. Indeed, there is evidence showing this correlation and the hypothesis is that a low pH can affect the normal functioning of several transporters (43) (see “importance of acid-base homeostasis” above). However this might be not the only factor involved as correction of acidosis in patients with RTA does not necessarily normalise electrolytes levels (39).

A study of *ATP6V1B1* knock out mice (44) provided new insights on how salt wasting may evolve in dRTA: the resulting rather elaborate hypothesis is that an apical sodium dependent chloride/bicarbonate exchanger called NDCBE ($1Na^+$ and $2HCO_3^-$ move to the intracellular space while $1Cl^-$ leaves for the tubular lumen) present in the β-intercalated cells of the collecting tubule has its function affected due to an impaired

activity of the basolateral H^+ ATPase (which does not only control intracellular pH in this tubular segment but also changes the membrane potential).

This H^+ ATPase in the β-intercalated cells appears to be the major driver of active transport and the main source of ATP consumption. The consequences of an altered function are: 1) a significant change on the cellular energetics and 2) ATP exiting the cell through specific anion channels. Those ATP molecules may now bind certain luminal receptors and stimulate PGE_2 secretion into the urine. Ultimately, PGE_2 will induce natriuresis and sodium wasting, as seen in cases of Bartter Syndrome and also Nephrogenic Diabetes Insipidus (45).

1.1.2.3.5. Urinary concentration defect

Polyuria has also been described as one of the symptoms in patients with dRTA at presentation and it may potentially be secondary to hypokalaemia. Within the loop of Henle, and more specifically in the thick ascending limb (TAL), an adequate potassium concentration (and recycling through ROMK) is essential for the effective working of the NKCC transporter. As seen in patients with Barter syndrome and loop diuretics, decreased sodium reabsorption in the TAL will consequently impair the maintenance of the medullary concentration gradient ultimately resulting in decreased urinary osmolality (46). In addition, hypokalaemia has also been correlated with a downregulation of AQP2 channel expression in the apical membrane of the collecting ducts in, hence an impaired water reabsorption (47).

1.1.2.3.6. Impaired ammoniagenesis

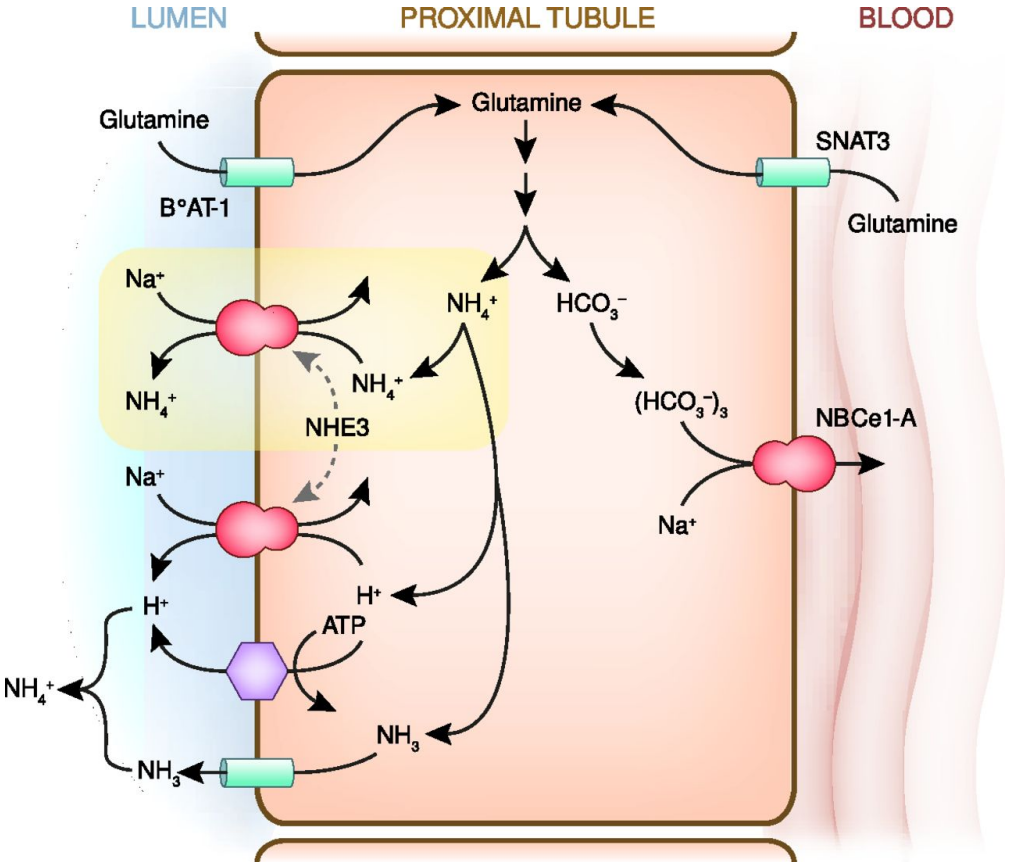
Another key factor in the physiopathology of dRTA is the presence of an impaired proximal tubular secretion of ammonia. Ammonium excretion accounts for the majority of basal bicarbonate generation and changes in its excretion rate are the primary response to acid-base disorders (48). Ammonia is delivered to the tubular lumen in different sections of the nephron but more importantly is generated in the proximal tubule. In this segment, the metabolism of glutamine provides 2 molecules of NH_3 and 2 of HCO_3^- (bicarbonate is subsequently transported to the basolateral membrane). Usually, half of the produced ammonia is excreted in the urine while the other half ultimately accesses the systemic circulation to produce urea in the liver (a process that consumes HCO_3^- and therefore the net effect on acid-base balance is neutral

under physiological circumstances). The proportional relationship between excreted ammonia in the urine and the one transported into the venous system, can be quickly modified by acidosis and in consequence urinary ammonia can transiently exceed ammoniagenesis (49).

1.1.2.3.6.1. Ammonia recycling

In the proximal tubule (Figure 1.2) NH_4^+ is secreted into the tubular lumen by NHE3 (Sodium Hydrogen exchanger 3, *SLC9A3*) but also parallel transport of H^+ (via NHE3) and NH_3 (which is membrane permeable) occurs, with subsequent "trapping" of NH_4^+ in the tubular lumen.

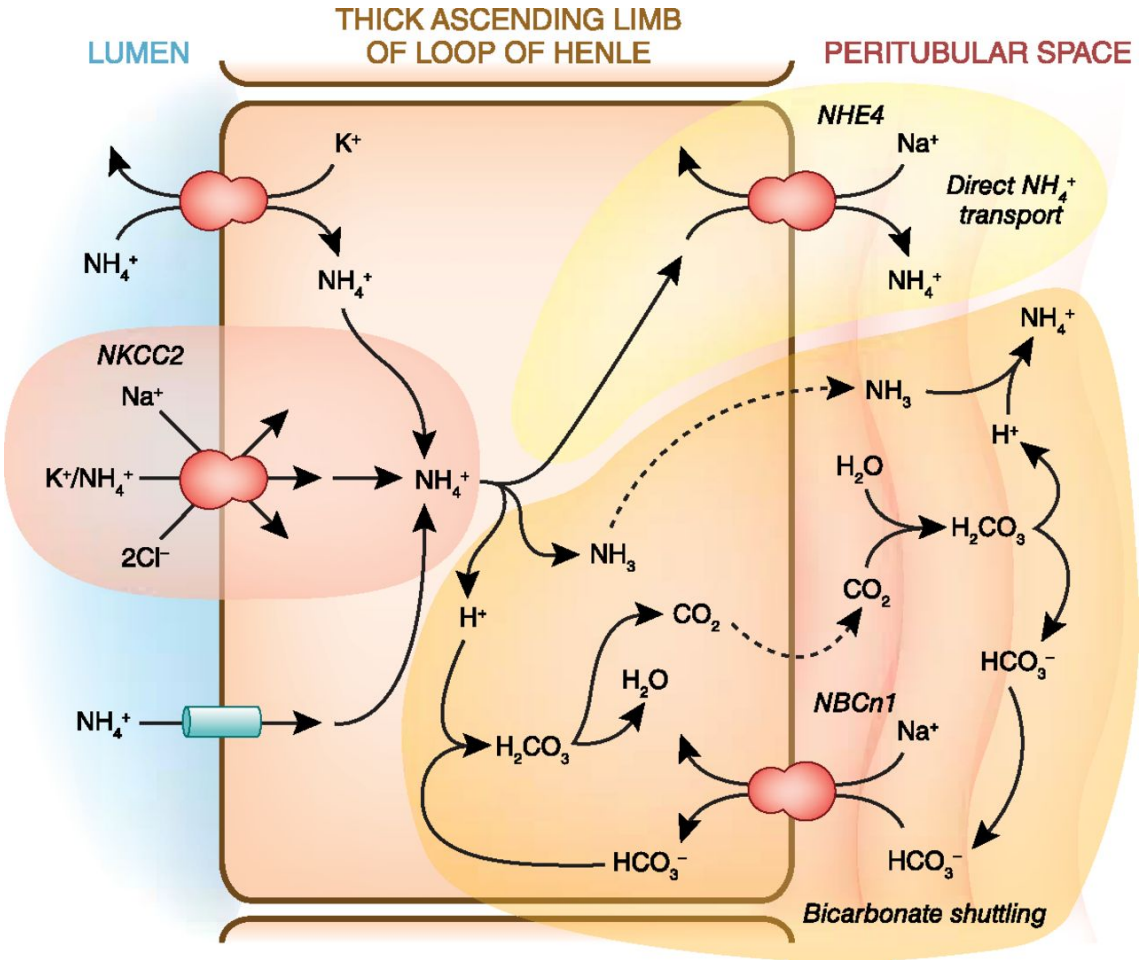
Figure 1.2. Model of proximal ammonia transport



Glutamine is the primary metabolic substrate for ammonia production. Proximal tubular glutamine uptake involves transport across both apical and basolateral membrane. Glutamine metabolism generates two NH_4^+ and two bicarbonate ions. Ammonium secretion across the apical membrane occurs primarily *via* NHE3-mediated Na^+/NH_4^+ exchange, with a lesser contribution by parallel H^+ and NH_3 transport. Reproduced from Weiner et al. 2015.

In contrast to the previous direction of movement, in the thick ascending limb (Figure 1.3), NH_4^+ is reabsorbed via different apical transporters: a NH_4^+ and Potassium antiporter (50) and, most importantly, NKCC2 (Sodium Potassium 2 Chloride cotransporter, *SLC12A1*, a transporter specifically inhibited by loop diuretics) leaving the basolateral membrane on its way to the medullar interstitial space as NH_3 but also via NHE4 (*SLC9A4*) (48).

Figure 1.3. Ammonia reabsorption in the Thick Ascending Limb

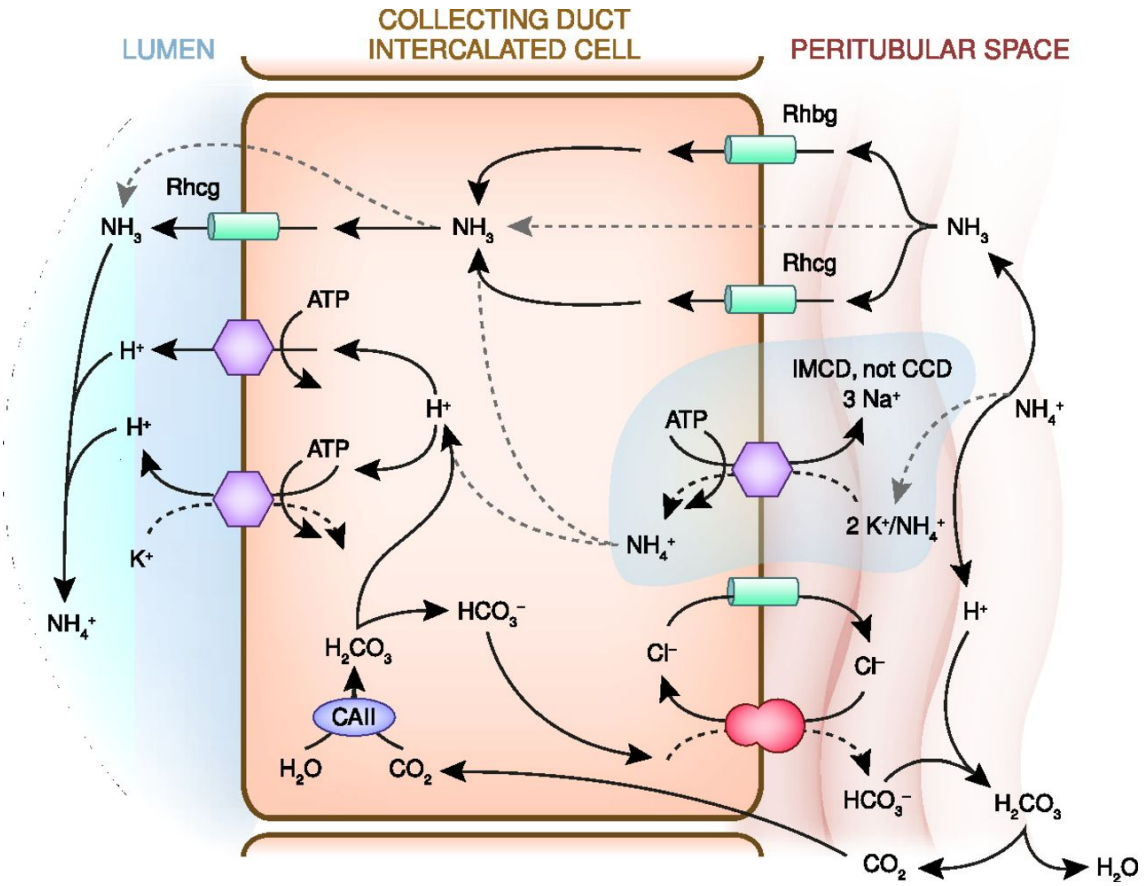


In TAL, ammonium absorption preferably happens through NKCC2 via substitution of NH_4^+ for K^+. Cytoplasmic NH_4^+ is transported across the basolateral membrane either *via* Na^+/NH_4^+ exchange mediated by NHE4 or *via* a bicarbonate shuttling mechanism involving NH_3 transport. Reproduced from Weiner et al. 2015

Finally, in the collecting duct ammonia (Figure 1.4) diffuses into the tubular lumen where it is again "trapped" by binding to H^+. Permeation of the apical membrane is further augmented by parallel transport of H^+ and NH_3 (51).

The transport of NH_3 through the basolateral membrane in this segment is facilitated by the ammonia-specific transporters Rhesus glycoproteins Rhbg and Rhcg and supported by the Na^+/K^+ ATPase. Cytosolic NH_3 is transported across the apical membrane by a combination of Rhcg and diffusive transport while apical H^+ secretion involves both H^+-ATPase and H^+-K^+-ATPase (52).

Figure 1.4. Ammonia handling in the Collecting Duct



Ammonia both diffuses and is transported first through basolateral membrane and final the apical to be lately secreted to tubular lumen where binds with secreted protons also from apical membrane to form ammonium which is later excreted through the urine. Reproduced from Weiner et al. 2015.

The ammonia secretion process is impaired in patients with dRTA (43). Impaired H^+ secretion into the lumen of the distal tubules reduces the formation of NH_4^+ with secondary diffusion of NH3 back into the cell and subsequently into the systemic circulation. Hyperammonaemia has been indeed one of the presenting features in patients with dRTA particularly during severe acidotic episodes (53); and it may be more common than reported, as ammonia levels are not routinely measured in episodes of metabolic acidosis.

From a small selected Japanese cohort of 11 patients with pathogenic variants in genes encoding for subunits of the proton pump, approximately one third (4) were found to have raised blood levels of ammonia at presentation (54).
Further reports on patients with dRTA have documented the same phenomenon in the absence of underline metabolic/hepatic disease and therefore implies a distal tubule defect to excrete the ammonia generated in the proximal tubules as result of glutamine metabolism (55). Urinary measurement of ammonia excretion can aid in the diagnosis of patients with dRTA, however this is not currently a routine test offered by laboratories. Instead, the urinary anion gap is more commonly used to estimate ammonium excretion. Further details on the measurement and estimation of urinary ammonia in dRTA are provided in the diagnosis section.

1.1.2.4. Aetiology/Genetics

Primary dRTA is caused by pathogenic variants in underlying genes and so far 5 different genes have been associated with dRTA: *SLC4A1(56), ATP6V1B1(57), ATP6V0A4(58), FOXI1(59)* and *WDR72* (60). Except for *SLC4A1* which can either have an autosomal dominant or recessive inheritance, all the other genes are inherited in a recessive fashion. There might be an exception for a specific variant (c.1181G>A; p.(Arg394Gln)) in *ATP6B1V1* where several individuals with dRTA from different families and countries have been found to be simple heterozygous carriers (61-63). If tested, these variants were *de novo*, providing an explanation for the absence of a family history. This scenario could be explained by the presence of a dominant negative effect of this variant due to incorrect assembly of the vacuolar ATPase, similar to what has been described in animal models (64). Indeed, a recent report provided strong genetic evidence for such dominant negative effect by showing that the p.(Arg394Gln) variant segregated with the disease in several families (65).

In another report, a single patient has been described with a homozygous variant in a subunit (*ATP6V1C2*) of the H^+ ATPase pump that had not previously been disease associated. Functional studies in yeast suggested a loss of function effect (66). Although this subunit had been previously described as a candidate gene for dRTA, it is important to note that the finding was only documented in a single individual with an unusual phenotype of acidosis and end stage kidney disease with death in infancy.

Moreover, the allele frequency of this variant in Africans is reported to be 0.8%, which is incompatible with the incidence of dRTA: if this variant was pathogenic, one would expect the frequency of dRTA in Africa from this variant alone to be around 1:15,000, or approximately 6 times higher than the usually assumed incidence of dRTA from all genetic causes (67). Thus, until further patients with convincing phenotype and variants are identified, *ATP6V1C2* remains a gene of uncertain significance with respect to dRTA.

1.1.2.4.1. Genotype-phenotype correlation

1.1.2.4.1.1. Sensorineural deafness

Certain extra-renal clinical features can be present depending on the causative gene. For example: the proton pump is not only expressed on the intercalated cells of the distal tubule but also in the inner ear with affected individuals being at risk of developing sensorineural hearing loss (68). *FOXI1* is a transcription factor controlling expression of several genes involved in acid secretion, including subunits of the proton pump and as such it does share a similar phenotype(59).

1.1.2.4.1.2. Haemoglobinopathy

Patients with specific variants in Anion Exchanger 1 (AE1, *SLC4A1*) are typically from Southeast Asian ethnic background and can present with haemolytic anaemia due to abnormal red blood cell morphologies known as ovalocytosis or spherocytosis, as this transporter is also expressed in red cell membranes (see below).

1.1.2.4.1.3. Amelogenesis imperfecta

And finally, individuals with *WDR72* pathogenic mutations have been described to present with Amelogenesis Imperfecta, a congenital enamel defect (69).

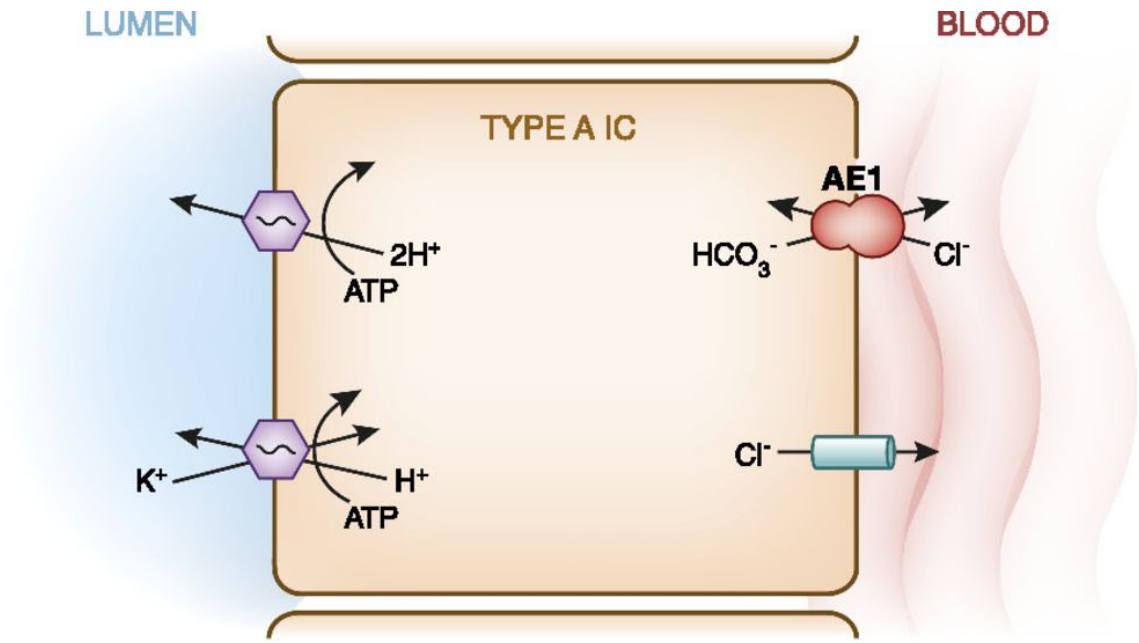
1.1.2.4.2. Molecular aspects of dRTA

1.1.2.4.2.1. SLC4A1

Pathogenic variants in *SLC4A1* (OMIM #109270) affect the structure and/or function of the anion exchanger (AE1), a Chloride/Bicarbonate antiporter (Figure 1.5) located in the basolateral membrane of the α-intercalated cells of the collecting duct and responsible for the transport of bicarbonate from the intracellular space to the systemic

circulation. Retention of bicarbonate in the cytosol will cause an increase in the pH and therefore impairs the ability of the α-intercalated cell to secrete protons to the tubular lumen.

Figure 1.5. Acid-base transport in in the Collecting Duct (Type A intercalated cell)



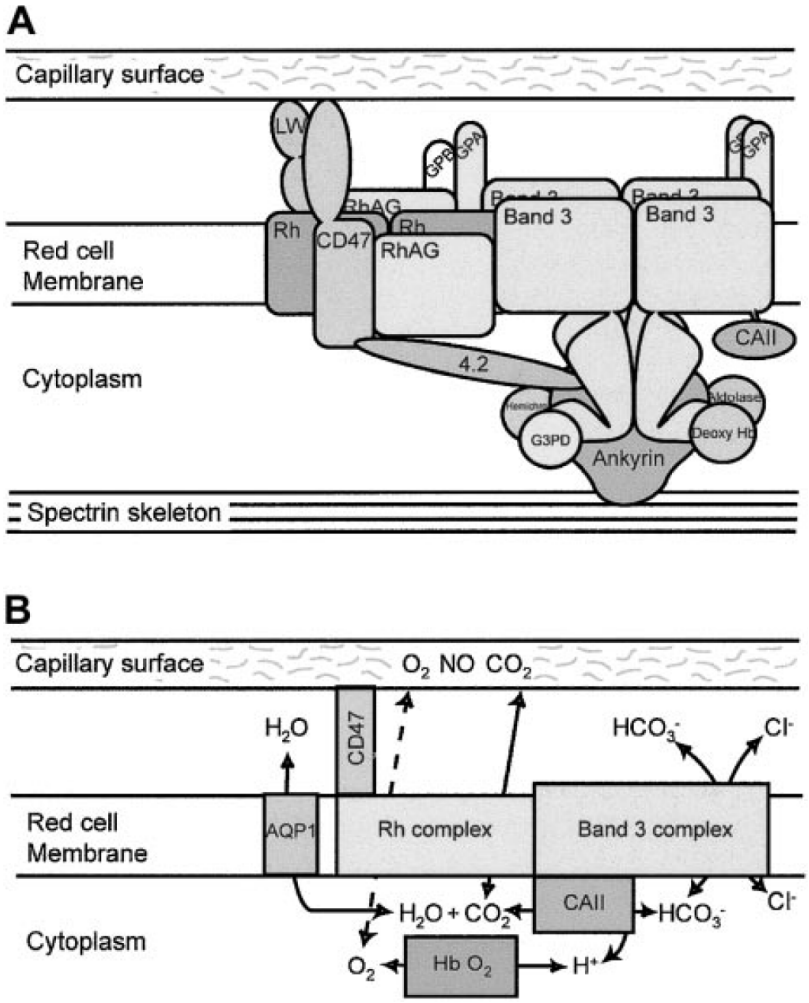
Summary of $H^+$and HCO_3^- transport in the type A intercalated cell (IC) of the collecting tubule. AE1, anion exchanger 1. Reproduced from Lee Hamm et al. 2015

A different AE1 isoform, including an extended N-terminus, is expressed in the membrane of red blood cells where it was first discovered as “band 3 protein” (56, 70). In the red cell, AE1 not only controls intracellular pH but also performs an important role in stabilising the membrane structure. A final macro-complex is formed by tetrameric AE1 in combination with the Rh complex (also in the membrane) and ankyrin which helps to establish a bridge with the cell cytoskeleton (Figure 1.6) (71). Joining in the macro-complex are RhAG (Rh-associated glycoprotein) and AQP1 (Aquaporin 1), which are involved in gas exchange.

AQP1 mediates transmembrane CO_2 transport, while RhAG facilitates NH_3 transport. Dysfunctional AE1 cannot adequately exchange anions and induces a monovalent cation leak from the erythrocyte (72). This event is thermo-sensitive with higher severity in lower temperatures and less intensity in tropical areas (73). In patients who are carriers (simple heterozygous) of a loss-of-function variant in *SLC4A1*, the red

blood cell phenotype is a mild haemolytic anaemia due to destabilisation of the erythrocytes membrane which acquires a morphology known as hereditary spherocytosis or Southeast Asian Ovalocytosis (SAO) (74).

Figure 1.6. Band 3 Macrocomplex



(**A**) Band 3 tetramers attach to the spectrin cytoskeleton through ankyrin. The N-terminal region of band 3 binds deoxy-hemoglobin, hemichromes, glyceraldehyde-3-phosphate dehydrogenase (G3PD), and aldolase. The C-terminal on the other hand binds CAII. (**B**) Proposed gas exchange metabolon in the RBC membrane. Reproduced from Bruce LJ et al. 2003.

Most patients that carry pathogenic variants in *SLC4A1* have either a renal or a haematological phenotype and there are different reasons:

1) variants in the N-terminal fragment of the transmembrane protein not transcribed in the kidney will only affect red cell function. So, if a patient carries a pathogenic variant located in this first segment of the Band-3 protein, there is

no consequence for the kidney isoform and both bicarbonate reabsorption and tubular luminal proton secretion will be intact.

2) Variants affecting intracellular sorting to the basolateral membrane will only affect kidney function, as the red cell is not polarised (75) and expresses chaperone proteins, such as Glycophorin A (76).
3) Variants causing dRTA typically have a dominant negative effect, such as mistargeting or retention of the dimeric AE1 in the ER, whereas variants causing haploinsuffciency are associated with isolated red cell problems (76).

Finally, and rarely seen in clinical practice except for certain tropical areas of South East Asia, some patients can develop dRTA and chronic haemolysis in what constitutes the most severe phenotype with an earlier disease onset (77). This can occur with bi-allelic variants in *SLC4A1* and in these cases, individuals are homozygous or compound heterozygous for loss-of-function variants, most commonly for a deletion of amino acids 400-408. Alternatively, patients can be compound heterozygous for a SAO and a dRTA variant.

The erythrocyte cation leak from SAO variants has been linked to protection from Malaria infection, which would explain why pathogenic variants are more prevalent in tropical areas (78).

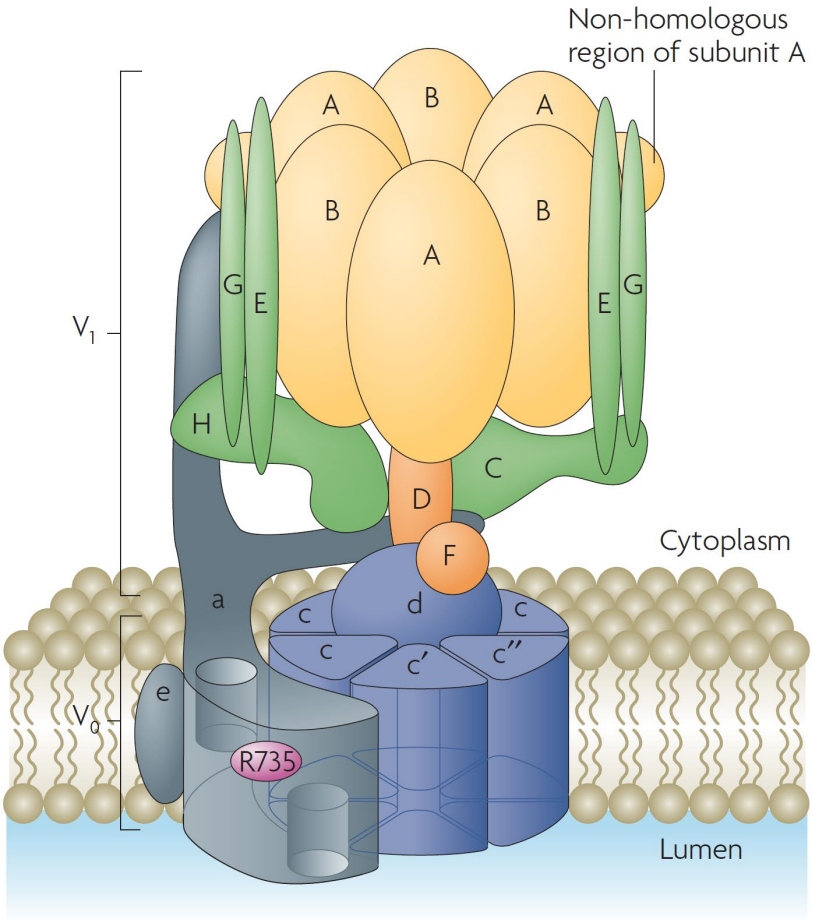
1.1.2.4.2.2. ATP6V1B1 and ATP6V0A4

The other two “classical genes” described with AR dRTA with/without sensorineural hearing loss (SNHL) were *ATP6V1B1* (OMIM #192132) and *ATP6V0A4* (OMIM #605239, formerly called *ATP6N1B*). They both encode for different isoforms of a multi-subunit enzymatic proton pump (vacuolar H^+ ATPase) which has two domains: a cytoplasmic or V1 domain, which hydrolyses ATP and a transmembrane or V0 domain facilitating the actual transmembrane transport of protons H^+, either to intracellular compartments or to the extracellular space (figure 1.7) (79).

Intracellularly, this vacuolar ATPases perform different tasks such as receptor-mediated endocytosis, membrane trafficking, pro-hormone synthesis, protein degradation and neurotransmitter uptake while it can also facilitate entry of various

viruses and toxins. In the plasma membrane they contribute to renal H^+ secretion, bone resorption and sperm maturation (79).

Figure 1.7. Structure of the V-ATPase



The vacuolar (V-)ATPase complex is composed of a peripheral domain (V1, shown in yellow and orange), which is responsible for ATP hydrolysis, and an integral domain (V0, shown in blue and grey), which is involved in proton translocation across the membrane. Reproduced from Forgac et al. (2007).

V1 is the larger domain formed by eight different subunits (A-H) while V0 contains six subunits (a, d, e, c, c′ and c″) (80, 81). Isoforms B1 and a4 encoded by these two classical genes are expressed in the kidney but also in other tissues such as inner ear and epididymis and this explains why patients with pathogenic mutations in these genes will have hearing loss. Of note, male infertility has not been documented in *Atp6v1b1* knock-out animal models nor in human patients with bi-allelic *ATP6V1B1* loss-of-function variants, despite seminal acidification being a key factor for sperm cell conservation in the epididymis (82). In contrast, *FOXI1* knock-out male mice are found to be infertile (83).

ATP6V1B1 was the first recessive disease gene identified in dRTA (57). The researchers at the time investigated a selected group (N=31) of patients with a high prevalence of consanguinity (87%) and a phenotype of dRTA and sensorineural hearing loss (61%). Linkage analysis identified the suspected locus on 2p13. Hearing assessments of patients with dRTA and SNHL showed a bilateral, symmetrical, progressive, and early onset pattern, in some instances associated also with conductive defects. Specific radiological studies also revealed abnormally enlarged vestibular aqueducts; vertigo has been described but appears to be rare (84, 85).

Finding a correlation of mutations in *ATP6V1B1* with dRTA and SNHL suggested to the researchers initially that individuals with dRTA but without SNHL may perhaps have mutations in a different gene and indeed a study focussing on a smaller cohort (N=13) of patients with this phenotype helped identify a second gene (located on chromosome 7q33-34) encoding a subunit of the V0 domain (58). This cohort also had a high prevalence of consanguinity, nephrocalcinosis and rickets with a very early age at presentation but all had normal audiometric studies providing then evidence in favour of this new type of “AR dRTA without SNHL”.

Two years later a new study provided evidence for some patients with mutations in *ATP6V0A4* also having hearing impairment, but with a tendency to a later onset at an age as old as 33 years old (68).

FOXI1 (OMIM #601093), reported for the first time as a dRTA disease gene in humans in 2018 (59), is encoding a forkhead transcription factor crucial in the expression of different proteins including: AE1, AE4, and the V-ATPase subunits B1, a4, A, and E2 (86). Pathogenic variants in *FOXI1* are linked to a severe phenotype of dRTA with early and profound deafness and also male infertility in mice due to its relevant role on keeping an appropriately acid environment within the epididymal lumen. Whether this phenotype will be relevant in humans still unknown as follow-up data for the only male individual reported so far are limited to his childhood (59). Of note, since the initial report in 2018, no further patients with *FOXI1*-associated dRTA have yet been described and therefore, there is currently only limited evidence for this being a

disease gene. If confirmed, variants in *FOXI1* appear to be an exceedingly rare cause of dRTA.

The most recent dRTA disease gene described (*WDR72,* OMIM #613214) was initially identified in several patients with amelogenesis imperfecta (AI)(87). The connection with dRTA was only very recently reported in the literature, illustrating the power of unbiased genetics to provide a completely unexpected discovery (69). The exact mechanism of how *WDR72* mutations are linked to dRTA remain unclear at the present. It is supposed that *WDR72* plays an important role in intracellular sorting of proteins involved in acid-base metabolism (AE1 and the apical H^+ pump) in the same way that it does with NCKX4 and intracellular calcium homeostasis in patients with AI (60).

Why some of these patients manifest either with AI, dRTA or both is still awaiting a clinical answer that further research studies will hopefully manage to reveal. However, “reverse phenotyping” in one report showed that all individuals with dRTA and variants in *WDR72* also had AI. Similarly, in some families with AI, affected individuals may or may not have overt acidosis, suggesting that dRTA phenotype can be mild or “incomplete” (see below).

Other candidate genes, not identified as dRTA disease genes in humans yet, may include the K/Cl cotransporter KCC4 (*SLC12A7*), the Cl/HCO3 exchanger *SLC26A7*, the ammonia channel RhCG (*SLC42A3*), the hensin (*DMBT1*) CXCL12 signal complex, or other H+ ATPase subunits (88).

1.1.2.5. Secondary dRTA

Secondary Causes of dRTA (89):

1. Autoimmune disease, especially Sjögren’s syndrome, but also systemic lupus erythematosus, rheumatoid arthritis, autoimmune thyroid disease and primary biliary cirrhosis.
2. Chronic kidney disease
3. Toxins and drugs, such as amphotericin B, lithium, toluene, amiloride, trimethoprim, pentamidine, vanadium.

4. Nephrocalcinosis (which can cause, as well as be caused by dRTA, presumably by mechanical disruption of the cortical collecting tubule). Primary hyperparathyroidism, hypercalcemic hyperthyroidism, vitamin D intoxication, idiopathic hypercalciuria with nephrocalcinosis, familial hypomagenesemia-hypercalciuria with nephrocalcinosis.
5. Dysproteinemic syndromes. Hypergammaglobulinemia, cryoglobulinemia and amyloidosis.
6. Sickle cell disease
7. Renal transplantation, either a toxic effect of calcineurin inhibitors or rejection.
8. Chronic urinary tract obstruction
9. Hyponatriuric states: liver cirrhosis and nephrotic syndrome

1.1.2.6. Incomplete dRTA

Incomplete dRTA refers to a disorder characterised by an impaired renal tubular acidification (as in dRTA) without overt systemic acidosis that is generally identified during the investigations for recurrent urinary stone disease. It was first reported in 1959 by Wrong and Davies while studying urine acidification in a large cohort of 68 individuals with the classic method of oral ammonium chloride administration (5). Although in the initial report the 3 identified patients with this condition did have nephrocalcinosis but no urolithiasis, subsequent studies have been highlighting also the high prevalence of recurrent stone disease in these individuals (90, 91).

Accurate estimations of the incomplete dRTA prevalence in general population are not possible to be established for several reasons including the lack of overt metabolic acidosis in normal situations plus the need of an acidification test for confirmation, a test with many associated difficulties, including nausea, vomiting, abdominal pain among others (92). However, there is available data published in selected population such as stone formers, where the prevalence varies from as little as 2% to as high as 19% (91, 93, 94).

It is not very clear why some patients despite a tubular acidification defect do not present with overt systemic acidosis as others with dRTA, but possible explanations include sufficient buffering produced by the skeleton via bone resorption and phosphate release. Indeed mineral bone disease and growth failure are both common

features of children with incomplete dRTA and alkali supplementation has been shown to reverse this effect in childhood (95). In adults incomplete dRTA has been associated with low bone mineral density ("osteoporosis") and risk of fractures in up to 20% of the studied individuals (96, 97).

The causes of incomplete dRTA may be the same as for the complete form, and some clinicians consider incomplete and complete dRTA as part of a continuum, where the former is preceding the latter or alternatively the expression of a milder phenotype. Different case reports in children support this hypothesis, particularly in patients with AD dRTA due to variants in AE1, where incomplete dRTA during childhood is followed by systemic acidosis in later years (9, 98). Heterozygous carriers of mutations in AR genes have also been associated with incomplete dRTA in families and also in animal models, perhaps denoting some degree of haploinsufficiency (99, 100). Incomplete dRTA has also been described in patients with radiological imaging compatible with medullary sponge kidney (101), Sjögren syndrome (102), non-specific nephrocalcinosis (103) and drug toxicity (92).

Patients with incomplete dRTA have a high risk of stone formation/nephrocalcinosis and share the same urinary metabolic profile (hypocitraturia and more alkaline urine) and stone composition is therefore similar as in those with complete dRTA (>95% of carbonate apatite in occasions) (104). Consistent with the notion of incomplete dRTA being a milder form of complete dRTA, hypercalciuria is only variably associated with incomplete dRTA (105).

The short ammonium chloride test (oral administration of 0.1 g/kg NH_4Cl) as protocolised by Wrong and Davies in the 1950's is still considered the standard diagnostic method to reveal incomplete dRTA, however, its considerably high rate of adverse effects (mainly gastrointestinal) makes its performance quite challenging in clinical practice (5).

Other alternatives like the F+F test (furosemide and fludrocortisone) seem to be better tolerated and provide an alternative tubular acidification assessment to the ammonium chloride test (106). In patients with recurrent urolithiasis, the F+F test has been reported to have a sensitivity of 85% and a specificity of 77%, compared to the short

ammonium chloride test (107). However, the positive predictive value was only about 30%, whereas the negative predictive value was 98%, using the ammonium chloride loading test as a gold standard (107). Thus, a positive F+F test may need to be confirmed by a formal ammonium chloride loading test.

As with the full form, the treatment of patients with incomplete dRTA and recurrent stone formation is alkali supplementation. For obvious reasons including a low prevalence of the disease in the general population, large-randomised control trials assessing treatment are lacking. Nevertheless, smaller cohort studies have been published in the medical literature. For example: citrate supplementation has been shown to improve bone health in longitudinal studies, while alkali therapy in paediatric population did have a positive impact on height SDS in both complete and incomplete dRTA cases (95, 108). Although potassium citrate is the most common salt prescribed in clinical practice, as it does help with both alkali and potassium replacement, bicarbonate salts are widely prescribed as well, particularly when gastrointestinal symptoms are present during citrate treatment. Sodium based salts are sometimes avoided by physicians due to a theoretical risk of increased calciuria, however there are no clinical data to support this (61).

1.1.2.7. Diagnosis

1.1.2.7.1. History and examination

In the evaluation of a patient with suspected dRTA as with other disorders, a clinical history and thorough examination are the essential first steps. A positive family history and its pattern of inheritance can provide the first clues about the underline genetic disorder. For example, in case of suspected AD disease, the most likely underlying gene will be *SLC4A1*. In contrast, the presence of sensorineural hearing loss suggests one of the H^+ pump subunits or the transcription factor *FOXI1* as underlying cause. Finally, enamel disorders can point towards a diagnosis of amelogenesis imperfecta with dRTA (*WDR72*). Early presentation, particularly in the first decade of life is very suggestive of an inherited form of dRTA, although up to 10% of patients may only be diagnosed in adult life.

Failure to thrive and rickets have been described in the paediatric age while mineral bone disease and increased risk of fractures can be frequently seen in adult cases (28).

1.1.2.7.2. Imaging

Medullary nephrocalcinosis is almost invariably present in patients and urolithiasis, although more prevalent in adulthood, can also present in early ages (61).

1.1.2.7.3. Biochemistries: blood

Blood tests can show haemolytic anaemia with dysmorphic erythrocytes particularly in patients from South East Asia and variable degrees of hypokalaemia, apart from the classical normal anion gap/hyperchloremic metabolic acidosis with an inappropriately non-acidic urine (consistent pH >5.3) described as a by Wrong and Davies in 1959 (5).

1.1.2.7.4. Biochemistries: urine

1.1.2.7.4.1. (Urine Anion Gap or UAG)

UAG=Na^++K^+-Cl^- (measured in urine), normal <0 with systemic acidosis (109). In the urine, H^+ are mostly buffered by NH_3 to form NH_4^+, hence a physiological (normal urinary acidification ability) response to systemic acidosis implies a significant increment in the concentration of NH_4^+ while a low concentration NH_4^+ indicates a defect in distal acidification. Ideally NH_4^+ should be directly measured, however in clinical practice this is rarely performed. Alternatively, urinary NH_4^+ can be indirectly estimated via the urine anion gap (UAG).

In a parallel way to how anion gap is calculated in plasma, an equivalent approach can be followed in the urine, however in this fluid Na^+, K^+ and Cl^- are the main ionic components. In contrast to the plasma anion gap, the UAG is usually negative due to an excess of unmeasured cations (in particular NH_4^+). For this reason, it has also received the name of “urine cation gap”.

In dRTA, as the ability to excrete protons is compromised, a low NH_4^+ concentration is expected, thus, the cation gap is absent and the UAG has a positive value (reviewed

in (109)). Calculation of the UAG can therefore be used as an indirect assessment of distal urinary acidification, with a positive UAG indicating dRTA.

Important factors to consider when calculating and interpreting the UAG calculation are:

1. It is only meaningful in patients with metabolic acidosis.
2. An increased UAG can be the physiological response to an alkali load.
3. Interpretations in advanced kidney disease should be done cautiously (110).
4. A positive UAG does not necessarily means increased presence of NH_4^+, other unmeasured cations could be present in excess as well, such as ketoacids, salicylates or penicillin (111).
5. Ammonium excretion may be preserved in incomplete dRTA (112).

When assessing urine and serum samples of patients with urinary acidification defects some difficulties arise when they present without overt acidosis (as an elevated urine pH does not seem to be inappropriate), i.e. with incomplete dRTA (see above). To confirm a suspicion of impaired urinary acidification, an acid loading test can be performed. Acidification tests with either hydrochloric acid (HCl) or ammonium chloride (NH_4Cl) were already proposed for at least 30 years before Wrong and Davies described their method, however their performance was very tedious and required sometimes up to 5 days of study, making the short NH_4Cl an easier and more practical test that subsequently became the gold standard in diagnosis (3, 5).

1.1.2.7.4.2. The short ammonium chloride test

The initial protocol was based on a single and slow (about 1 hour) oral administration of 100 mg/kg ammonium chloride. Tolerance to the test tended to be quite challenging due to early development of nausea and vomiting which was making some patients quit before the entire procedure could be finished. Alternative doses of 75mg/m^2 in infants diluted and administrated via nasogastric tube have been described (113). The entire test takes about 6-8 hours to be completed and includes a baseline serum sample and a control 2-4 hours post NH_4Cl is given to confirm that metabolic acidosis has been induced (tCO_2 <21.0 or 18.0mmol/L in infants) plus serial urines. Individuals with normal urinary acidification capacity can drop urine pH below 5.3 and generally between 4.6-5.0. Urinary NH_4 if measured is expected to rise.

A prolonged test using a lower dose (50 mg/kg/d), but repeated over 3 days to improve tolerance, has also been described (114).

1.1.2.7.4.3. Sodium sulphate testing

First described as a test to evaluate urinary acidification in 1955 (115) its concept was based on the idea that under circumstances where sodium reabsorption is stimulated in the distal tubule the administration of a neutral salt (pH = 7.0 in solution) containing sodium with a non or poorly reabsorbed anion such as sulphate will generate an exchange between Na and H^+ and due to sulphate being a weak base the net effect on urinary pH will be towards acidification. This is indeed an indirect mechanism and crucially different from the classic acid load challenge. Urine pH usually drops as low as 4.0 meaning that potentially it can drop urinary pH further than what systemic acidosis could do physiologically. Therefore, this uncertainty and the inconvenience of needing intravenous administration has made it a very rarely performed test in clinical practice.

1.1.2.7.4.4. Urinary pCO2

The use of pCO2 in the assessment of urinary acidification was first protocolised in 1974 (116). The rationale behind it was that urinary pCO_2 should be greater in urine than in blood in normal individuals while in patients with impaired acidification this difference tends to minimise. Unfortunately, low urinary pCO2 can be found in both proximal renal tubular acidosis and advanced chronic kidney disease therefore specificity is not adequate (117, 118).

1.1.2.7.4.5. Furosemide testing

Based on a similar principle to the sodium thiosulfate test, it was hypothesised by different authors during the 80's that the administration of loop diuretics like furosemide would increase distal sodium delivery and reabsorption in this segment with secondary generation of a negative transmembrane voltage which will favour H^+ to the tubular lumen, hence generating a more acid urine (119-121).

The usual furosemide dose is 1 mg/kg and is administered by either intravenous or oral route with urine samples being collected during a period of 4 hours. Urinary pH in

normal individuals should drop below 5.3 whereas urine NH_4^+ and potassium excretion increase 2–3 times with respect to baseline values (114, 121).

1.1.2.7.4.6. Furosemide + Fludrocortisone testing

While the furosemide test was easier to perform in clinical practice than the sodium sulphate test, later experiences showed it not to be reliable. Exogenous administration of mineralocorticoids can increase the acidification ability of the distal tubule (120). As aldosterone takes between minutes and less than 1 hour to potentiate sodium reabsorption (through ENaC) in the distal nephron, the protocol for adults recommends simultaneous administration of furosemide (40mg) and fludrocortisone (1mg). Performance is better tolerated by the patients with no gastric irritation and acidification capacity is equivalent to the ammonium chloride test, but significantly quicker (106).

1.1.2.8. Treatment

The principle for therapeutic management of dRTA is that in order to compensate the daily acid load generated by the diet and in case of growing children their more active bone metabolism, alkali supplementation should be administered and the dose titrated according to signs of adequate disease control (hypercalciuria and metabolic acidosis).

When the decision to treat is made there are multiple factors to consider:

1. Type of alkali (bicarbonate and/or citrate)
2. Type of salt (sodium, potassium but also other alternatives as calcium or magnesium are available)
3. Doses (generally requirements are higher at youngest ages)
4. Formulation (liquid, tablets, short vs prolonged released).

In general, citrate forms are more frequently prescribed. At the present there is a lack of evidence to support this practice and in fact previous studies looking at the effect on urinary composition have failed to show a more favourable impact of citrate supplementation against bicarbonate sodium and potassium salt (122, 123). Available evidence has shown no differences in metabolic control between patients taking sodium bicarbonate and sodium/potassium citrate. However, as dRTA tends to

present with hypocitraturia and this seems to be one of the key risk factors for stone formation most clinicians still prescribe it with more frequency.

Another factor to consider when choosing between both types of alkali is the gastrointestinal tolerance. With short release formulations of citrate patients tend to complain of dyspepsia and general discomfort while bicarbonate tends to increase gas production in the stomach. Recent trials on a new combined formulation of bicarbonate and citrate delivered in prolong release granules have shown a better gastrointestinal tolerance compared to standard treatment and have also allowed to delivered higher alkali dose with only two administrations per day (124, 125).

Citrate may have potentially another advantage against bicarbonate as it requires metabolization in the liver (citric acid cycle) with the subsequent delayed alkali release into the systemic circulation perhaps providing a longer and steadier alkalinisation. Whether a combination of both citrate and bicarbonate has a better long-term profile than citrate or bicarbonate alone remains unknown at the moment and future studies still need to be performed.

Prescription of Calcium or Magnesium salts are anecdotal. In practice, potassium and sodium salts are most commonly prescribed and particularly potassium has the extra benefit of providing treatment for concomitant hypokalaemia. Very commonly clinicians prefer the use of the potassium salts due to the usual presence of hypercalciuria in dRTA and the theoretical risk of urinary tract calcification including nephrocalcinosis and urolithiasis, as extra sodium intake has been associated with increased urinary calcium excretion. However, in dRTA, hypercalciuria is more likely to be related to systemic acidosis with increased calcium release from the skeleton rather than secondary to provided sodium with therapy and our recent study (discussed in Results) did not show significant differences between individuals taking potassium vs sodium salts (61). Variability across the world but even regions of the same country is high and indeed in our study, at least 30 different types of formulations were identified.

In clinical practice, pragmatic considerations, such as local availability, affordability and palatability are most important to support effective treatment and compliance. If a

Na^+-containing formulation is used, separate K^+ supplementation may be necessary to maintain normal plasma levels.

Dosage and frequency of alkali supplementation depend on multiple factors, but generally the age of the patients does have the biggest impact, with little children having higher doses prescribed, which can reach 8-10mEq/kg/day while older children and adults may achieve control with average doses around 2-3 mEq/kg/day (9, 126). The total daily dose needed to control acidosis can vary considerably between individuals. Factors contributing to this variability include the underlying molecular basis, as well as diet (see below).

As the acid load occurs throughout the day, the ideal administration of alkali supplementation should be several times a day to provide consistent control. Moreover, infrequent high doses generate transient alkalosis, which, if the bicarbonate level in the blood exceeds the proximal tubular threshold for reabsorption leads to urinary losses with consequent loss of buffering capacity.

In infants, who are fed very frequently and as much as every 2-3 hours it is indeed a recommended practice to split the total dose in small amounts administered with each feed, helping not only to keep a more sustained therapy but also minimising the risk of aversion/side effects. In older children, less frequent dosing (3-4 times daily) is likely to help compliance and allows uninterrupted sleep. With the new extended release formulation, 2 daily administrations have been found to be safe and effective with potentially better compliance (125).

1.2. PROJECT 1. LONG-TERM OUTCOME IN PRIMARY DISTAL RENAL TUBULAR ACIDOSIS. AN INTERNATIONAL COLLABORATION.

1.2.1. BACKGROUND AND OBJECTIVES

As a very rare condition with an estimated prevalence of < 1:100.000, gathering and presenting large informative data for the better understanding of distal renal tubular acidosis has been a challenge. Nevertheless, in the most recent years there has been a growing interest, among groups focused on the study of rare tubular disorders, in reporting on different aspects of this condition. Either large tertiary centres on their own (9, 127) or in national collaborations various cohorts have been presented with as many as 89 patients with primary distal renal tubular acidosis (128). These large efforts have helped considerably to expand knowledge not only in relation to its phenotype spectrum but also on genotype, providing multiple new mutations in both previously described causative genes as well as in the two new documented genes (*FOXI-1* and *WDR72*) (59, 69, 128).

Despite all above-mentioned recent efforts, there are still multiple clinical questions that remained unanswered regarding primary distal renal tubular acidosis:

1. Epidemiological data is still very vague.
2. Presentation in patients with pathogenic mutations in genes encoding proton pump subunits (*ATP6V0A4* and *ATP6V1B1*) has been reported as considerably earlier compared to those in the anion exchanger (*SLC4A1*). However, how much earlier and if there are significant differences between those with impaired synthesis/function of subunits a4 vs B1 is not yet fully known.
3. Could pathogenic mutations in specific genes have a different impact on growth or kidney function?
4. Does alkali supplementation with sodium containing salts increases the risk of developing hypercalciuria compared to potassium salts?
5. Is alkaline supplementation with citrate offering a better metabolic control compared to bicarbonate salts?
6. Are there differences in the degree of metabolic acidosis according to genotype?
7. Could age impact on daily alkali requirement, and if it could by how much?

8. Could a better metabolic control have a positive effect on long-term growth as a surrogate marker of bone health but also on kidney function?
9. Could differences in countries' health care resources have a potential impact on long-term outcome in dRTA?
10. Is there any significant difference in prevalence of nephrocalcinosis and/or urolithiasis according to genotype?
11. What is the prevalence of hearing loss and the severity degree among patients with primary distal renal tubular acidosis?

It was clear to me when confronting these questions that unless a larger scale study could be put in place most of them will continue to remain unsolved for a long period of time. Therefore, at this point and with the support of the ERA-EDTA (European Renal Association – European Dialysis and Transplant Association) and the ESPN (European Society for Paediatric Nephrology) Working Groups for Inherited Kidney Diseases plus the European Rare Kidney Disease Network (ERKnet) the decision to design an international collaborative study for the study of primary distal renal tubular acidosis was taken. Further details on how this project was developed are explained in the methods section of this chapter.

In summary, and with the support of multiple collaborators, this project reports on clinical and genetic features in a multinational cohort of 331 patients with inherited dRTA. The published manuscript (61) did provide important data that directly informs the clinical management of affected patients based on the underlying gene, such as age of onset, age-specific doses for alkali supplementation required and prevalence of complications, such as nephrocalcinosis, nephrolithiasis, chronic kidney disease and sensorineural hearing loss. Most importantly, it has been the first study on this condition to report on the prevalence of adequate metabolic control and its association with long term kidney function and growth. Finally, correlations between economic status of the different countries and their patients long term outcomes are also investigated, questioning whether resource availability might be a relevant factor in relation to the prognosis of this rare disease.

1.2.2. METHODS:

1.2.2.1. Designing the study

Initially a group of international experts in distal renal tubular acidosis was created for the design of a questionnaire with multiple key questions about epidemiological, clinical, biochemical and prognostic features of patients with diagnosis of primary dRTA. With the support from ERKNet and through the online platform SurveyMonkey® (www.surveymonkey.com) an online questionnaire was created. Subsequently, electronic correspondence was sent to the membership of ERA-EDTA and ESPN sharing an invitation (for adult and paediatric nephrologists) to include anonymised data on their patients with a confirmed diagnosis of primary dRTA. The online platform was open for data inclusion from the 6th to the 31st of August 2017 and it could be accessed directly via a link provided in the email.

1.2.2.2. Clinical data

Required data was collected by answering a total of 28 questions (Table 1.1), focused on patients' retrospective clinical history review for demographics, kidney function, dRTA related comorbidities such as nephrocalcinosis, urolithiasis and hearing loss and clinical management.

Considering the potential risks for inaccurate/incomplete information to be included in data analysis a direct communication was established between myself and the corresponding clinicians making sure that not only outlier results and missing information, but the entire database was double checked and confirmed. Only patients with 1) no more than 5 missed questions and 2) confirmed information from submitting clinician were included for final data analysis.

Table 1.1. Data points collected

Renal Unit details	**Laboratory**
Treating physician	Serum creatinine (µmol/L)
Email address	Serum bicarbonate (mmol/L)
Centre – City	Urinary calcium/creatinine (mmol/mmol)
Country	**Treatment (mmols/day)**
ERK-Net centre (y/n)	Bicarbonate (sodium +/- potassium)
Demographic data	Citrate (sodium +/- potassium)
Patient ID	Others
Gender (F/M)	**Comorbidities**
Age at presentation (years)	Nephrocalcinosis (y/n)
Genetic information	Age at diagnosis (years)
Gene	Urolithiasis (y/n)
Mutation details	Age lithiasis was first diagnosed (years)
Auxiometry	Hearing loss (y/n)
Current age (years)	Hearing aids prescribed (y/n)
Current height (cm)	Age at prescription (years)
Current weight (kg)	Cochlear implantation (y/n)
	Age when implanted (years)

1.2.2.3. Genotype-phenotype correlation analysis

To investigate potential associations between reported genotypes and phenotypes, the studied population was classified in 4 different groups according to the available genetic information, these groups were: 1. *ATP6V1B1*, 2. *ATP6V0A4*, 3. *SLC4A1* and 4. *Unknown*. While the first 3 groups included cases with confirmed genetic mutation in one of the three classical known genes, the fourth group was heterogenous and included patients with non-completed/non-performed genetic screening but also those who despite having a full genetic screening were not found to carry causative mutations in the classically reported 3 genes (as in groups 1-3). Considering the physiological differences between the anion exchanger 1 (encoded by *SLC4A1*, mostly autosomal dominant inheritance) and the proton pump subunits (*ATP6V1B1*

and *ATP6V0A4,* all autosomal recessive) mutations, further genotype-phenotype analysis was carried on among these individuals with proved pathogenic mutations.

During the initial period for data gathering, the most recently reported dRTA associated genes *FOXI1* and *WDR72* were not discovered yet, hence this study could not take them into consideration for analysis. Considering that specific genetic information was reported by the respective clinicians according to their best knowledge in addition to some results possibly been originated from research projects with no confirmation in a clinical laboratory; a screening strategy was developed to review all suggested causative mutations. Mutation details, as provided, were reviewed to ensure they accorded to American College of Medical Genetics and Genomics' standard (129).

1.2.2.4. Growth

Patients height (reported in cm) and weight (reported in kg) underwent normalisation to finally be reported as SDS (Standard Deviation Score), in agreement with criteria set by World Health Organisation (WHO) for children (130, 131) or the 2000 Centre for Disease Control for adults (132). Adequate height was defined as a calculated SDS ≥ -2.00 while for weight it was defined by an interval between -2.00 and 2.00. These measurements, timewise, were recorded at the last clinical appointment. According to international WHO BMI Classification criteria, prevalence data on Obesity of children and adults from this cohort was compared to large European epidemiological reference populations (Feel4Diabetes study and EU28-eurostat) (19).

1.2.2.5. Kidney function

Kidney function assessment was performed via estimated glomerular filtration rate (eGFR). For adult patients (age ≥ 20 years old) the MDRD equation was used [175 × Serum Creatinine$^{-1.154}$ × age$^{-0.203}$ × 1.212 (if patient is black) × 0.742 (if female)]. No data on ethnicity was available, according to demographics in the participating countries and only a small number of black patients were expected to be included in this cohort, therefore no correction was performed (133) for black individuals. More importantly it is appreciated that following international concerns on unequal access to medical care for black patients, since 2021 the new NICE guideline on management of chronic kidney disease has recommended stopping this specific correction (134). It is worth mentioning that this MDRD formula is used with units of creatinine of mg/dL,

considering this study measured creatinine in µmol/L, the reported results had to be divided by a factor = 88.4 for conversion to mg/dL.

For the rest of the cohort (children and young adults), the formula used instead was the modified "Schwartz" [36.5 × height (cm) / serum creatinine (µmol/L)] (135). Only individuals with age 2-20 years were included in this group, this was due to the physiological ascend of kidney function in early life which peaks and stabilise approximately at 1-2 years of age (136). As per KDIGO Guidelines recommendations, CKD was classified in stage 1-5 and defined in this population by either a history of chronic tubular disorder and/or abnormal radiological imaging of the kidney; no data on urinary sediment abnormalities, proteinuria or histopathology was collected in this project (137).

To compare the burden of CKD in this cohort of patients with dRTA with the general population, data from a large population group (3rd National Health and Nutrition Survey, NHANES III) was used as a reference (138). For equivalent matching of age groups, 20 years intervals were used (20-40 and 40-60 years, N=61) when assessing CKD prevalence. This dRTA cohort only had 2 patients older than 60 years, hence this age group was not included in the CKD prevalence's analysis.

1.2.2.6. Metabolic control

Considering that therapeutic targets in clinical practice are 1) correction of metabolic acidosis and 2) normalised urinary calcium excretion; a satisfactory metabolic control was defined as a plasma or serum bicarbonate level ≥22.0mmol/L in the absence of hypercalciuria. All bicarbonate results in this study were considered to come from serum samples in order to simplify calculations, whether it came from serum, plasma or calculated from blood gas analysis was not taken into consideration. While published normal ranges define the lower limit of normal as 20 mmol/l in young children, increasing to 22.0mmol/L during adolescence, we arbitrarily decided to use 22.0 mmol/L in both children and adults to simplify assessment.

Hypercalciuria was here defined as a urine calcium/creatinine ratio result above upper limit of normality for age range. The specific cut-off values were: <1 year: 2.2 mmol/mmol; 1-2 years: 1.5 mmol/mmol; 2-3 years: 1.4 mmol/mmol; 3-5 years: 1.1

mmol/mmol; 5-7 years: 0.8 mmol/mmol; 7-17 years (and adults): 0.7 mmol/mmol (139). No 24-hour urine samples were recorded in this project.

1.2.2.7. Nephrocalcinosis and Nephrolithiasis

Nephrocalcinosis and nephrolithiasis were reported as binary categorical variables (yes/no). There was also specific information regarding age at diagnosis for both comorbidities. Meaningful analysis was not possible regarding lithiasis as only few patients had their age of first diagnose reported. Only past or current events of lithiasis were recorded, there was no specification on number, size, location within the urinary tract neither stone composition.

1.2.2.8. Gross Domestic Product per capita

Patients were also classified in three different groups according to the Gross Domestic Product (GDP) per capita of their country of residence, the specific information was extracted from The World Bank (140). Countries with GDP per capita >$35.000 were classified as “high GDP”, $10.000 and 35.000 as “medium GDP” and <$10.000 as “low GDP”. Table 1.2. collects exact information regarding countries and their GDP.

1.2.2.9. Statistics

Analysis was done in IBM SPSS Statistics for windows version 24.0 (Armonk, NY: IBM Corp.). Kolmogorov-Smirnov test was performed to assess normality of the data. Data following a normal distribution was expressed as mean (± Standard Deviation [SD]) and non-normally distributed data as median (range or Interquartile Range [IQR]). Statistical significance for categorical binary variables was analysed through the Pearson Chi-square test. The Student-t, as a parametric test, was employed when comparing means between two groups with normal distribution of data, when doing so with three or more different groups the choice was the one-way ANOVA test. When studying medians, two different non-parametric tests were used: 1) Mann-Whitney U-test for binary and 2) Kruskal-Wallis test for other nominal variables. A p-value of < 0.05 was used to define a statistical association between certain variables as significant.

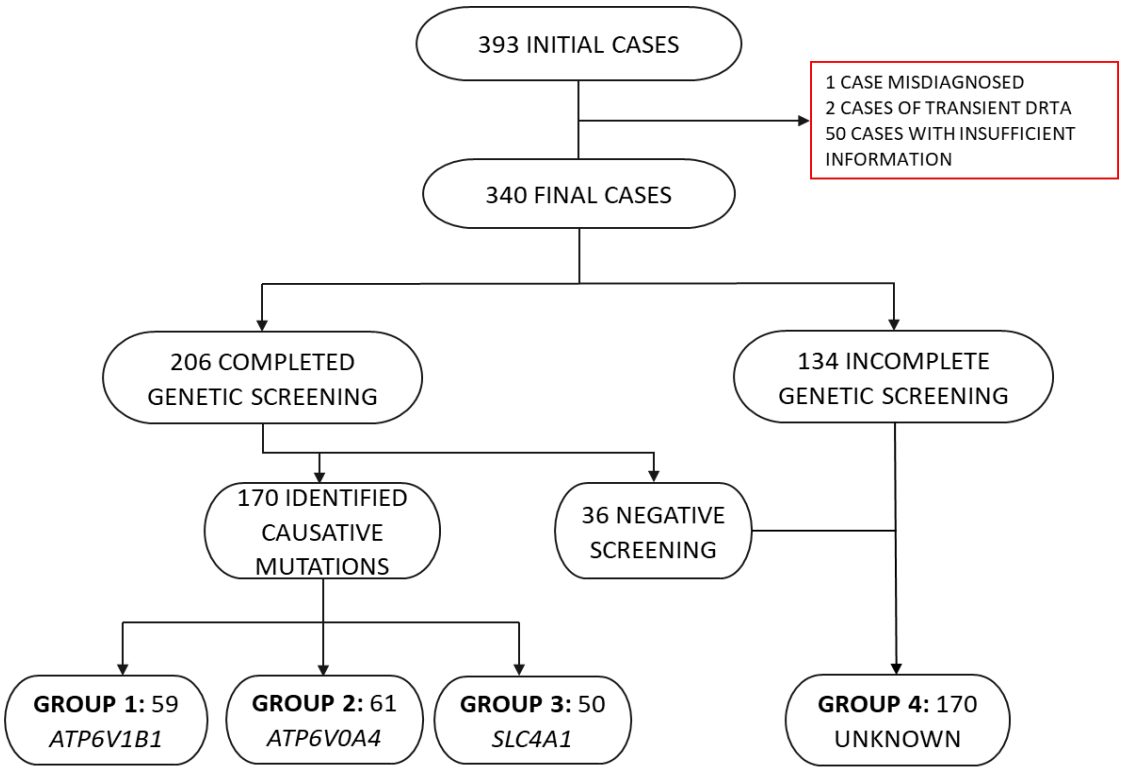
Table 1.2. GDP per capita 2016

Country Name	GDP per capita (US$)	GPD (low/medium/high)
India	1709.59	LOW
Egypt, Arab Rep.	3477.85	LOW
Kosovo	3661.43	LOW
Belarus	4989.43	LOW
Iran, Islamic Rep.	5219.11	LOW
Macedonia, FYR	5237.15	LOW
South Africa	5274.55	LOW
Serbia	5426.20	LOW
Montenegro	7028.93	LOW
Russian Federation	8748.37	LOW
Turkey	10862.60	MEDIUM
Croatia	12149.19	MEDIUM
Poland	12414.10	MEDIUM
Lithuania	14912.69	MEDIUM
Greece	17890.57	MEDIUM
Portugal	19871.72	MEDIUM
Saudi Arabia	20028.65	MEDIUM
Spain	26616.49	MEDIUM
Italy	30668.98	MEDIUM
France	36857.12	HIGH
Israel	37180.53	HIGH
United Kingdom	40412.03	HIGH
Belgium	41271.48	HIGH
Germany	42161.32	HIGH
Netherlands	45637.89	HIGH
Australia	49755.32	HIGH
Sweden	51844.76	HIGH
Denmark	53578.76	HIGH
Switzerland	79887.52	HIGH

1.2.3. RESULTS:

1.2.3.1. Responses and demographic data:

Figure 1.8. The patient cohort.



Patients were grouped for analysis according to underlying gene. Patients without genetic testing or with no causative mutations in the tested 3 genes were classified as "*Unknown*".

A total of 340 cases (from 29 countries) were included in final analysis. From these, 201 patients were from seven western European countries within the top 10 contributing countries. An overview of the cohort distribution according to their genetic group classification is given in figure 1.8, including details from all excluded cases (n=53) in final analysis. Main reason for exclusion was insufficient clinical information available and only three were not included due to not meeting criteria for primary distal renal tubular acidosis.

There was no significant difference on gender distribution with 177 (52%) females and 163 (48%) males. At the time of last follow up, the median age (range) was 11.0 (0-70) years, the number of adults (≥18 years old) were 83 (24%). Furthermore, the adult group was classified in several subgroups according to age ranges of 20 years, with

a majority included within the interval of 20 to 60 years. The exact number of individuals per age group was:

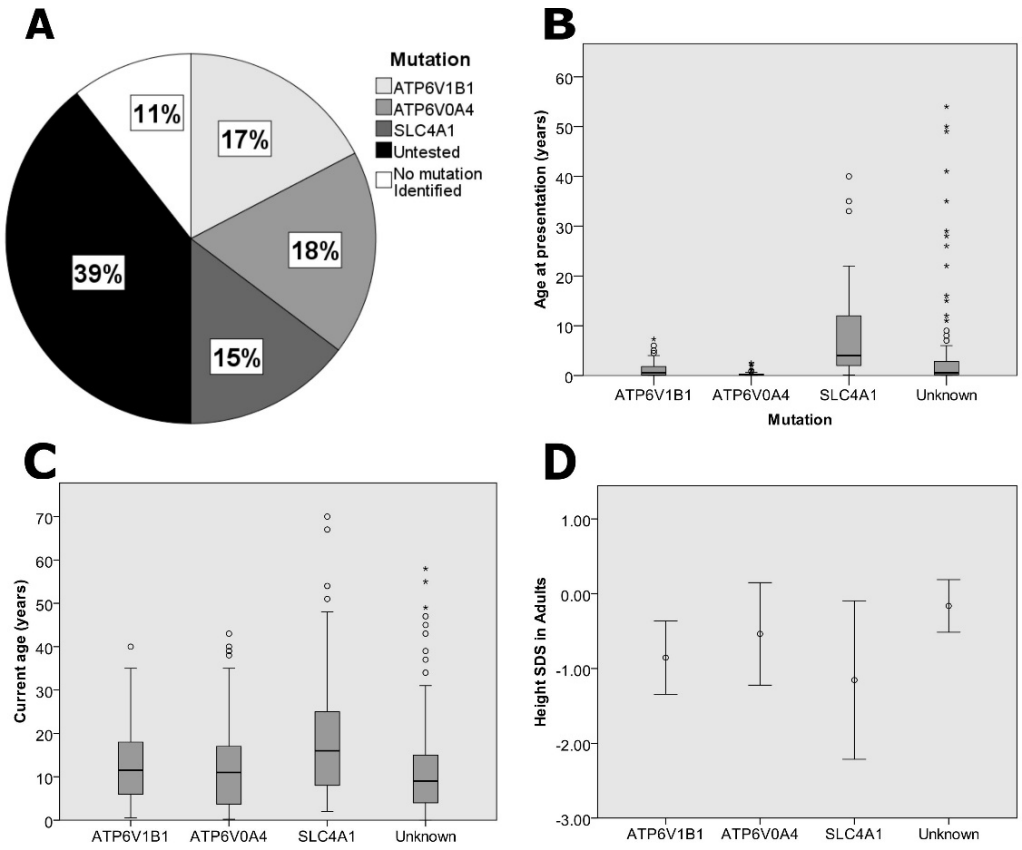
- 18-20 years: 20
- 20-40 years: 45
- 40-60 years: 16
- Above 60 years: 2.

1.2.3.2. Genetic information:

Complete genetic screening in the three-classical known dRTA genes (*SLC4A1, ATP6V0A4* and *ATP6V1B1*; while *FOXI-1* and *WDR72* were excluded) had been performed in 206 cases (61%) and of these 170 (83%) were found to have causative mutations. The overall distribution of the patients as per the outcomes of genetic testing is represented in Figure 1.9a.

A particular variant (*ATP6V1B1*: c.1181G>T, p.(Arg394Gln)) of interest was identified in 6 individuals from the group of 36 total who underwent full genetic testing and were not identified as having a confirmed genetic diagnose during the study time. This was the case as all the patients were simple heterozygous thus a second mutation could not be identified at the time.

Figure 1.9. Demographic, genetics and growth.



Box plot graphs represent the median and interquartile range; the upper and lower whiskers include data points within 1.5 x IQR. Outliers are plotted individually. **A)** Population distribution according to genetic testing. **B)** Age at presentation in years. Note that patients with proton pump mutations all present below the age of 10 years and the significantly ($p<0.001$) older age at presentation in the SLC4A1 group. **C)** Age at last clinic visit. Note again the significantly ($p=0.019$) older age in the SLC4A1 group. **D)** Adult height (mean and SDS) according to genetic group. No significant difference was seen between the genetic groups.

1.2.3.3. Age at presentation:

The median (IQR: interquartile range) age at presentation was 0.5 (0.1-2.5) years with a significantly ($p<0.001$) later onset in patients with *SLC4A1* mutations (Figure 1.9b): *ATP6V1B1* 0.5 (0.1-1.9), *ATP6V0A4* 0.2 (0.1-0.3), *SLC4A1* 4.0 (1.9-12.0) and *Unknown* 0.5 (0.2-2.8). Patients with mutations in *ATP6V0A4* tended to present earlier than any other group, in fact 93% (57/61) of individuals in this group were diagnosed in the first year of life. Conversely, only 8% of individuals from the *SLC4A1* (4/50) presented earlier than 1 year of age. Finally, from the patients in the *ATP6V1B1* group, 57% (34/59) did present at that young age.

A total of 307/336 (91%) cases were diagnosed within the first decade of life. None of the patients with mutations affecting the proton pump presented during adulthood, while 12% (6/50) of the cases from the *SLC4A1* group were diagnosed after 18 years of age.

1.2.3.4. Age at last consultation:

Median (IQR) age at last follow-up in years was 11.0 (5.0-17.5) years and for the specific subgroups: *ATP6V1B1* 11.5 (6.0-18.0), *ATP6V0A4* 11.0 (3.6-17.2), *SLC4A1* 16.0 (7.9-26.3) and *Unknown* 9.0 (4.0-15.0) (Figure 1.9c). Consistent with the older age at presentation, patients with *SLC4A1* mutations were also significantly ($p<0.019$) older at last clinic visit.

1.2.3.5. Long-term outcome: growth

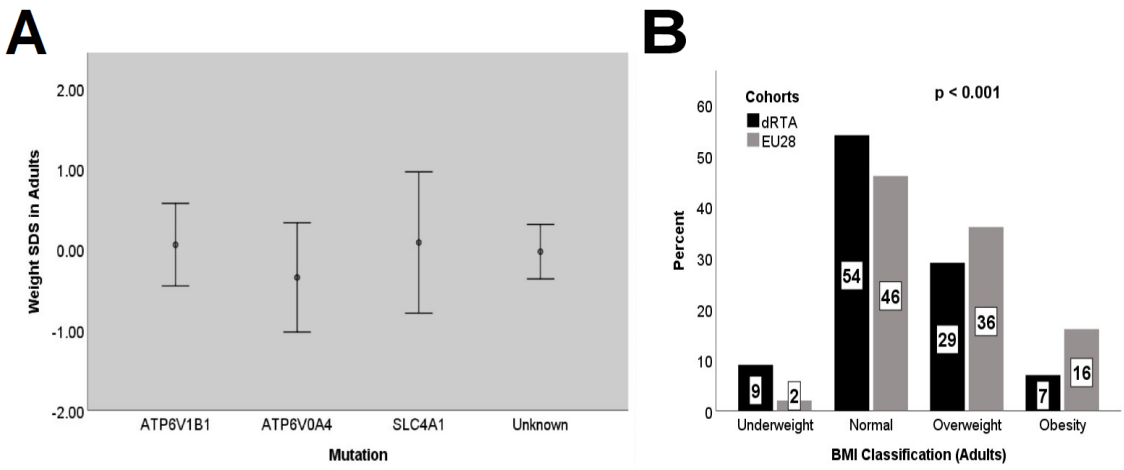
The most recent height standard deviation score (SDS) mean (±SD) for the adult population was -0.57 (±1.16), there were no significant differences ($p=0.059$) between the different groups according to their genetic classification: *ATP6V1B1* -0.86 (±0.92), *ATP6V0A4* -0.54 (±1.19), *SLC4A1* -1.15 (±0.67) and *Unknown* -0.16 (±0.89). (Figure 1.9d).

Weight mean (±SD) in the adult population was -0.04 (±1.20), as per figure 1.10a there was not significant difference between the groups ($p=0.75$) with specific results per genetic group being: *ATP6V1B1* 0.06 (±0.96), *ATP6V0A4* -0.35 (±1.18), *SLC4A1* 0.08 (±1.77) and *Unknown* -0.03 (±0.89). Secondary analysis for adult patients from EU28 countries ($n = 77$) showed a significantly elevated proportion of underweight individuals in the dRTA cohort compared with the reference population (9% versus 2%) with also a reduced prevalence in both overweight and obesity groups as described in figure 1.10b ($p = 0.025$).

In children, weight SDS did have a trend towards lower values of the normal range with a mean (±SD) of -0.86 (±2.86). There were no significant differences between the different genetic groups, although a marked distance ($p = 0.055$) was noticed between the Unknown group with a mean of -1.31 (±3.63) and the other 3 groups with means oscillating between -0.47 and -0.22. The combined prevalence of overweight (18%)

and obese (4%) children (age 2-18 years) in this dRTA cohort was similar to that described in recent epidemiological studies in Europe (25.5%) (19).

Figure 1.10. Weight and BMI



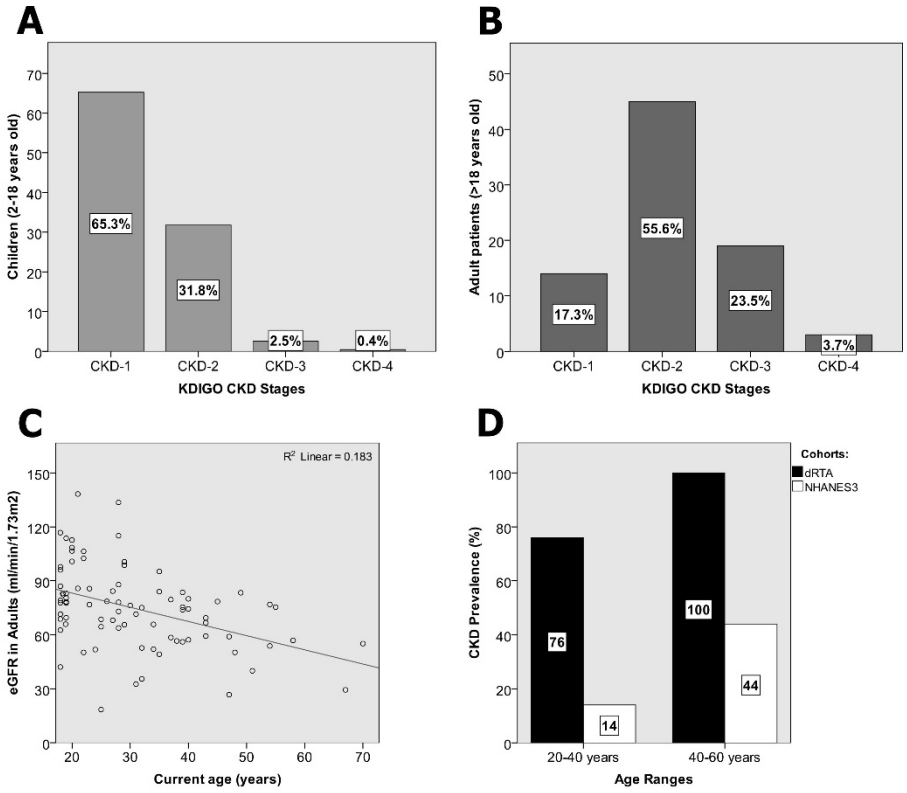
A) Adult weight (mean ± SD) according to genetic group. No significant difference was seen between the genetic groups. **B)** BMI of adult patients (underweight <18.5, normal 18.5–24.9, overweight 25.0–29.9 and obese ≥30.0 kg/m^2). Particularly note the increased prevalence of underweight patients in this dRTA cohort in relation to European reference population, also a general trend towards lower BMI in most of the groups.

1.2.3.6. Long-term outcome: kidney function

A third (34.7%) of the children (age 2-18 years) had impaired eGFR (<90ml/min/1.73m^2), most of them classified as stage 2 CKD (Figure 1.11a), 2.9% were either stage 3 or 4, and none in end stage kidney disease.

Mean (SD) eGFR at last follow up in adults (N=83) was 75ml/min/1.73m^2 (±23) and was broadly similar across the genetic groups: *ATP6V1B1* 81 (±27), *ATP6V0A4* 79 (±26), *SLC4A1* 66 (±20) and *Unknown* 75 (±20) (p = 0.2). No patient with end stage renal disease was noted neither, yet of the 83 adult patients (≥18 years) eGFR was < 90ml/min/1.73m^2 in 68 (82%) as shown in Figure 1.11b. In adults the overall rate of eGFR decline was 0.8ml/min/1.73m^2/year (Figure 1.11c). The prevalence of CKD stage ≥2 was significantly higher (50/61 = 82%) in dRTA patients age 20-60 years compared to the NHANES III reference group (2729/10444 = 26%) (Figure 1.11d).

Figure 1.11. Kidney function and CKD



A) Prevalence of CKD stages in the paediatric age group. Note that 35% have CKD stage ≥2. **B)** Prevalence of CKD stages in the adult age group. Note that 82% of the adult patients analysed had CKD stage ≥2. **C)** Plot of eGFR versus age at last clinic visit. The solid line represents a regression line suggesting a linear correlation between age and loss of renal function with a calculated decline of 0.8ml/min/1.73m^2/ year. Note that mean eGFR at age 18 years is already impaired, consistent with CKD stage 2. **D)** Comparison of CKD stage ≥2 prevalence in adults between our cohort (dRTA) and controls (NHANES 3) according to 2 different age ranges (20-39 years and 40-59 years old). Note the significantly ($p<0.001$) increased prevalence of CKD stage ≥2 in the dRTA cohort.

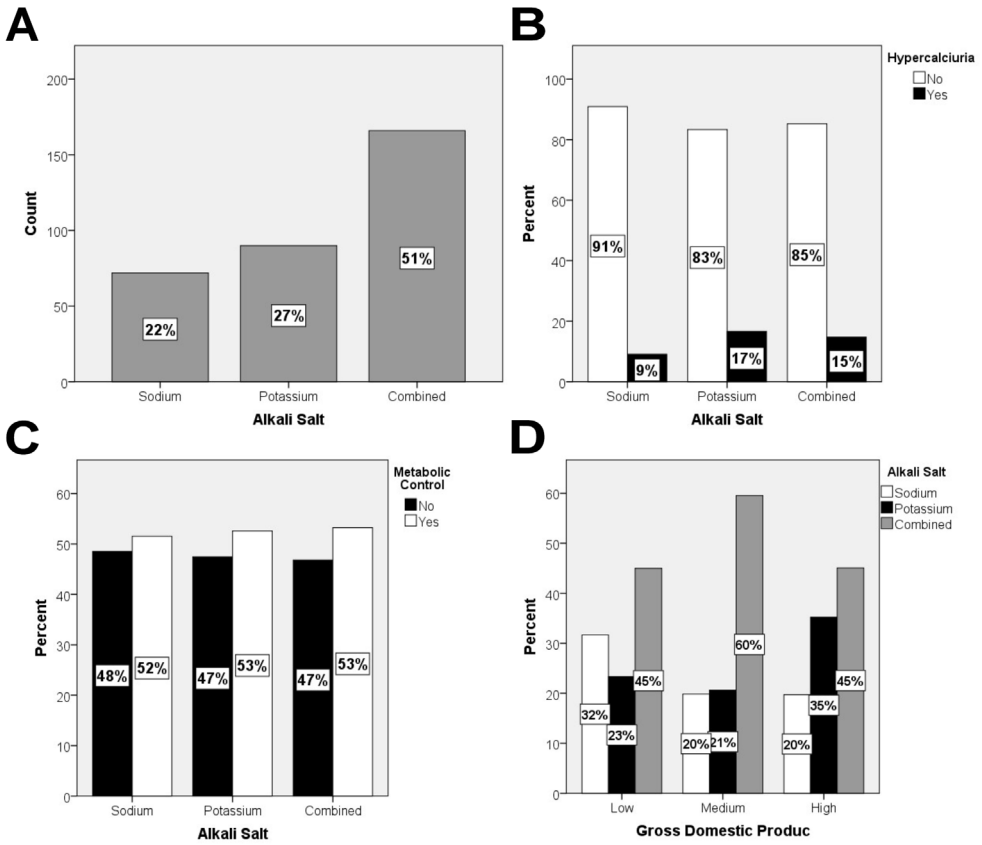
1.2.3.7. Treatment

As reported by referring clinicians, more than 30 different oral alkali formulations were used for treatment of acidosis. The distribution of the main type of salts used were: 25% of the patients (N=84) were treated with bicarbonate only, 40% (N=132) with citrate and 34% (N=113) with different types of combination of both.

Two patients (1%, both with mutations in the *SLC4A1* gene) were maintaining a borderline normal serum bicarbonate level without needing alkali supplementation, this particular phenotype is described as incomplete dRTA. Half of the treated patients (51%) were receiving an alkali salt that combines sodium and potassium, exclusively

sodium was reported in 22% of the cases while potassium was prescribed in 27% (figure 1.12a).

Figure 1.12. Alkali salt and metabolic control



A) Distribution of the whole cohort according by the type of salt currently administrated. **B)** Hypercalciuria vs type of salt. Note that there is no difference between the groups. **C)** Adequate metabolic control in relation to type of salt. No statistically significant difference was found. **D)** Use of different type of salt in relation to gross domestic product of the countries, inverted relation sodium/potassium between low and high GDP (p = 0.03).

Specific analysis was performed to determine whether the use of sodium vs potassium salts as alkali therapy could have an impact on urinary calcium excretion and overall metabolic control, figure 1.12b and figure 1.12c specifically represent this; dedicated statistical analysis failed to show a significant difference despite a noticeable reduced prevalence of hypercalciuria in the sodium group. The single factor identified with a potential influence on the prescription of sodium vs potassium was the GDP per capita, in this cohort sodium salts were more commonly offered in countries with lower income (Figure 1.12d).

The median (IQR) prescribed dose of alkali treatment in milliequivalents per kilogram daily (mEq/kg/day) was 1.9 (1.2-3.3) and similar across groups: *ATP6V1B1* 1.7 (1.1-2.3), *ATP6V0A4* 1.9 (1.2-3.3), *SLC4A1* 1.5 (0.9-2.7) and *Unknown* 2.2 (1.4-4.1). Yet, median (IQR) prescribed doses of alkali equivalent were significantly higher in younger patients compared to older ones (p value < 0.001) (Figure 1.13a). After classifying the entire cohort according to age groups, it was noted that median (IQR; range) in mEq/kg/day of alkali for each group was: <6 years: 3.3 (2.3-5.0; 0.9-12.3), 6-18 years: 1.8 (1.1-2.6; 0.0-7.3) and ≥ 18 years: 1.1 (0.6-1.6; 0.0-8,1).

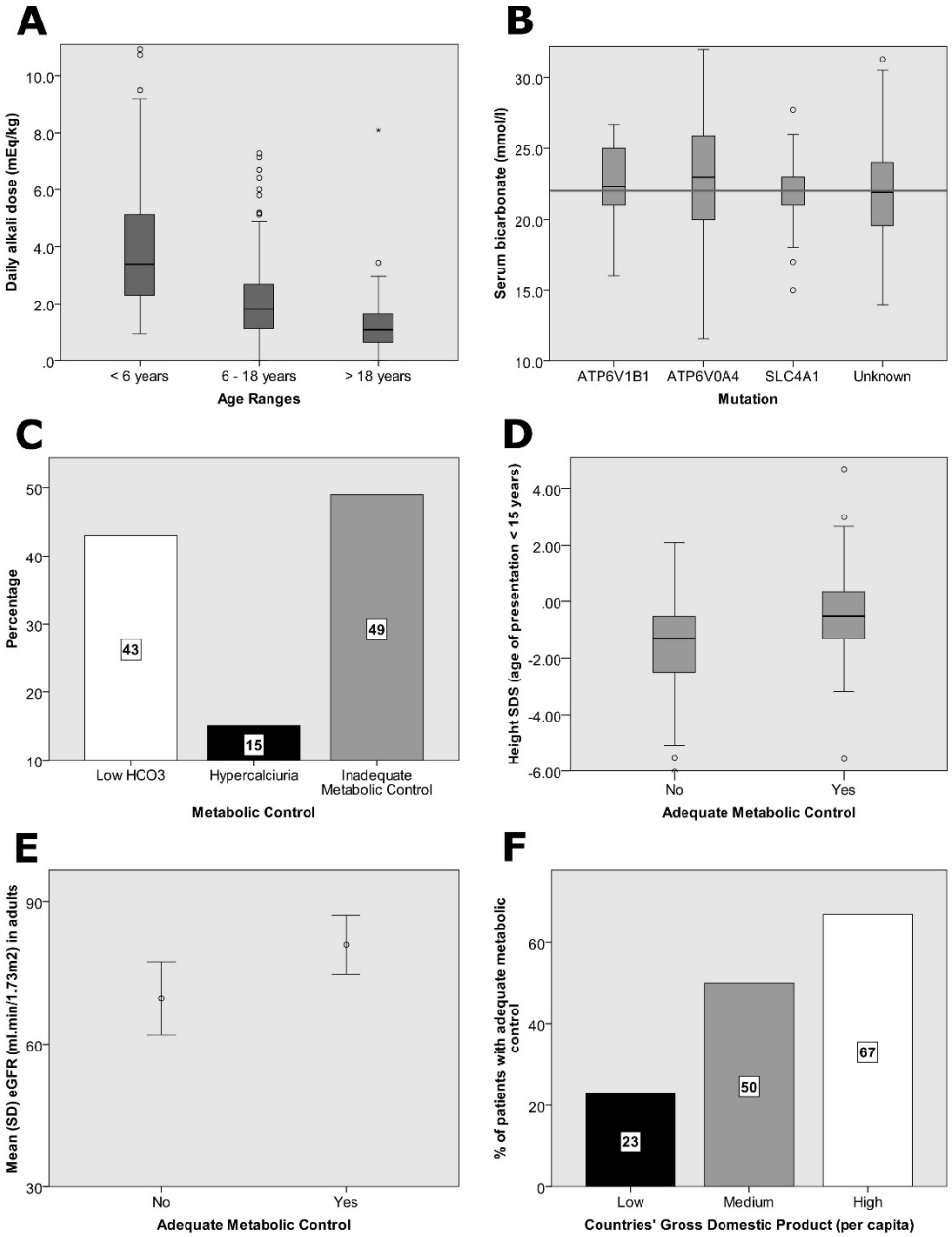
1.2.3.7.1. Metabolic control

Serum bicarbonate (mmol/L) levels and urine calcium/creatinine ratio (mmol/mmol) at last follow-up were used as markers for assessment of metabolic control. Data for both items were available in 312 patients. Median (IQR) serum bicarbonate level at last follow up was just at the lower limit of the normal range at 22.0 mmol/l (20.0-24.0) with no significant difference between the genetic groups (Figure 1.13b). The proportion of patients with hypercalciuria was very similar among the patients with proved pathogenic mutations, at 12% in ATP6V1B1 (7/58), 14% in ATP5V0A4 (8/56), 11% in SLC4A1 (5/47) and slightly increased at 19% in the Unknown group (28/151), however this difference was not significant at statistical level.

From the entire cohort, 43% of cases had metabolic acidosis (serum bicarbonate < 22 mmol/L) and 15% had increased urinary calcium excretion. When these two factors were analysed in combination, overall, 49% of the patients had one or both abnormalities and therefore were classified as not having an adequate metabolic control at last clinic visit (Figure 1.13c). To assess the potential impact of this combined factor, further analysis of its correlation with growth and kidney function was performed.

For growth, the analysis was focused on the last documented height from patients who presented and thus started treatment at an age with presumed growth potential (defined as <15 years of age). Median (IQR) height SDS was significantly (p < 0.001) higher at -0.52 (-1.32 to +0.36) in those with adequate metabolic control compared to -1.31 (-2.50 to -0.52) in those without (Figure 1.13d).

Figure 1.13. Treatment and metabolic control at last follow-up.



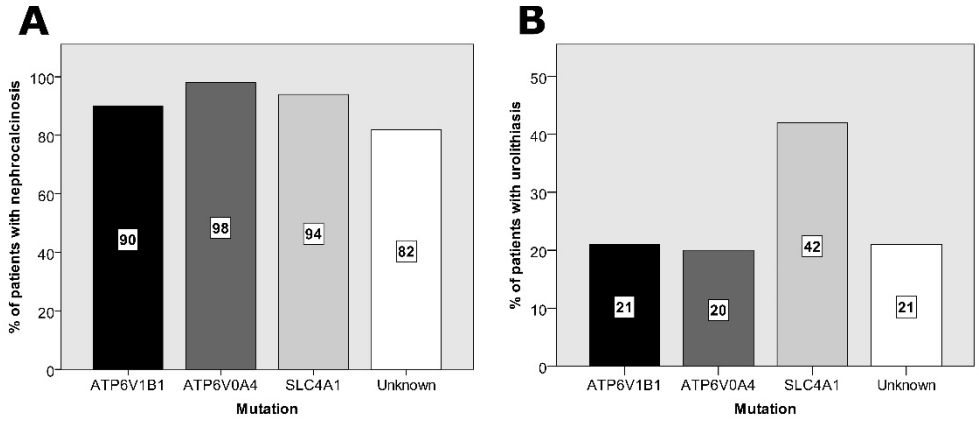
Box plot graphs specifics are as detailed in Figure 1.9. **A)** Daily weight-adjusted alkali dose according to age group (under 6 years, 6-18 years and adults). Note that prescribed weight-adjusted dose of alkali supplement decreases with age ($p<0.001$). **B)** Serum bicarbonate level at last clinic visit according to the different genetic groups. The horizontal line indicates the lower limit of the normal range (defined as 22.0mmol/L) **C)** Prevalence of metabolic acidosis (white), hypercalciuria (black) and inadequate metabolic control (grey) within the whole cohort. Note that 49% of the cohort had low bicarbonate and/or hypercalciuria, thus having inadequate metabolic control. **D)** Significantly ($p<0.001$) different height SDS (in patients who presented under 15 years) in relation to metabolic control. **E)** eGFR in adults (≥18 years) and metabolic control. Note the significant ($p<0.023$) difference in eGFR between those with and without adequate metabolic control (for details, see text) **F)** Prevalence of metabolic control in relation to GDP group. Note the significant ($p<0.001$) difference in achievement of adequate metabolic control in countries with high GDP compared to those with lower GDP.

To look for long-term effect on kidney function, eGFR was compared in adults with or without adequate metabolic control: mean (±SD) eGFR was significantly higher (p = 0.023) in those with adequate metabolic control at 79 (±19) compared to those without at 67 (±22) ml/min/1.73m^2 (Figure 1.13e). Adequate metabolic control was achieved in a significantly (p value < 0.001) higher proportion (67%) of patients in the countries with high GDP, compared to 50% in countries with medium and 23% in countries with low GDP (Figure 1.13f).

1.2.3.8. Nephrocalcinosis and Nephrolithiasis:

Nephrocalcinosis was common in all groups but had a significantly higher (p value = 0.004) prevalence in patients with *ATP6V0A4* mutations 98% (59/60) compared to *ATP6V1B1* 90% (53/59), *SLC4A1* 94% (47/50) and *Unknown* 82% (140/170). Nephrocalcinosis was already noted at presentation in a vast majority of the patients (229/261 = 88%) (Figure 1.14a). Nephrolithiasis was significantly (p = 0.014) more common in patients with *SLC4A1* mutations 42% (21/50) compared to *ATP6V1B1* 21% (12/57), *ATP6V0A4* 20% (12/59) and *Unknown* 21% (34/165) (Figure 1.14b).

Figure 1.14. Nephrocalcinosis and urolithiasis



Presence of **A)** nephrocalcinosis and **B)** history of urolithiasis at last clinic visit. Note that nephrocalcinosis is significantly (p = 0.004) more common with *ATP6V0A4* mutations, compared to the other genetic groups. Note also the significantly increased prevalence of nephrolithiasis in SLC4A1 group.

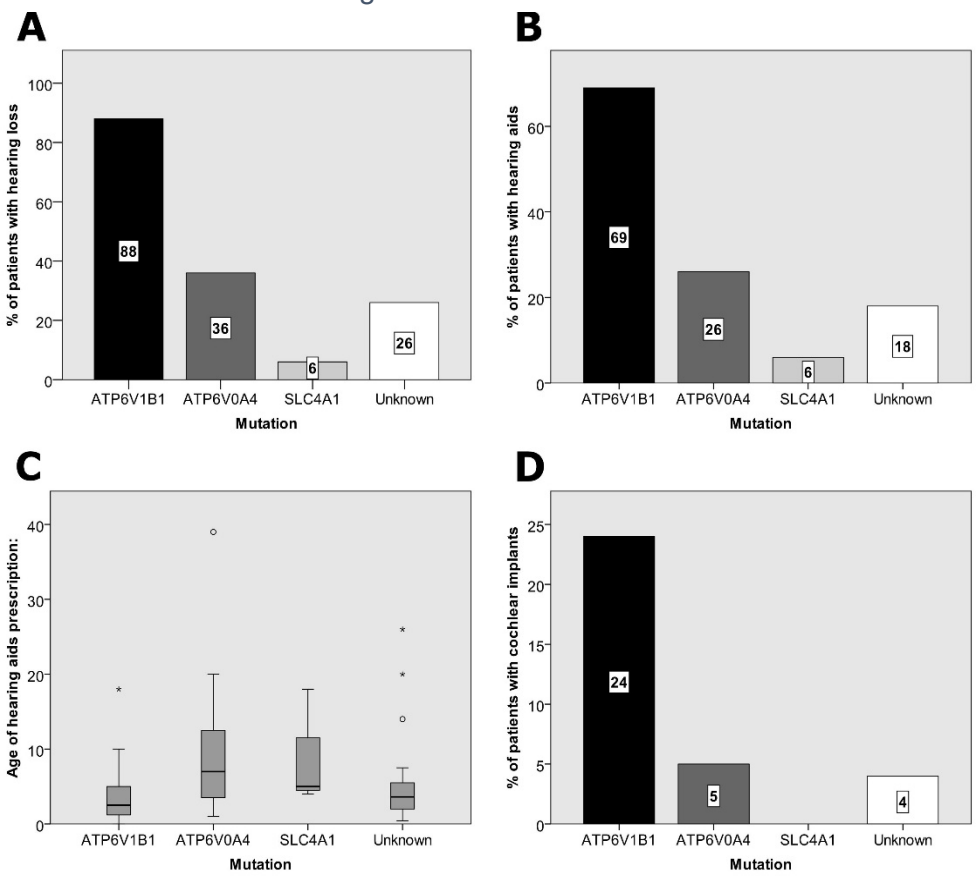
From the cohort of patients with declared medical history of nephrolithiasis (n=79) in at least 61 (77%) this was documented as one of the presentation signs. According to genetic group distribution, the proportion of patients with nephrolithiasis on presentation was: ATP6V0A4 0% (0/11), AT6V1B1 27% (3/11), SLC4A1 50% (5/10)

and Unknown 41% (12/29); this unequal distribution was significant on analysis (p = 0.032).

1.2.3.9. Hearing loss:

Hearing loss was significantly (p < 0.001) more prevalent in patients with *ATP6V1B1* mutation 88% (50/57) compared to *ATP6V0A4* 36% (21/59), *SLC4A1* 6% (3/49) and *Unknown* 26% (42/161) (Figure 1.15a). Hearing aids were prescribed in a total of 90 (27%) patients and most commonly in patients with *ATP6V1B1* mutations (N=40, 69%), compared to *ATP6V0A4* (N=16, 26%), *SLC4A1* (N=3, 6%) and *Unknown* (N=31, 18%) (p < 0.001) (Figure 1.15b).

Figure 1.15. Sensorineural hearing loss



Shown is the prevalence and treatment of sensorineural hearing loss across the genetic groups. **A), B)** and **D)** Prevalence of hearing loss, history of hearing aids prescription and history of cochlear implantation. Note the significantly (p<0.001) increased prevalence of hearing loss, prescription of hearing aids and cochlear implants in patients with *ATP6V1B1* mutations. **C)** Patients' age at prescription of hearing aids. Note the significantly (p<0.021) younger age at prescription in the ATP6V1B1 group.

Median (IQR) age (years) of hearing aids prescription was significantly lower ($p < 0.021$) in the *ATP6V1B1* group at 2.5 (1.2-5.0) compared to 7.0 (3.1-13.3) with *ATP6V0A4*, 5.0 (4.0-5.0) with *SLC4A1* and 3.6 (1.9-5.9) with *Unknown* (Figure 1.15c). Similarly, cochlear implants were significantly ($p < 0.001$) more commonly performed in patients with *ATP6V1B1* mutation 24% (14/59) compared to *ATP6V0A4* 5% (3/61), *SLC4A1* 0% and *Unknown* 4% (7/170) (Figure 1.15d).

1.2.4. DISCUSSION:

This work reports on the treatment and long-term outcome in patients with a clinical diagnosis of primary dRTA. This cohort is also the largest reported so far for this disorder and arguably the most complete, with the inclusion of not only extensive clinical and demographic data but also providing information on genotypes when available. Indeed, while approximately half of the cohort did not have an identified genetic diagnosis (including 39% who were not genetically screened), the large number (N=50-61) in each genetically classified group allows for meaningful interpretation of data according to genotype. Moreover, the lack of obvious differences with regards to treatment, long-term outcome and complications between the groups with unknown genetics and those with defined mutations, suggest that the presumed clinical diagnosis of primary dRTA must be accurate also in most of the patients without genetic conformation. Of those who had genetic testing performed, causative mutations were identified in about 83%, roughly comparable to other recent reports (9, 128, 141).

Of particular interest is the recurrent identification of the heterozygous variant in *ATP6V1B1* p.(Arg394Gln), which has been reported previously in heterozygous form in patients with clinical diagnosis of dRTA (141, 142). A subsequent study has since provided strong evidence that this variant indeed causes a dominant form of dRTA (65).

1.2.4.1. Genotype-Phenotype analysis

Data from the study has shown some genotype-specific characteristics, comparable to previous reports (14 ,15). In general, patients with mutations in the proton pump subunits have a more severe phenotype, compared to those with *SLC4A1* mutations: age of presentation is younger, with the vast majority (91/118, 77%) presenting in the first year of life and all before 10 years of age, whereas 12% (6/50) of the patients with *SLC4A1* mutations presented during adulthood (Figure 1.9b).

1.2.4.2 Treatment and metabolic control

Control of the acidosis, as assessed by plasma bicarbonate concentration and urine calcium excretion was achieved in only about half of all patients. Of course, there are

limitations to this analysis: biochemical data were only available from the last clinic visit, which may not be representative of the entire follow-up period. Moreover, bicarbonate determinations varied, as in some instances total CO_2 was measured, whereas in others it was calculated from a blood gas. Most importantly, bicarbonate levels in this condition depend heavily on the timing of the last alkali dose taken. Yet, these limitations reflect routine clinical practice. Considering the above mentioned concerns and the fact that urinary calcium excretion is generally raised when acidosis is present, calciuria was taken as well into consideration as another indicator of metabolic control in dRTA (143).

This cohort analysis showed that a concerningly high proportion (~half) of the managed patients with dRTA do not have an adequate metabolic control as per our defined criteria. This group is at increased risk of complications, such as poor growth and faster progression of chronic kidney disease (later discussed in this thesis) (144-146). Although the importance of an adequate management in chronic metabolic acidosis is very well known and persistently highlighted in CKD guidelines recommendations (137), this large dRTA cohort of patients has shown that further work still needs to be taken.

Interestingly, adequate metabolic control was also significantly associated with per capita GDP (Figure 1.13f). Whether this reflects the affordability and/or availability of specialised medical care and medications cannot be deduced from this data. Of note, median (IQR) age at diagnosis was not significantly different among the three GDP groups (during the statistical analysis), however some tendency towards early diagnose was observed as GDP increments:

1. Low: 0.6 (0.3-3.7)
2. Medium: 0.6 (0.2-2.5)
3. High: 0.3 (0.1-2.0)

Forms of supplementation varied widely and overall, a total of 34 commercialised different alkali formulations were used, plus some patients also received compounded versions. This may reflect clinician preference, patients' preferences as well as country-specific availability of these alkali supplements. Citrate formulations are not widely available in every country and in some cases can be associated with increased

gastrointestinal adverse effects (such as dyspepsia, reflux, nausea, or diarrhoea). While slow-release formulations are even more difficult to obtain, particularly for their high cost, many physicians also traditionally have gathered more experience with the use of bicarbonate salts, particularly sodium. Concerns regarding the use of sodium bicarbonate are:

1. The potential increment on urinary calcium excretion; however, in this cohort whether patients were under treatment with bicarbonate, citrate, or a combination of both was not significantly associated with hypercalciuria prevalence. Also, in the group treated with sodium salts the presence of hypercalciuria was lower than in the potassium/combined one (9% vs 19%), this difference did not reach statistical significance in the analysis (Figure 1.12b).
2. Urinary citrate (low in dRTA) may not be equally increased. This is likely a misconception. Citrate does not follow a straight pathway from intestinal absorption to bloodstream and filtering into the renal tubular lumen. Instead it is metabolised in great proportion in the liver where it enters the citric acid (Krebs) cycle and provides a net amount of 2 bicarbonate ions (147). Urinary citrate depends mainly on systemic pH and proximal tubular citrate reabsorption more than on specific citrate intake, indeed several studies have shown that both oral citrate and bicarbonate have equivalent effect on urinary pH and citrate content (122, 123).

The prescribed alkali dosage is highest during the first years of life, presumably reflecting the increased metabolic rate of younger children, which necessitates a larger caloric intake relative to body size and thus an increased acid load (Figure 1.11a). This cohort specifically informs on the treatment dose for dRTA patients, with young children (< 6 years old) needing up to 10 mEq/kg/day (median 3.3, IQR: 2.3-5.0), whereas older children and adults can achieve acid-base balance with typically 1-3 mEq/kg/day of alkali supplementation. There was no difference with regards to metabolic control between the age groups, consistent with the notion that physicians adjusted alkali supplementation accordingly.

1.2.4.3. Long-term outcome: growth

Distal RTA when untreated is known to have a severe impact on growth with improvement in patient's height and weight once alkaline treatment has been established (148, 149). Modest data on failure to thrive in patients with primary dRTA is mainly focused on disease's presentation and scarce information is available on medium-long term (late childhood or adulthood age) (128, 150, 151). This large cohort presented here is particularly informative as it provides data from a large adult population regarding height and weight.

Adult height was mildly reduced at -0.57 SDS and, again, the majority (90%) of adult patients had achieved a final height within the normal range (SDS > -2.0). Paediatric height data also showed a reduced average SDS, however this was not largely lower compared to adults (mean -0.92). Considering that no data on presentation was collected in this project and also that evidence available in the literature shows a rapid and significant improvement in height and weight in paediatric patients following the start of alkali therapy (9); one can hypothesize that the long mean follow up period (6 years and 8 months) for children has been sufficient for catch up growth. In parallel to this dRTA cohort, a recent large and heterogenous cohort of Spanish patients with inherited tubulopathies has also shown a high prevalence of short height on presentation (38%) which reduces by at least 24% after a median age of follow up of 5 years (151).

Another important correlation revealed in the data analysis is the fact that height SDS was significantly ($p < 0.001$) better in those patients with an adequate metabolic control compared to those without, suggesting that growth can be optimised with adequate treatment indeed. A recent study looking at long term follow up of patients presenting during childhood with primary dRTA did fail to reproduce this correlation between metabolic control and growth, however the number of cases was very small with only 13/16 patients having sufficient data available (150).

Regarding weight analysis, parallel findings to height were noticed. While mean SDS in childhood was slightly reduced this may have potentially be corrected when reaching adult period. Detailed interrogation to BMI classification in the adult cohort did also show an increased prevalence of underweight cases with reduced proportion

within the overweight and obese groups taking as reference European epidemiological data (Eurostat: EU-28). This is one of the few studies in the literature to specifically look at BMI in adult patients with primary dRTA, and by far the largest in terms of number of patients analysed. A 2001 publication from Thailand looking at this specific question reported an underweight proportion of 5/14 (36%) in adult dRTA patients that was markedly increased compared to the average underweight prevalence in their general population of 6% (152), highlighting once more the deleterious impact that this inherited tubulopathy can have on patients anthropometrics.

1.2.4.5. Long-term outcome: kidney function

A reduced eGFR in adult cases (mean of 75 ml/min/1.73m^2) with a prevalence of CKD stages II-IV in more than 80% in this population suggests that dRTA has indeed a negative impact in kidney function. Furthermore, several other studies on dRTA in the literature have reported also a raised prevalence of CKD with rates fluctuating between 30-67%, particularly in relation to aging (9, 128). Potential factors reportedly involved in this increased risk of CKD have been: 1) nephrocalcinosis, 2) new formation of renal cysts in relation to the so-called “hypokalaemic nephropathy” and/or 3) recurrent episodes of acute kidney injury for example secondary to dehydration (153).

With the intention of having a better understanding of how much of this CKD burden could be related to the dRTA, a direct comparison was established between the CKD prevalence in this cohort and the one from a large reference population study also known as the third National Health and Nutrition Survey (NHANES III). Conveniently, NHANES III also estimated GFR by using the MDRD equation (154). This analysis demonstrated a CKD prevalence 3 times higher in the dRTA cohort, a finding that again highlights the importance of adequate treatment and management of patients with dRTA when aiming to reduce the long-term burden of this disorder.

The average eGFR decline in adult patients (Figure 1.11c) was about 0.8ml/min/1.73m^2/year. This progressive reduction is equivalent to the one in the general population (155, 156). However, for the healthy individuals in the general population, the physiological decline of the kidney function usually begins during the fourth decade of life, and more importantly it does so from a starting eGFR point of 130-140ml/min/1.73m^2 (157).

Unlike in the general population, this study has shown that dRTA patients are already having an impaired kidney function earlier in life with average function at age 18 years already equivalent to CKD stage 2. This suggests that the permanent kidney damage has already initiated during childhood, a point previously suggested by other authors (128).

Although data from large-scale epidemiological studies of CKD in children is lacking, registry information is estimating a prevalence of CKD around 70 per million of the age-related population (<0.01%) (158, 159). This highly contrasts with the 35% prevalence of CKD stage 2 among paediatric patients from this thesis. Considering this large CKD prevalence and the potential negative impact of an uncontrolled disease during this period, further analysis was performed to evaluate the impact of adequate metabolic control on eGFR in children. Parallel to height SDS, an adequate metabolic control was associated with better kidney function (median 103 vs 94 ml/min/1.73m^2; p = 0.008), highlighting again the importance of adequate management.

1.2.4.5. Nephrocalcinosis and Nephrolithiasis:

Nephrocalcinosis and nephrolithiasis are two classical clinical features of distal renal tubular acidosis. Multiple risk factors typically play together in the urinary tract of this patients: such as increased calcium excretion, abnormal (high) urine pH, reduced excretion of anti-lithogenic factors like citrate, etc (160).

Regarding hypercalciuria a high prevalence was noted in this cohort at presentation, particularly in cases with identified pathogenic mutations (90-98%) with the highest proportion found in the *ATP6V0A4* group. Similar findings have been reported in other studies with large number of cases (9, 128). Patients with pathogenic mutations in *ATP6V0A4* have been described as particularly at risk of secondary proximal tubular dysfunction with presence of low molecular weight proteinuria, worse metabolic acidosis and/or full renal Fanconi phenotype. Whether this is only secondary to the systemic acidosis or alternatively a consequence of a dysfunctional proton pump in the apical membrane of the proximal tubular cells is still uncertain (9). Nephrocalcinosis has also been proposed as a potential contributing factor to dRTA's

related decline in kidney function (9), however statistical analysis did fail to show any significant influence, hence hypercalciuria does not seem to be one of the major associated risk factors, at least in this particular cohort.

In relation to stone formation an interesting finding was documented in this project, despite a lower prevalence of hypercalciuria in the *SLC4A1* group (11%), these patients had the highest prevalence of nephrolithiasis (42% vs 20% in the other genetic groups). Yet, it is important to note, that hypercalciuria could only be captured at last follow-up. Moreover, other measurements such as phosphaturia or urinary citrate excretion were not captured. In addition, as patients with *SLC4A1* mutations typically present later in life, they presumably have had a longer time with undetected and untreated hypercalciuria (among other lithogenic factors), favouring stone formation within the urinary tract.

1.2.4.6. Hearing loss:

Sensorineural hearing loss is a classic associated feature of dRTA, first described in 1971(161). This study confirms the close association of deafness with mutations in *ATP6V1B1*(57). Yet clinically relevant deafness was also seen in almost a third of patients with *ATP6V0A4* mutations, with hearing aids or cochlear implants present in 26 and 5%, respectively (Figure 1.13). Incidentally, we found a 6% rate of hearing aids prescription in patients with pathogenic mutations in *SLC4A1*, the youngest at 4 years of age. As *SLC4A1* is not known to have a functional impact in the cochlea, interpretation of this finding was done with caution and after a review of audiology literature it was found that these results are simply reflecting the usual prevalence of hearing loss in the general population (162, 163).

1.2.5. LIMITATIONS OF THIS STUDY:

This study is a retrospective review based on a limited number of results from the last clinical follow up of the patients and captured via an online form. With any such study there needs to be a balance between feasibility and the comprehensiveness of the data collected. If a large number of data is requested, clinicians may be reluctant to participate, as data entry is time consuming. Upon conception it was therefore decided to focus only on aspects deemed most important, including up to 28 main variables for initial assessment.

Other specific aspects of dRTA's phenotype were not specifically reviewed in this thesis, such as hypokalaemia and haematological disorders. Regarding the latest, it is understood that autosomal recessive *SLC4A1* mutations may be associated with mild defects in red cell morphology, particularly if there is coincident acidosis (77). Given the rarity of this form of dRTA in patients of European ancestry, making meaningful statements would not be possible. Indeed, only 5 such cases were included in the study. Investigations into this particular issue are more appropriate in Asian cohorts, as has been reported before (78).

Finally, no specific data on diet were requested. As diet determines the acid load, meaningful data on dietary patterns may explain some of the variability in the treatment doses (164). Yet, details of dietary habits are rarely collected by clinicians in routine practice. The high number of patients entered validates this study design of limited data gathering, even though it only allowed a focused investigation of selected clinical aspects of dRTA.

Yet, the key limitation is the assumption that data from last clinical follow-up adequately reflect treatment throughout the lifetime of the patients. This obviously is not necessarily the case, which may explain some of the variability in the reported results. Moreover, the "long term" outcome information is based on the data from adult patients, whose previous treatment may not necessarily reflect current treatment of newly diagnosed patients. These limitations clearly show the need for prospective gathering of granular data in an international registry for this rare disorder, a currently

ongoing international project that has been greatly potentiated by the development of this study.

1.2.6. CONCLUSIONS:

Data from this cohort of patients with dRTA suggests an overall favourable outcome in agreement with previously published reports. However detailed analysis of this large number of patients have revealed interesting points, some of whom are providing new insights into the paradigm of this rare disorder:

1. Chronic kidney disease is considerably more prevalent than what has previously been reported. This has been particularly striking in the paediatric age and further studies are needed to assess if this and other complications, such as nephrocalcinosis and urolithiasis can be ameliorated by early diagnosis and treatment. Our results also suggest that adequate metabolic control may slow down the progression of CKD. Therefore, clinicians should aim to maintain serum bicarbonate and urine calcium in the normal range.
2. Despite certain limitations this study did not detect any clinical advantage certain types of alkali over others (eg: citrate vs bicarbonate), nor a difference in hypercalciuria rates between sodium and potassium containing salts. Yet, almost half of the studied individuals did not achieve adequate metabolic control, suggesting a high prevalence of suboptimal treatment in common practice. Our data suggest that clinicians should use whatever alkali supplement is tolerated best by their patient to optimise metabolic control.
3. Until now there has been no reported data regarding long term growth of patients with dRTA, a factor of significant concern as chronic metabolic acidosis is known to have a negative impact on bone health. This project has shown that growth is associated with adequate metabolic comtrol, again highlighting the importance of appropriate treatment.
4. The presence of failure to thrive on presentation but also the newly reported higher proportion of adult patients with low BMI supports the notion of considering dietetic support throughout the entire follow up of the patients and not only during childhood.

5. Hearing loss can be profound and occur at very early age. This study confirms the high burden of this specific phenotype in patients with pathogenic mutations in *ATP6V1B1*. Moreover, it also provides an estimated prevalence in patients with mutations in *ATP6V0A4*. In addition, it provides detailed clinical information about age at diagnosis but also at prescription of both hearing aids and cochlear implants, which can be very useful for clinicians when planning follow-up as well as counselling patients and families about the different comorbidities and disease prognosis.

CHAPTER 2. NEPHROGENIC DIABETES INSIPIDUS

2.1. INTRODUCTION

2.1.1. Diabetes Insipidus and Historical Context.

Evolution started in the sea and since then water has played a critical role in biology; from the simplest first microorganisms to the most complex plants and animals currently inhabiting our planet. Indeed, the philosopher Aristotle described it as one of the four building block elements of the Universe.

Even though for a long period of time water has been recognized as essential to life on earth, the discovery of the molecular mechanisms of its homeostasis occurred only in the 20th century; with studies of diabetes insipidus being an important contributor(165).

The term diabetes insipidus is better understood if looked at each word individually. The word *Diabetes* is Greek in origin and refers to a high urine excretion, also described with the term polyuria. The concept of diabetes was introduced in medical literature as far as the 1st-2nd century BC by Demetrius of Apameia and it derives from the Ionic, literally meaning: “to pass or run through,” as in a siphon. What Demetrius described at the time was the fact that certain individuals were afflicted by a condition causing a large and continuous production of urine (166). Considering the rarity of diabetes insipidus compared to other disorders that can present with polyuria as well, it is more likely that those patients did suffer from osmotic diuresis secondary to hyperglycaemia, a disease called diabetes mellitus in reference to the sweet or honey-like taste of the urine as specifically described by the Oxford Professor Thomas Willis in 1674 (167).

Although Prof. Willis did not discuss the differences between polyuric patients with and without sweet urine (the later less frequently seen), he set the basis for the studies that William Cullen (Scotland) and Matthew Dobson (England) performed a century later (1769). These two researchers were the first to show with their experiments that A) the sweet substance highly present in the urine of patients with Diabetes Mellitus was at the same time elevated in the blood and B) there was a subset of patients with

diabetes (polyuria) that did not have sweet urine and on the contrary the urine was characteristically tasteless (they called it “urina potus” in reference to the medieval concept of urine produced after large fluid intake). Finally, in 1794, the term “insipidus” (from the Latin word for tasteless) was introduced by Johann Peter Frank from Pavia University in a condition that he described as “a long continued abnormally increased secretion of non-saccharine urine which is not caused by a diseased condition of the kidneys”. At last, diabetes insipidus was described as a specific entity. However, the mechanisms involved in its pathogenesis remained unknown. During the following century the study of some of these patients (and their families) provided new insights in what appeared to be a familial disorder; first Lancombe in 1841 (168) and later McIlraith in 1892 reported on different kindreds of affected relatives (169).

Analogous to diabetes mellitus being a non-primary kidney disorder, for a long time this was considered to be the case also for diabetes insipidus. Indeed this concept was reassured in 1901 when Magnus and Shaffer demonstrated the antidiuretic effect of posterior pituitary extracts and later on in 1913 when Farini and Von den Velden used those extracts to successfully treat patients with diabetes insipidus (170). This therapeutic approach took the name of “hypophysiotherapy”. Yet, following its introduction some clinicians started reporting certain cases of therapeutic failure or resistance, suggesting different subforms of diabetes insipidus. Indeed, recent attempts at renaming diabetes insipidus to avoid confusion with diabetes mellitus focus on the response to antidiuretic hormone (ADH, also called Arginine vasopressin or AVP) by calling it AVP-deficiency (cranial DI) and AVP-resistance (NDI) (171).

The first kidney related variants of diabetes insipidus were described in 1945 by two different groups: Forssman (172) in Europe (Sweden) and Waring *et al.* in America (USA) (173). Shortly after (1947), Williams and Henry introduced the term “nephrogenic diabetes insipidus” for the congenital syndrome characterized by polyuria and a renal concentrating defect unaffected by antidiuretic hormone (ADH) at usual therapeutic doses (174). Their theory was reaffirmed once subsequent analysis showed the presence of ADH in high quantity in both serum and urine samples in these patients, confirming a normal response from the neurohypophysis to a hypovolemic state. While reflecting on the inheritance pattern, scientists realized that this disorder

tended mostly to be transmitted by asymptomatic females to their male offspring (169). Finally, *AVPR2* and *AQP2* the currently known genes associated with primary nephrogenic diabetes insipidus were both identified in 1992 and 1993 respectively (175, 176).

2.1.2. Physiopathology of NDI

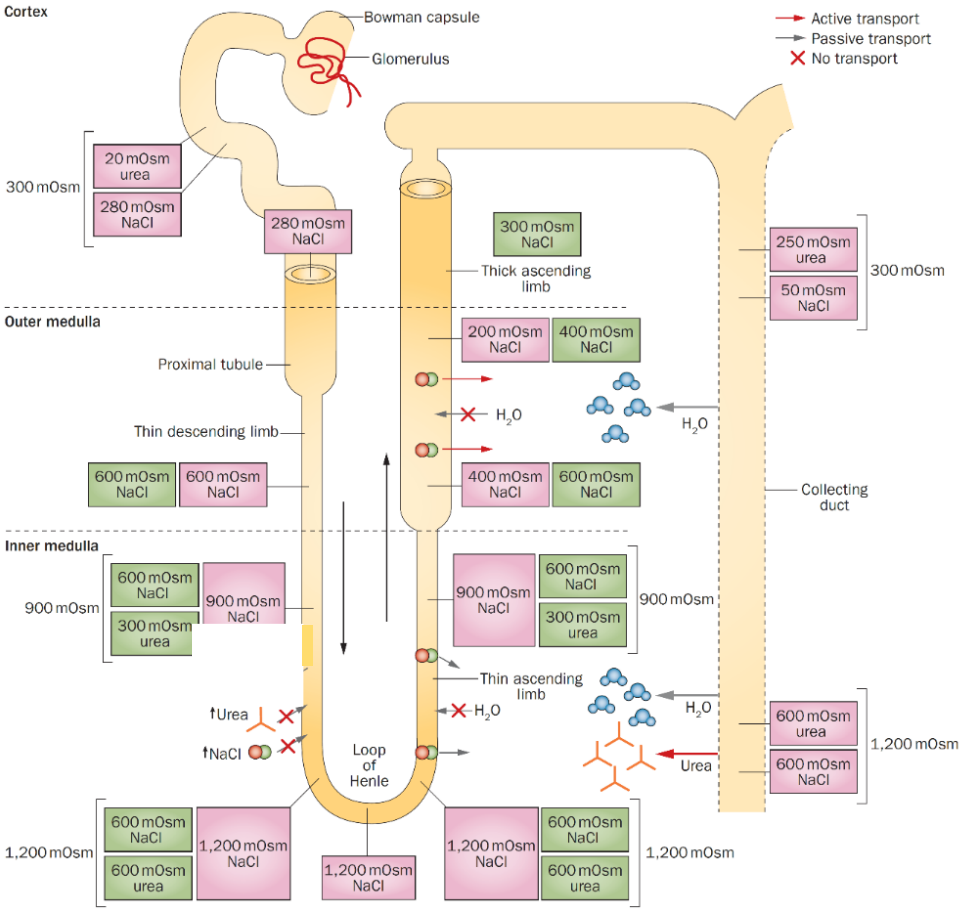
2.1.2.1. Urinary concentration:

Healthy adult kidneys produce approximately 180 litres of primary glomerular filtrate per day (177) with the majority of this filtrate being reabsorbed in the proximal tubule (approximately 2/3 of total water). This segment of the nephron is freely permeable to water due the constitutive presence of AQP1 water channels and while solutes are reabsorbed, water passively diffuses through the epithelial layer following an osmotic gradient (178). Further down in the tubular lumen this early and isotonic "urine" reaches the loop of Henle which has a very important role in the urinary concentration process thanks to its unique counter-current mechanism (Figure 2.1).

Urinary concentration starts in the thin descending limb (TDL). At this segment the mechanisms of water absorption will differ according to the type of nephron (179, 180). In short-loop nephrons (SLN), animal and human studies have failed to prove expression of AQP1 with indeed a lower osmotic water permeability compared to long-loop nephrons. The exact mechanisms involved in water reabsorption here are still under debate, some hypothesis point to the presence of alternative water channels (yet not described), paracellular water reabsorption, influx of sodium into the tubular lumen or alternatively, a process of intrarenal urea recycling with tubular secretion mediated by urea transporters UT-A1, UT-A2 and UT-A3 (181, 182).

In the long loop nephrons (LLN) on the other hand, the high expression of AQP1 plays an important role into water transmembrane passive transport (183, 184). Urine then enters the thick ascending limb (TAL, "the diluting segment"), which is impermeable to water, while salt (NaCl) is reabsorbed in an active fashion via the co-transporter NKCC2, resulting in urinary dilution (185).

Figure 2.1. Mechanism of urinary concentration and dilution



Isotonic tubular fluid accesses the Loop of Henle to undergo first concentration (Thin descending limb, TDL) and subsequent dilution (Thick ascending limb) with hypotonic fluid exiting to the final segments of the nephron. While mechanism involving tubular fluid concentration in the TDL are not completely clear in the present (passive water reabsorption *vs* solute influx), active sodium reabsorption in the TAL is mediated by apical NKCC2 and driven by the basolateral N/K ATPase pump. Final urine concentration in the collecting duct is mediated by Aquaporin 2 channels and dependant on ADH. Extracted and modified from Bockenhauer and Bichet, 2016 (20).

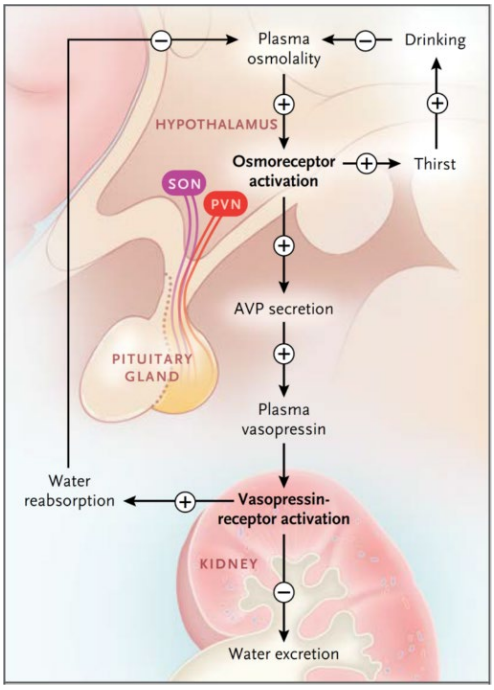
In the interstitium, solutes are accumulated following an osmotic gradient from smaller to larger concentrations in direct relation to the medullar depth. Further removal of sodium chloride occurs in the distal convoluted tubule via SLC12A3 (also known as NCC). By the time this almost “final urine” enters the segments sensitive to the antidiuretic hormone (connecting and collecting tubules) the osmolality is approximately 50-100mOsm/kg. The final osmolality of the urine is solely dependent on the availability of water channels. If these channels are present and functional, water exits the tubule following the interstitial concentration gradient and the urine is concentrated. If no functional water channels are present, dilute urine (Osm: 50-100mOsm/kg) will be excreted (186).

2.1.2.2. Antidiuretic hormone (ADH):

ADH (also known as vasopressin or arginine vasopressin, AVP) is a basic cyclical nonapeptide with a disulphide bridge between cysteine residues at positions 1 and 6 that is critical for biological action. The majority of ADH is synthesized and stored in a biologically inactive from in the magnocellular neurons from two hypothalamic nuclei: the supraoptic (SON) and paraventricular nuclei (PVN). Within storage granules, the hormone is cleaved into the biologically active form and transported down the long neuronal axons to the posterior pituitary and stored there. Following appropriate stimuli, ADH is secreted from the posterior pituitary into the circulation as a biologically active hormone (Figure 2.2) (187, 188).

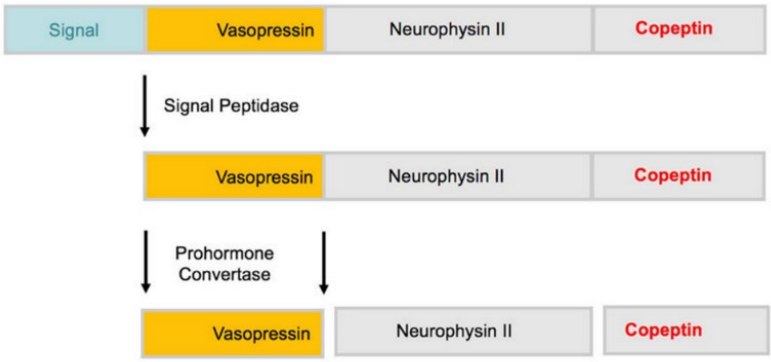
Figure 2.2. ADH Physiology

A raised plasma osmolality stimulates specific hypothalamic osmoreceptors that subsequently induce ADH secretion by the posterior pituitary gland. Increased levels of ADH ultimately stimulates water reabsorption in the distal segments of the nephron with the aim to reduce plasma osmolality. The hypothalamic osmotic sensors do also stimulate thirst to help restore plasma osmolality. Extracted and modified from Knepper, Kwon and Nielsen, 2015 (22).



ADH's encoding gene is located on chromosome 20 (OMIM 192340). It is formed of 3 exons (and 2 introns) and the initial product from its subsequent translation is a 145 aminoacids pre-prohormone that includes an aminoterminal signal peptide, ADH, another molecule called NPII (neurophysin-II) and a carboxyterminal peptide without biological function called Co-peptin which will be later discussed (Figure 2.3) (189).

Figure 2.3. Structure of prepro-vasopressin-neurophysin II



The polypeptide is stored in neurosecretory granules of magnocellular neurons and during its axonal transport to the posterior pituitary enzymatic cleavage of the prohormone results in the final products: vasopressin (ADH), neurophysin II and the glycoprotein copeptin. Extracted from Refardt and Christ-Crain, 2020 (23).

ADH does have multiple and diverse actions in relation to the targeted cell and the type of receptors (V1-V3, all G-protein coupled receptors) that they may express on plasma membrane (187).

Table 2.1. ADH V1-3 receptors and their specific characteristics

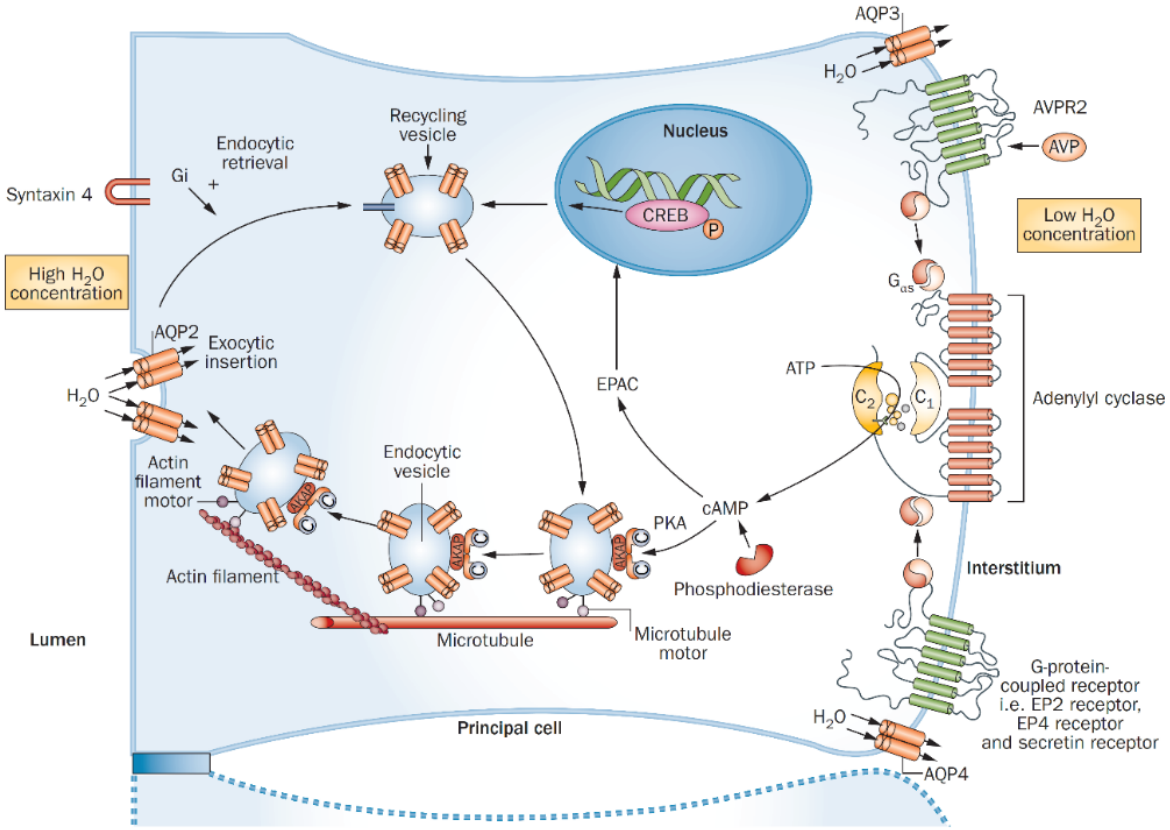
Receptor	V1 (a/b)	V2	V3
Expression	Vascular smooth muscle, liver, platelets and CNS	Basolateral membrane of distal nephron and vascular smooth muscle.	Pituitary
Amino acid structure (humans)	418 amino acids	370 amino acids	424 amino acids
Second messenger system	Gq/11 mediated PLC activation: Ca2+, inositol triphosphate and diacyl glycerol mobilization	Gαs mediated adenylate cyclase activation: cAMP production and PKA stimulation	As V1
Physiological effects	Smooth muscle contraction, stimulation of glycogenolysis, enhanced platelet adhesion, neurotransmitter and neuromodulatory function	Increased production and action of AQP-2 and smooth muscle traction.	Enhanced adrenocorticotropic hormone release

1. Cardiovascular: mainly mediated by the type 1 receptors (V1R). ADH is a potent pressor agent as its other name (vasopressin) suggests, although marked effects on systemic blood pressure are apparent only seen at plasma

concentrations significantly higher than those observed under physiological conditions (190).

2. Pituitary and CNS: a group of PVN parvocellular neurons co-expressing ADH and corticotropin releasing factor (CRF) terminate on the hypophyseal-portal bed feeding the anterior pituitary. ADH on its own has a weak effect on stimulating ACTH secretion, however this action is enhanced by CRF. The effects of ADH on pituitary ACTH release are mediated by the corticotrophic specific V3R. Vasopressinergic nerve fibers and ADH receptors are also widespread elsewhere in the central nervous system (CNS), outside the neurohypophysis. In rodents for example, central vasopressinergic systems have been shown to have important roles in mediating complex social behaviour (191).
3. In the kidney, ADH binds to vasopressin type-2 (V2 or AVPR2) receptors on the basolateral membrane of the principal inner medullary collecting duct cells and in the DCT (Figure 2.4). Upon binding of ADH the V2 receptor (already coupled to a trimeric G protein) is activated, causing dissociation of the G-protein from the receptor. GTP-aGs can then bind to and activate the membrane associated adenylate cyclase (AC), which results in an increase in intracellular cyclic adenosine monophosphate (cAMP). The elevated cAMP levels stimulate protein kinase A (PKA), leading to phosphorylation of aquaporin-2 channels (AQP2) which in turn initiates a redistribution from intracellular vesicles to the apical plasma membrane, rendering this membrane water permeable (192). The increase in apical membrane permeability allows water to flow from the tubular lumen to the hypertonic medullary interstitium, via AQP2 in the apical membrane and via AQP3 and AQP4, constitutive water channels in the basolateral membrane. This then leads to the formation of concentrated urine. Upon fluid intake, ADH release into the blood decreases, AQP2 is redistributed into intracellular vesicles, and water reabsorption is reduced (193).

Figure 2.4. Water reabsorption in principal cells within the distal nephron



AVPR2 receptor (expressed on the basolateral side of the principal cell) as a classic GPCR will ultimately stimulate cAMP production via activation of adenylyl cyclase. cAMP as a key intracellular messenger will have several functions both at transcriptional level but also at intracellular trafficking increasing exocytosis of endocytic vesicles carrying water channel AQP2. Water flow through principal cells will start at apical level with AQP2 and finalise at basolateral side with others such as AQP3 and AQP4. Notice also that alternative GPCR receptors such as EP2, EP4 and secretin are also able to increase intracellular cAMP levels and could offer potential therapeutic alternatives in the future for treatment of NDI secondary to AVPR2 dysfunction. Extracted from Bockenhauer and Bichet, 2015 (20).

ADH release is regulated by changes in plasma osmolality (as little as 2%) but can also occur in response to non-osmotic stimuli. These non-osmotic stimuli are generally related to changes in either total blood volume or the distribution of extracellular fluid. Patients with depleted effective circulating volume may secrete ADH even in the presence of low plasma osmolality. In addition, physical pain, emotional stress, and certain drugs (e.g., nicotine) influence the release of ADH.

i) Osmoregulation: in healthy individuals, increments in plasma osmolarity stimulate the secretion of ADH and this positive relationship is defined by two characteristics: a) the osmotic threshold for ADH release (284mOsm/L) and b) the sensitivity of the osmoregulatory mechanism (194). Some exceptions to these points are: exaggerated ADH released after rapid increments in plasma osmolarity; the lowered threshold for ADH release as consequence of hormonal influences (pregnancy, luteal phase of ovulation and ovarian hyperstimulation syndrome); enhanced response to osmotic stimulation with aging and the negative effect on ADH secretion that non-osmosensitive oropharyngeal afferents can have after drinking fluids (187).

ii) Baroregulation. Reductions in volemia and/or systemic blood pressure also trigger both an autonomic and endocrine cascade which includes ADH release and at the same time this effect can be influenced by certain neurohormonal factors. For example, catecholamines like norepinephrine potentiate the baroregulated release of ADH while the atrial natriuretic peptide has an inhibitory effect. In addition, the baroregulatory mechanism when triggered, for example by hypovolemia, has the potential to stimulate ADH release despite significant hyponatremia (195).

iii) Apart from the above two main mechanisms, other factors can also stimulate ADH release. Nausea, abdominal surgery, neuroglycopenia, systemic immune stressor and mediators like histamine and bacterial lipopolysaccharide (187).

2.1.2.3. Aquaporin type 2

AQP2 is 1 of the 13 members of the aquaporin family. After transcription AQP2 is folded into its native monomeric conformation in the endoplasmic reticulum followed by a process of homotetramerization (196). The tetramers move to the Golgi apparatus and undergo complex N-glycosylation. Now the channels are fully functional and ready to be transferred to the apical membrane, but until the right stimuli will come they will be stored in endosomal vesicles (197).

For the AQP2 water channels to be successfully translocated to the apical membrane several critical steps must be taken. One of the most relevant is the phosphorylation of the aminoacid 256 (serine) located in the carboxy terminal domain, in what constitutes a PKA (Protein Kinase A)-consensus site (198). Another critical event for successful translocation of AQP2 is the association of PKA to PKA-anchoring proteins (AKAPs), which facilitates targeting of PKA to AQP2-bearing vesicles (199). Studies using oocytes as a model system indicated that for plasma membrane localization three out of four subunits in an AQP2 tetramer need to be phosphorylated (200). PKA is the main kinase for AQP2 phosphorylation, but other kinases may potentially participate in the regulation of AQP2 trafficking. Apart from PKA sites, putative phosphorylation sites for PKG, PKC, and casein kinase II are also present in the AQP2 sequence.

Even after a correct tetramerization, N-glycosylation and phosphorylation, the channels still need to be able to take the last two steps: docking and fusion of AQP2-containing vesicles with the apical membrane. The way how this process happens is similar to exocytosis and involves vesicle v-SNAREs (soluble NSF attachment protein receptors) and target membrane t-SNAREs. The apical membrane-specific t-SNARE is syntaxin 4, which interacts specifically with the v-SNARE protein VAMP2 located on the cytoplasmic side of AQP2-containing endosomal vesicles (201-204). V- and t-SNAREs are recycled by the AAA-type ATPase NSF.

Another important factor involved in the successful transport of AQP2 to the apical membrane is in fact the correct organization of the actin cytoskeleton (205, 206), providing a functional network that anchors the AQP2 bearing vesicles during the resting periods. ADH has been found to induce the depolymerization of apical F-actin in rat inner medullary collecting duct, and as a result the fusion of AQP2-carrying vesicles with the apical membrane occurs (207), hence the reorganization of the apical actin network may be critical in promoting the trafficking of AQP2 bearing vesicles. Rho inhibition through PKA-mediated phosphorylation of Rho-GDP dissociation inhibitor (Rho-GDI) is shown to be a key event for actin reorganization inducing AQP2 translocation (205, 206).

Counterbalancing increased expression on the plasma membrane, AQP2 is internalized by endocytosis. During this process, AQP2 accumulates in clathrin-coated pits and is internalized via a clathrin-mediated process. Endocytosis is regulated by short-chain ubiquitination at lysine 270 (K270) in the AQP2 terminal tail (208-210).

Recycling of AQP2-containing vesicles implies that these need to be redistributed to the perinuclear region in a process mediated by dynein-dependent transport along microtubules (211). Initial endocytosis is mediated by the specific protein Rab5 (from the Ras superfamily of GTPases) and subsequently the endosomes or multivesicular bodies (MVBs) can follow a slow recycling pathway (mediated by Rab11) or marked for lysosomal degradation (212). Prolonged K270 ubiquitination induces MVB trafficking and localization to internal vesicles of MVBs followed by lysosomal degradation, while deubiquitination increases localization to early endosomes and the limiting membrane of MVBs and enables AQP2 recycling (210).

Long-term adaptation to circulating ADH levels, for instance, in a dehydrated state, is accomplished by increasing the expression of *AQP2* mRNA and protein. PKA-mediated phosphorylation of a cAMP-responsive element-binding protein 1 (CREB-1) stimulates synthesis of AQP2 by binding to the *AQP2* gene promotor and activating its transcription, which increases intracellular AQP2 levels (213).

2.1.3. Genetics and Nephrogenic Diabetes insipidus

To date three different inheritance patterns of primary NDI have been described.

I. Most cases (up to 90% in most of the published series) are recognised to be X-linked recessive (MIM 304800). In these families, female carriers tend to be generally asymptomatic, however their affected male sons display the complete clinical picture (174, 214). With the expansion of molecular biology, the major NDI locus was first mapped to the distal region of the long arm of the X chromosome (Xq28)(175), and shortly after mutations in the *AVPR2* gene were shown to be causative of X-linked NDI (215-217).

II. In a minority of families (about 10 %) inheritance was found to not follow an X-linked pattern. In these families, females are equally symptomatic, and their clinical features were equal to the affected male members of the family (218-220). Pedigrees were showing autosomal inheritance patterns, mostly

recessive (MIM 222000) but some also dominant negative (MIM 125800). These two autosomal forms of NDI are actually caused by mutations in the same gene, *AQP2*, the ADH-sensitive aquaporin-2 water channel (221, 222).

Due to NDI being a very rare disease, its exact prevalence remains unknown although is estimated to be around 1:100.000 individuals. Efforts have been taken to better define this, for example the estimated prevalence of NDI in Quebec (Canada), is 8.8:1,000,000 males (223). Whether this is representative for the rest of the world is unclear as genetic events in specific populations (e.g., a founder effect), can influence the incidence of NDI in different regions (eg: Utah- USA and Nova Scotia- Canada where it is elevated) (186).

2.1.3.1. X-Linked Nephrogenic Diabetes Insipidus: Mutations in the AVPR2 Gene

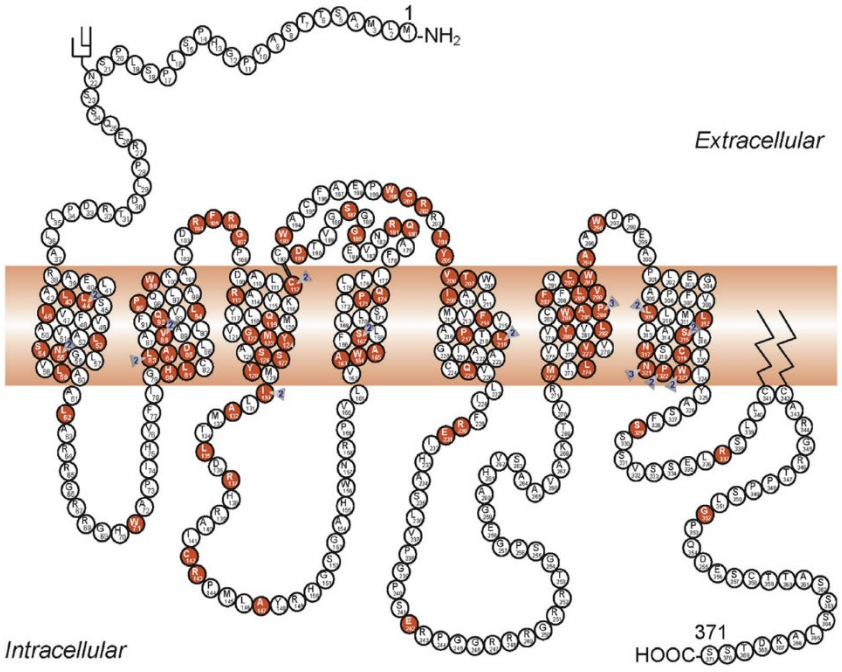
The X-linked form of NDI is caused by loss-of-function mutations in the *AVPR2* gene (OMIM 300538). *AVPR2* is a short gene, made of three exons separated by two introns, with two isoforms known to be generated by alternative splicing (224). *AVPR2* is localized on the X chromosome on locus Xq28. The cDNA encodes a protein of 371 amino acids with a predicted molecular mass of ~ 40 kDa and shares the general structure of a G-protein-coupled receptor (GPCR) with seven hydrophobic transmembrane (TM1-7) α-helices, connected by 3 extracellular (EL1-3) and 3 intracellular (IL1-3) loops (Figure 2.5) (225). The receptor's tertiary structure resembles a barrel, with the seven transmembrane helices forming a cavity within the plasma membrane that serves a ligand-binding domain (often covered by EL-2).

The receptor contains one unique consensus sequence site for N-linked glycosylation in the extracellular amino-terminus and multiple phosphorylation sites for G-protein-coupled receptor kinases (GPCRKs) represented by a serine cluster in the carboxy-terminus (226-228). The N-terminal part of the protein including the TM-1 domain and the positively charged IL-1 are important for the adequate insertion and orientation in the membrane (229). A conserved glutamate-dileucine $E(X)_3LL$ motif in the intracellular carboxy-terminal part of the receptor has been found to be essential for receptor transport from the endoplasmic reticulum (ER) to the Golgi apparatus (230)

and therefore membrane expression. Two conserved adjacent cysteines $C(X)_3C$ in the C-terminus are palmitoylated, thereby anchoring the carboxy tail to the plasma membrane and controlling the tertiary structure of this region of the receptor (231).

Almost 300 distinct disease-causing mutations in *AVPR2* have been identified and the number is constantly increasing (232). Mutations are equally distributed along the entire sequence rather than clustered in a single domain, although fewer in the N- and C-terminus. Almost two thirds of the mutations are missense/nonsense (61%). Nucleotide deletions and insertions causing frameshifts (27%), nonsense mutations (13%), large deletions (8%), large in-frame insertions/duplications (1%), splice-site mutations (1%), and complex rearrangements (1%) account for the remainder of mutations according to Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php ; accessed 23rd March 2025).

Figure 2.5. AVPR2 secondary structure



Aminoacids marked in red have been found mutated in patients with NDI, as per Bockenhauer and Bichet, 2016 (58).

Several mutations are recurrent as evidenced by the fact that these mutations were found on different haplotypes in ancestrally independent families. From these, the most common ones seem to be allocated in potential mutational hot spots (D85N,

R106C, R113W, R137H, S167L, and R337X). *AVPR2* mutations seem to have a similar distribution among diverse ethnical groups with no preference (233, 234).

There are also known polymorphisms in AVPR2, including G12E (in the N-terminal tail), described in non-affected individuals with proper functioning of the V2 receptor (235).

The molecular mechanism underlying the lack of response to AVP differs according to the specific mutation. One way of classifying the different pathogenic mutations is to do it according to the cellular fate of the V2R. This practical and simple approach can help to link physiology and pharmacological research (236, 237):

I. Class I includes those mutations with a deleterious effect on processing and/or stability of mRNA. For example: promoter alterations, exon skipping, or aberrant splicing. Class I also includes frameshift and nonsense mutations, resulting in truncated proteins, some examples are W71X, ?161X, and R337X.
II. Class II are missense or insertions/deletions of one or more nucleotide triplets, resulting in fully translated proteins. Because of these changes, mutant receptors are misfolded and retained in the endoplasmic reticulum (ER: the organelle that has the cellular quality control over adequate folding and maturation of proteins). Misfolded proteins are subsequently mostly targeted for proteasomal degradation (238). Taking in account that more than 50% of the V2R mutations are missense type, intracellular entrapment of mutant V2R and their rapid degradation are the most frequent cause of NDI, indeed cellular expression revealed that the consequence of most of these mutations is ER-retained proteins. The amount of retention and degradation varies within this class, since different mutations affect protein folding to a different extent, sometimes allowing partial transport of at least partially active receptors to the plasma membrane (239).
III. Class III. In these cases a full-length receptor is expressed at the cell surface, however receptor-ligand interaction is impaired and as a consequence the signaling process is reduced or absent (240). Class III mutations are divided into two minor groups.
    a. III-a mutations interfere with binding of or signal transduction to the coupled trimeric G-protein, leading to a reduced activation of adenylate

cyclase (AC) and thus formation of cAMP. Mutations in this group are missense mutations and inframe deletions, mostly located in transmembrane and intracellular domains. Examples are D85N and P322S (241).

b. III-b mutations impair the binding to AVP. These mutations, which are also mostly missense and small inframe deletions or insertions, especially involve residues thought to be in or close to the AVP-binding pocket, of which delR202 is a classic example (242).

IV. Class IV is assigned to all mutations that affect protein function without interfering with protein synthesis or ligand binding. One good example is the R137H mutation, changing the well-conserved DRY/H motif of GPCRs. The effect of this mutation is constitutive internalization of V2, leading to reduced expression of the receptor in the plasma membrane and thereby reduced adenylate cyclase-dependent cAMP signaling upon AVP binding (243, 244).

Occasionally, mutations can share features of different classes and the V2R mutants can be partially retained in the ER (Class II) but also expressed in the plasma membrane either showing a reduced G-protein coupling (Class III-a) or impaired AVP binding (III-b). This can explain for example partial responses to DDAVP when administered in high doses (245).

2.1.3.2. Genotype-Phenotype Correlations in X-Linked NDI

Most pathogenic mutations in the *AVPR2* gene are associated with complete loss of the ADH-mediated urinary concentrating ability, yet there are also some with a milder phenotype. In this later group patients tend to present later in childhood rather than during the neonatal or early infancy period and they do not necessarily have an impaired growth. This milder phenotype sometimes is defined as “partial” NDI and some of the known variants associated with it are: D85N, V88M, G201D, M311V, N317S, P322S, and S329R (246-249).

An interesting example is P322 where mutation to serine (P322S) results in partial NDI, whereas mutation to Histidine (P322H) is associated with a severe phenotype. This particular case has been study *in vitro* by Ala and collaborators (242) by expressing both P322H and P322S in COS-7 cells and comparing receptor function

between each other and against wild-type. Consistent with the clinical phenotype, P322H mutant had totally lost the ability to stimulate the Gs/adenylate cyclase system, whereas the P322S mutant was able to stimulate adenylate cyclase (although less than the wild-type receptor). 3D modelling of the P322H and P322S mutant receptors suggests that P322H receptor loss of function could be related to the formation of hydrogen bonds between Histidine 322 and the carboxyl group of Aspartate in position 85 (D85), which does not occur in the P322S receptor (242).

Intrafamilial variability of the X-linked NDI phenotype has also been described. A very instructive example is a Belgian family case published by Kalenga and collaborators where the same reported variation (R137H) was associated with a severe phenotype in a member and at the same time with a milder presentation in his brother (250). Genetic and/or environmental modifying factors are likely to account for this intrafamilial phenotype variability.

Interestingly, when this arginine in position 137 is substituted to cystine/leucine, it results in gain of function with a complete opposite phenotype known as nephrogenic syndrome of inappropriate antidiuresis (NSIAD) (251).

2.1.3.3. The Autosomal Recessive and Autosomal Dominant Forms of NDI: Mutations in AQP2.

Both autosomal (recessive and dominant negative) types of NDI are caused by mutations in the AQP2 water channel gene (OMIM 107777).

The *AQP2* gene is a small gene consisting of 4 exons and comprising 5 kb of genomic DNA. The product of the gene transcription is a 1.5 kb mRNA which is translated into a 271 amino acids protein with a predicted molecular mass of 29 kDa (176). AQP2 belongs to a family of integral membrane proteins, aquaporins, which predominantly act as selective water transporters. Aquaporins can be found in both animal and plant biological kingdoms and specifically in mammals, 13 different aquaporins have been identified to date, 8 of which (aquaporins 1-4, 6-8, and 11) are highly expressed in the kidney. AQP2, like other aquaporins, are assembled in the membrane. The 29kDa monomers, containing 6 membrane-spanning α-helical domains with intracellular N-

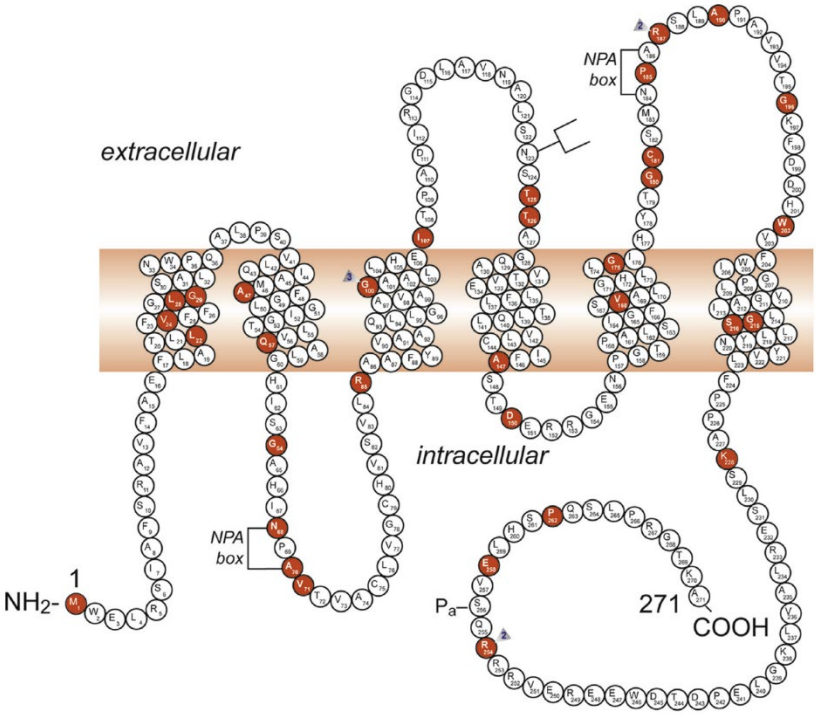
and C- terminals, must adopt a quaternary structure of homotetramers to reach a functional conformation.

The 6 transmembrane domains are connected by 5 loops (A-E). Initial studies on AQP1 have allowed to better understand the mechanism of selectivity for water of aquaporins while more recent works on AQP2 have confirmed the previous model (252, 253). The water pore is formed between the first and sixth transmembrane domains and is lined by the intracellular B-loop and the extracellular E-loop. AQP2 is exclusively localized in the apical membrane and a subapical compartment of collecting duct cells and insertion is promoted by ADH.

To date, about 65 putative disease-causing mutations in *AQP2* have been identified in families with autosomal recessive NDI (232, 254). These include 51 missense/nonsense, 11 small insertions/deletions and 3 splicing mutations; according to Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php , accessed 23rd March 2025). Most mutations are found between the first and last transmembrane domain of AQP2 (Figure 2.6).

In vitro studies in Xenopus laevis oocytes have revealed that most *AQP2* missense mutations that are causative for autosomal recessive NDI are class II mutations, leading to misfolding of the mutant protein, retention in the endoplasmic reticulum (ER), and rapid degradation of AQP2 (255). Indeed, AQP2 in the urine of patients with recessive NDI tends to be absent (256).

Figure 2.6. AQP2 schematic representation of secondary structure



Secondary structure and its recognised putative mutations (red marked aminoacids) as per Bockenhauer and Bichet, 2016 (58). Notice highlighted (red) known putative mutation points, NPA boxes (involved in apical membrane targeting), N-glycosylation point (N123), Pa phosphorylation point (S256) and Ub ubiquitinisation (K270).

Other in vitro studies, looking at overexpression of certain AQP2 mutants in oocytes and CHO (Chinese Hamster Ovary) cells (A147T, T126M, G64R, L22V, A47V, and T125M), have shown a certain degree of water permeability (257, 258). This suggests that at high expression levels, some AQP2 mutants can escape from the ER and be routed to the plasma membrane, where they can be functional. Such projects provide highly valuable information regarding potential new therapeutic options. Indeed, subsequent projects have already assessed the possibility of restoring mutant trafficking and allowing functional channels to reach plasma membrane, this mechanism is discussed in detail in the treatment section (259).

A missense mutation in *AQP2* that would be of interest to discuss is P262L, located in the C-terminal tail. Pathogenic variants in this region were initially thought to be associated with dominant NDI inheritance, however subsequent studies did find it was associated with AR NDI instead (260). In vitro studies have shown that cells which are

simple heterozygous for P262L express functional water channels in the apical membrane, and this happens as P262L mutants and wild-type monomers can associate in functional hetero-tetramers. Therefore, the apical sorting signal of the wild-type is dominant over the missorting signal of P262L. This is fundamentally different from dominant NDI, where mutants retain wild-type subunits in intracellular locations.

Further analysis in those patients who were single carriers of P262L have shown that they were instead compound heterozygous and therefore carriers of a second mutation on the opposite allele. These specific mutations were R187C and A190T. *AQP2*-R187C and *AQP2*-A190T mutants tend to be retained in the ER and therefore cannot interact with P262L, in consequence P262L mutants will only be able to assemble with themselves in homo-tetramers, and these ones tend to be retained in intracellular vesicles because of the mutated apical sorting signal. Thus, there is lack of sufficient AQP2 proteins in the apical membrane, resulting in NDI (260).

To date 13 pathogenic variants have been described causing autosomal dominant NDI. The identified mutations in *AQP2* comprise 6 small deletions, 1 insertion, and 6 missense mutations (261). All mutations causing dominant NDI are present in the coding region of the C-terminal tail of AQP2. Although this domain is not part of the pore-forming segment, it does contain important sorting signals for an adequate intracellular transport of the protein (241, 262).

The relevance of affecting only the C-terminal tail relies on the fact that indeed all dominant NDI mutants can fold with wild types and create functional channels, however these hetero-tetramers tend to be missorted to wrong locations within the cell (eg: late endosomes/lysosomes and the basolateral membrane). This is the case for example for E258K, where expression studies in polarized cell lines showed that hetero-tetramer AQP2-E258K/wild type is routed to the Golgi complex or late endosomes/lysosomes (221, 263). In addition, mistargeting to the basolateral membrane has been reported in association to the following indel mutations: 721delG, 763-772del, 812-818del and 779-780insA (262, 264). Another deletion (727delG) has also been shown to cause mistargeting of AQP2 to both basolateral membrane and late endosomes/lysosomes (264).

Several mechanisms have been proposed to explain inappropriate AQP2 hetero-tetramers trafficking in dominant NDI. One of the best studied is the impaired phosphorylation of serine 256 which serves as an introducible apical sorting signal; its inactivation implies that adequate trafficking signal is overruled by basolateral sorting signals, or reprogrammed to induce basolateral sorting, all causing intracellular misrouting (265-268). It is interesting how the S256A variant has been associated with reduced apical presence of AQP2 channels while on the contrary S256D induces constitutive membrane expression of AQP2 (269). While the S256D variant could in theory be associated with the nephrogenic syndrome of inappropriate antidiuresis, a normal ubiquitylation process plays an essential role in controlling the cellular distribution of AQP2 (270).

Lastly, patients with dominant NDI typically have a milder phenotype than the recessive forms, suggesting that some wild type homo-tetramers are still able to be assembled and reach the apical membrane (263).

2.1.4. Nephrogenic Diabetes Insipidus in Females

Classical clinical and biochemical features of NDI have been described within some female members of affected families. With the identification of the *AQP2* gene it was thought that finally all these cases could have an explanation and indeed a proportion of them were later identified as carriers of recessive or dominant mutations in this gene.

Subsequent studies however, described several cases where symptomatic females were not carrying pathogenic variants in *AQP2*, but were heterozygous for *AVPR2* (271-274). Some of these women could not achieve a maximal urinary osmolality of >200mOsm/L after desmopressin administration. Interestingly, family studies were showing that the same pathogenic mutation present in different female individuals could either be associated with polyuria or sufficient urinary concentration capacity (274, 275).

Most likely, the observation of such different clinical features in carriers of the same *AVPR2* mutation is caused by skewed X-inactivation (276). The X-chromosome

inactivation is a physiological process that takes place during early embryonic development in female individuals. The expected random inactivation of the paternal or maternal X chromosomes in every cell should theoretically result in mosaics with a 50-50 inactivation of parental X chromosomes across all cells. However, this inactivation process can diverge from an equal distribution and as a result a female patient that carries a pathogenic mutation in a single X chromosome can manifest a certain phenotype due to a significantly greater inactivation of the non-mutated chromosomes in a given tissue (276).

X-inactivation patterns can differ between tissues, the *AVPR2* gene for example is expressed in kidney, liver and blood vessels, among others; this is the reason why female patients can have different patterns of response to ADH for example in vascular tone, production of Factor VIII and urinary concentration capacity (277).

This hypothesis has been specifically tested in X-inactivation patterns studies performed in peripheral blood leukocytes of female carriers by using methylation analysis of the polymorphic CAG repeat in the androgen receptor gene (*AR*) (278). Random X-inactivation (50-65%) was found in asymptomatic women, while most carriers with clinical features of NDI had skewed X-inactivation patterns favouring the mutated X allele.

Interestingly, random X-inactivation has been identified in a few females with overt clinical NDI, highlighting the fact that although NDI phenotypes may correlate with the X-inactivation patterns in women with heterozygote *AVPR2* mutation, the clinical phenotype cannot always be predicted by evaluation of X-inactivation patterns in peripheral blood cells, probably due to the fact that X-inactivation ratios within an individual can vary between different tissues (279).

2.1.5. Acquired Nephrogenic Diabetes Insipidus

Although the hereditary forms of NDI are relatively rare, a wide range of pathologic conditions and drug treatments can lead to acquired NDI. The urine osmolality obtained after DDAVP administration in these acquired disorders is generally higher than in congenital NDI. As with primary NDI, the most common mechanism in acquired

NDI is decreased expression of AQP2 or deregulated AQP2 trafficking to the apical membrane (47, 280-282).

Prolonged treatment with lithium for example, the drug of choice for treating bipolar disorder (prescribed to 1 in 1000 of the population), leads to the development of NDI in at least 20% of treated individuals (283). The development of lithium related NDI is believed to occur in two phases:

1. First, reduced AQP2 expression (284). Lithium enters the cells via epithelial sodium channel (ENaC) and accumulates in principal cells (285, 286). How lithium downregulates AQP2 is not clear but likely involves glycogen synthase kinase type 3 (GSK3), which is important in ADH-regulated antidiuresis and is inhibited by lithium (287-289). Lithium also influences AQP2-mediated water reabsorption by increased tubular release of prostaglandin E2 (PGE_2) (288).
2. Second, it decreases the percentage of principal cells in the collecting duct, increasing the proportion of intercalated cells, involved in acid-base homeostasis (290). The exact contribution of this collecting duct remodelling in the lithium-induced resistance to ADH remains to be elucidated.

A rare but interesting cause of secondary NDI is indeed Bartter syndrome. Bartter syndrome is characterised by a salt reabsorption defect in the TAL (thick ascending loop) also known as the diluting segment. Though patients with Bartter syndrome typically present with polyuria, the impairment of the counter-current mechanism is expected to result in isosthenuria (similar osmolarity in urine to plasma) rather than hyposthenuria which is the phenomenon observed in patients with NDI (291). Nevertheless, multiple reports in the literature have provided evidence that some individuals with pathogenic mutations in either *SLC12A1* or *KCNJ1* can indeed present with a phenotype of NDI (182, 292).

The reasons why a minority of patients with Bartter syndrome type 1 or 2 present with NDI remains unclear and it seems that other factors, genetic or environmental may be leading to this complication. Some of those which have been proposed are:

Hypokalaemia: linked to reduced AQP2 expression (47). Arguably, it cannot be a factor on its own as patients with other similar conditions like Gitelman syndrome do not develop a severe urinary concentration defect. Moreover, patients with Bartter type like 3 usually have more severe hypokalaemia, yet do not usually present with NDI (293).

a. Hypercalciuria/Nephrocalcinosis. Hypercalciuria is thought to affect renal concentrating ability via activation of the calcium-sensing receptor CaSR, which is expressed at the luminal aspect of the collecting duct (294). Nephrocalcinosis could potentially alter the physical relationship between tubular lumen, interstitium and vasa recta, interfering with water reabsorption (292).

High urinary flow and pressure may cause changes in the expression of AQP2 (295).

2.1.5.1. Causes of secondary nephrogenic diabetes insipidus

1. Monogenic diseases associated with secondary NDI (192):
   - Renal Fanconi Syndromes (RFS)
   - Bartter Syndrome (type 1 or type 2)
   - Familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC)
   - Distal renal tubular acidosis (dRTA)
   - Apparent mineralocorticoid excess (AME)
   - Ciliopathies (nephronophthisis, Bardet-Biedl syndrome, etc.)
2. Other renal diseases:
   - Obstructive uropathy
   - Renal dysplasia
   - Post-ischaemic damage
   - Amyloidosis
   - Sarcoidosis
   - Chronic kidney disease
3. Sickle-cell disease
4. Drug induced:
   - Lithium

- Ifosfamide
- Amphotericin B
- Tetracyclines

5. Biochemical abnormalities:
   - Hypercalcemia, hypercalciuria and nephrocalcinosis
   - Hypokalemia

2.1.6. Clinical Manifestations

The most common clinical presentation is dehydration secondary to excessive urinary loss of free water and secondary electrolyte disbalances in particularly hypernatremia.

In contrast to other congenital polyuric disorders, such as antenatal Bartter syndrome, pregnant women who carry a baby with NDI do not develop polyhydramnios, as the osmotic load is cleared by the placenta. The urinary concentrating defect in NDI manifests postnatally, so clinical features tend to emerge as early as the first weeks of life. As urinary volume is directly proportional to urine osmolality and osmolar intake, babies who are breastfed exclusively from birth typically have a later presentation. This is explained by the fact that breast milk provides a lower renal osmolar load (lower protein and salt content) compared to formula feeds (mainly cow's milk derived). Formula administration therefore results in a higher demand for free water and babies are at increased risk of hypernatraemic dehydration, especially if there are additional fluid losses, such as with diarrhoea or impaired fluid intake (vomiting or neurological impairment).

Affected babies typically present with irritability, poor feeding, and weight loss/failure to thrive (296). Patients are very thirsty and suck avidly, their high fluid intake typically aggravates gastroesophageal reflux, vomiting tends to occur during or shortly after feeding.

Hypernatraemic dehydration in general facilitates a better preservation of extracellular volume compared to normo- or hyponatraemic dehydration. Yet, with severe dehydration, patients may present anyways with classical signs of dehydration, such as dryness of the skin, loss of normal skin turgor, recessed eyeballs, increased periorbital folding, depression of the anterior fontanel, and a scaphoid abdomen.

Another symptom typically used for the assessment of dehydration is urine output. As diuresis is typically high in NDI, the presence of a maintained large urine output often confounds paediatricians and emergency physicians (most of them unfamiliar with this rare disorder), so this is misinterpreted as absence of severe dehydration.

Another common feature in hypernatraemic dehydration is an intermittent rise of body temperature (occasionally even fever), predominantly in very young children and particularly under 1 month of age leading to intravenous administration of antibiotics and misleading diagnosis of sepsis. Body temperature is usually normalized by rehydration. Rarely and in the most severe cases, seizures can manifest at presentation but they are more likely to happen during rehydration, particularly if this is administered quickly with a consequent rapid drop in plasma sodium concentration (>10-12mmol/L/day) (297).

Constipation is another common symptom, as the gut maximises water extraction. Nocturia and bedwetting are common complaints later in childhood.

Failure to grow is common in untreated patients. Indeed in a retrospective study (N=30) of male patients with NDI, the majority of boys' height was below the 50th centile with most of them having standard deviation scores (SDS) below -1 (298). In contrast, appropriately treated patients, can potentially reach normal adult height SD scores, as normalization of water and electrolytes balance helps to facilitate catch-up in growth. Bone maturation is generally not delayed (299). Weight SDS are generally more decreased in comparison to height ones but tend to equalise by school age (298). Initial feeding problems are mostly secondary to a preference of the patient for water over food, result in a hypocaloric diet with failure to thrive. Feeding issues can be addressed by tube feeding (either nasogastric or via gastrostomy) (300).

Severe learning disabilities have long been considered a significant complication of untreated/delayed treated NDI and assumed to be the consequence of recurrent alternating episodes of brain dehydration acutely followed by cerebral oedema during rehydration phase (234, 301, 302). In addition to these studies, other reports have provided evidence of the presence of intracranial calcifications in patients with NDI which are thought to have developed after previous injuries such as haemorrhages

and/or necrosis (303, 304). Cerebral calcifications have been found to correlate with mental impairment (305).

Although even nowadays learning disabilities can still be found in some patients with NDI, the development of specialised hospital units and subsequent earlier diagnosis compared to the past makes severe mental impairment an extremely rare feature. Exact estimates of the current frequency of mental impairment under modem treatment are unknown, particularly as NDI is a very rare condition and to get a better idea of this has been one of the most important motivations for the development of this project.

In recent years, smaller cohort of patients have been analysed, and as such a study (n=17) reported on 2 individuals with an intelligence quotient (IQ) below 2 SD from the mean. The rest of the cases were within or above the normal range with only one individual scoring between -1 and -2 SD (305). The psychological development of NDI patients is thought to be influenced by a persistent desire for drinking and the need for frequent voiding, which strongly compete with important activities such as playing and learning. Thus, many NDI patients are characterized by hyperactivity, distractibility, short attention span, and restlessness. In the psychometric study mentioned earlier, criteria for attention-deficit/hyperactivity disorder were met in 8 of 17 tested NDI patients (305).

Persistent polyuria can result in the development of flow uropathy: megacystis (markedly enlarged bladder), trabeculated bladder wall, hydroureter and/or hydronephrosis (298, 306, 307). Urinary tract dilatation may be seen on ultrasound examination already in infants and young children (308, 309). Potential complications of urinary tract dilatation are rupture of the urinary tract, infection, pain, bladder dysfunction, and/or chronic kidney disease. These complications may occur as early as the first or second decade of life. Patients should be trained to void regularly to assure that maximal urinary bladder capacity remains within normal range and reduce the risk of bladder dysfunction. Large-capacity hypotonic bladder dysfunction might benefit from clean intermittent catheterization (CIC) or performing a continent catheterisable channel (Mitrofanoff) (307).

A less known feature accompanying primary NDI is the risk of developing CKD, even at paediatric age. Various factors have been linked to the development of CKD in this condition; among them, recurrent AKI, chronic use of NSAIDs and abnormalities of the urinary tract have been frequently suggested (310, 311). In addition to the above factors there is also some evidence suggesting that the persistently high circulating levels of AVP typically seen in NDI may also contribute to an accelerated deterioration of kidney function. There are several lines of evidence coming from animal research studies:

- Mice with surgically reduced renal mass showed reduced proteinuria, blood pressure and glomerulosclerosis when using AVP receptor antagonists (312).
- In contrast, there is increased albuminuria and salt-induced hypertension in rats with Adriamycin induced nephropathy after chronic infusion of DDAVP (313).
- Reduced albuminuria, hyperfiltration and glycosuria in diabetes mellitus rat models with central DI compared to no DI rats (314).

2.1.7. Diagnostic Procedures

To diagnose diabetes insipidus and subsequently differentiate between a central and a kidney related form, it may be necessary to perform two complementary tests.

2.1.7.1. Water deprivation test:

The first procedure is called a water deprivation test and is indicated in those patients that despite large polyuria can maintain normal serum levels of sodium/osmolarity. In fact, the aim of the water depravation test is to distinguish between primary polydipsia and primary polyuria. Patients presenting with polyuria and inappropriately dilute urine in the context of elevated serum osmolarity/hypernatremia do not benefit from a water deprivation test as they have already demonstrated an abnormal urinary concentration capacity. Exacerbating dehydration by water deprivation is unsafe for these patients and therefore contraindicated.

Yet, in patients with initially normal plasma biochemistries, the persistence of polyuria (despite impending dehydration from water deprivation), together with an inappropriately diluted urine (Uosm<Posm), provides evidence for diabetes insipidus (DI). In children with normal serum sodium where polydipsia is suspected, a first

morning Uosm is often sufficient as an informal water deprivation test, unless the child gets up at night to drink. In general, a Uosm > Posm rules out a severe form of DI.

2.1.7.2. DDAVP test:

To distinguish between a kidney related and a central form of diabetes insipidus, a test to stimulate the AVPR2 receptor is performed. This test includes the administration of 1-deamino-8-D-arginine vasopressin (DDAVP), a synthetic analogue of the native arginine vasopressin, that is highly selective for the AVPR2 receptor. There are multiple ways of administrating DDAVP (intramuscular, intravenous or intranasal) and as such different doses and formulations (table 2.2) have been recommended. Ideally all urine aliquots passed in the following 4-6 hours should be collected and analysed urgently for osmolality.

Table 2.2. Routes and dosing for DDAVP administration

	Intranasal(315)	**Intramuscular**(139)	**Intravenous**(316)
Infants	10 µg	0.4 µg	0.3 µg/kg
Older children/adults	20 µg	2 µg	0.3 µg/kg (max dose of 24 µg)

After DDAVP administration, NDI patients are unable to increase urinary osmolality, which typically remains below 200 and as low as 30-100 mOsm/kg in the most severe cases (normal values: under 1 year of age is > 300 mOsm/kg, for 1-2 years old between 600-800 mOsm/kg and for older than 2 years old > 800 mOsm/kg), and cannot reduce urine volume or free-water clearance.

2.1.7.3. Copeptin:

Derives from the precursor protein pre-provasopressin and although it was described almost 50 years ago its current biological function remains unknown. It has better stability and longer half-life than ADH and hence is more easily measured. Plus, it has a good correlation with serum ADH levels and osmolarity (317). Physiological levels of copeptin have been established between 1.0 and 13.8 pmol/L based on two large trials (n=359 and n=706) with healthy individuals. One study (n=55) that analysed patients with polyuria-polydipsia have suggested reliable cut-offs such as >21.8pmol/L as a single measure without prior thirsting for the diagnosis of NDI with 100%

sensibility and specificity; while values >4.9pmol/L showed a 94% sensibility and specificity for the differential diagnosis between primary polydipsia and partial central DI (318). Plasma ADH levels are difficult to measure and also generally unreliable in clinical practice due to the requirement of complex pre-analytical requirements (319).

The primary congenital form of NDI has to be differentiated from central diabetes insipidus (due to lack of ADH) and from the secondary or acquired forms, which are much more common (320). In clinical practice, the urinary osmolality obtained after DDAVP administration in secondary disorders is typically higher than in NDI.

2.1.8. Treatment

2.1.8.1. Supportive treatment of NDI:

Symptomatic treatment of NDI is focused on establishing and maintaining euvolemia by ensuring adequate water intake to replace urinary water losses and by reducing urine output. The latter is based on dietary and pharmacological interventions. Dietary modification to reduce urine output is aimed at providing a diet with a low renal osmotic load to reduce obligatory water excretion (321). Comparison between low and high osmotic diets have revealed differences in daily urine output between 50-250% (322). As the osmotic load consists primarily of salt and proteins (which are metabolised to urea), these should be limited their diet but continue to meet the recommended dietary intake allowing for sufficient growth in children. The usual target for renal osmotic load in the diet is around 15 mOsm/kg/day, (139).

Thiazide diuretics were the first class of drugs shown to be effective in lowering the urine volume in NDI (323), when combined with a reduction of salt intake, they can decrease urine output by 20-50% from baseline. Thiazides are generally well tolerated however they can also be associated with side effects, mainly hypokalaemia, hyperuricemia, alterations in plasma lipid profile and glucose intolerance. Treatment of hypokalaemia with administration of potassium salt however increases the osmotic load. Potassium-sparing diuretics such as amiloride can help to counterbalance the potassium loss from prolonged use of thiazides. Since amiloride appears to have a safe profile, the combination of hydrochlorothiazide with amiloride (0.3 mg/kg/daily) is a common choice of treatment. The use of medications appears to be particularly

effective during childhood, although amiloride is sometimes not well tolerated in young children because of persistent nausea. Particularly in this age range another group of medicaments has shown high effectivity in reducing urine output: prostaglandin synthesis inhibitors also known as NSAIDs (Non-Steroidal Anti-Inflammatory Drugs).

Clinical observations have shown that the combination of hydrochlorothiazide with either a prostaglandin-synthesis inhibitor, such as indomethacin (2 mg/kg/day), or the potassium-sparing diuretic amiloride, is much more effective in reducing urine volume than the thiazide diuretic alone (324-328).

Prolonged use of NSAIDs, however, is often complicated by gastrointestinal, haematological or kidney related adverse effects. Gastrointestinal issues include anorexia, nausea, vomiting, abdominal pain, ulceration, perforation, and hemorrhage (329). Complications involve both upper and lower gastrointestinal tract. Bleeding (the most severe complication) has been less associated with the use of ibuprofen/celecoxib (Relative Risk / RR < 2.0) compared to others such as indomethacin/ketorolac (RR > 4.0) (330). Hematopoietic reactions include agranulocytosis, thrombocytopenia, haemolytic anaemia and (rarely) aplastic anemia (331).

In addition, renal dysfunction has been described during chronic treatment with NSAIDs therapy. Some of the documented events are: electrolyte disbalances such as hyponatraemia and hyperkalaemia; acute kidney injury, papillary necrosis and interstitial nephritis (332). In patients, who are not tolerating non-selective cyclooxygenase (COX) inhibitors, COX-2 selective inhibitors drugs such as celecoxib might be helpful (333). Acute events can potentially have a full recovery if identified early and the drug is promptly discontinued, however, chronic exposure can produce permanent damage.

The most frequent mechanism of NSAIDs related nephrotoxicity involves the blockage of prostaglandin’s synthesis, which are generated in hypovolaemia to protect renal perfusion. Patients with NDI are at a increased risk of hypovolemia due to polyuria, hence they may have a theoretically higher sensitivity to NSAIDs nephrotoxicity (332). Conversely, by reducing the urine output, the drugs decrease the risk of hypovolaemia.

The balance between reduction of polyuria and risk of chronic kidney damage is challenging and management by a nephrologist is advised.

The exact mechanisms how thiazides and NSAIDs reduce urinary losses are still uncertain nowadays. The most accepted theories for each one are:

- Thiazides. Their primary action is to reduce sodium reabsorption in the distal tubule by inhibition of the NaCl cotransporter (NCC). The direct consequence is an increased sodium excretion through the urine, which is followed by extracellular volume contraction, decreased glomerular filtration rate and increased proximal tubular reabsorption of sodium and water. Subsequently less water and sodium reach the collecting tubule and a lower volume of water is excreted (334, 335). This hypothesis, based on an indirect effect of thiazides on tubular water and sodium reabsorption, has been accepted for more than 50 years, however in more recent times this idea has been challenged by others like Magaldi and collaborators.

  The new insights involve a direct effect of thiazides on distal tubular reabsorption and are based on microperfusion studies in rat inner medullary collecting duct (336). What these studies were specifically showing was that even in the absence of vasopressin, hydrochlorothiazide (when added to the luminal side) was able to increase water permeability by increasing *AQP2* expression in collecting duct cells, therefore decreasing water excretion (337). However, if this was the only mechanism, then thiazides would not decrease urine output in patients with autosomal recessive NDI, as these have no functional AQP2.

- NSAIDs. Initially, NSAIDs where thought to decrease urinary losses due to their theoretical hemodynamical changes on renal blood flow with consequent reduction of the glomerular filtration rate. Multiple experiments in animals and humans have shown several interesting points:
  - 1) prostaglandin inhibitors in fact do not have a significant effect on renal hemodynamics and its blood flow in humans or animals (338); 2)

prostaglandins have been shown to specifically inhibit ADH-stimulated adenyl cyclase in the kidney (339), so that ADH mediated water absorption is enhanced in the absence of prostaglandins (338); 3) the failure to observe an antidiuresis in animals receiving NSAIDs in the absence of ADH points to an intrarenal mechanism rather than one mediated by central release of the hormone presumably in the form of 4) an increased ADH-independent expression of *AQP2* (340).

- Others include: 1) prostaglandins have been shown to decrease sodium reabsorption by the isolated perfused medullary thick ascending limb of Henle. Thus, prostaglandin inhibition increases sodium reabsorption in this segment, with a secondary increase in passive water reabsorption from the descending limb (341). 2) Increased sodium and water reabsorption by the proximal tubule. Both of these tubular effects of indomethacin therapy result in decreased fluid delivery to the collecting duct, and thus a reduction in urine volume (342).
- Lastly, prostaglandins have been shown to reduce the antidiuretic effect of thiazides in diabetes insipidus. This finding may offer one explanation why NSAIDs potentiate the effect of thiazides in NDI (336).

Although symptomatic treatment can substantially reduce urine output, achieving normal volumes as in healthy individuals is not possible. Moreover, the efficacy appears to decrease with age, whereas the risk of complications from cumulative exposure increases. Consequently, there is a trend to stop these medications during childhood and adolescence.

There clearly is an unmet need for novel therapies, ideally by targeting the underlying pathomechanism.

2.1.8.2. Therapeutic Strategies for Treatment of X-Linked NDI

2.1.8.2.1. Rescue/activation of intracellularly trapped V2R.

In vitro expression studies have shown that most of the *AVPR2* described mutations result in a normal protein that is retained within the endoplasmic reticulum (ER), so an interesting approach is the use of agents that restore intracellular routing. Some of the most promising therapies are based on the administration of cell permeable V2R antagonists and agonists; agents that in vitro have been able to rescue several intracellularly retained V2R mutants (343-345).

One of the issues that presents with the use of antagonists is that they need to be displaced from the rescued V2R once it is present in the basolateral membrane. A particular way to achieve this displacement is by administration of high doses of DDAVP to compete against the antagonist. Low affinity antagonists can potentially be dislodged easier and therefore are considered the most promising option, but on the other hand their rescuing efficiency seems to be lower, and the highest doses required for treatment are a concern in terms of potential risks for adverse effects. A different type of agonists (non-peptide) can bypass the previous problem as they don't need displacement to activate V2R. High-affinity agonists have been shown to induce receptor maturation as well as translocation to the plasma membrane with positive cAMP response (346).

Pharmacologic chaperones have also been tested in vivo with primary NDI patients secondary to *AVPR2* mutations. Results were indeed satisfactory with reduced urine output and increased urinary osmolality, in particular with the use of V1a receptor antagonists as showed by Bernier and collaborators (343). However, this study was terminated early due to a potential interaction with the P450 cytochrome metabolic pathway and therefore long-term effects could not be assessed.

Interestingly some non-peptide V2R agonists, such as OPC51, VA88, and VA89, were able to activate the V2R in the intracellular compartment with subsequent increment in cAMP production and AQP2 translocation to the apical membrane (347). Unlike pharmacochaperone-assisted folding and rescue of the receptors; the localization and maturation state of the V2R did not change upon activation, indicating that these

alternative compounds do not act as molecular chaperones. The exact mechanism how intracellularly trapped receptors can still stimulate their coupled G-protein and how this activates adenylate cyclase has not been elucidated yet. Whether pharmacological chaperones and non-peptide agonists will prove to be effective and safe in the long-term management of patients with NDI is still to be found.

2.1.8.2.2. Bypassing the V2R

Another potential alternative to treat patients with X-linked NDI is to bypass the V2R and directly activate the intracellular signalling pathway.

Prostaglandin E2 (PGE_2) does have up to 4 different types of receptors with different tissue expression and functions (EP 1-4) and stimulation of the E-prostanoid receptor EP4 has been reported to reduce polyuria in a conditional AVPR2-deletion mouse model (348). Administration of the EP4 agonist resulted in increased production of cAMP. Secondary EP2 receptor activation also increased intracellular levels of cAMP and indeed a similar effect has been described with the used of its specific agonist, butaprost (349). Although EP2 agonists have been shown to have a safe profile in previous clinical studies, its experience in NDI is very limited and specific clinical trials are still lacking.

Not only cAMP but also activation of the cGMP signalling pathway appears to be a potential therapeutic alternative. Tried approaches of either increasing production of cGMP (mediated by nitric oxide or atrial natriuretic peptide) or decreasing its degradation (phosphodiesterase inhibitor, sildenafil) have been shown in vitro and in vivo to increase the membrane presence of AQP2 in renal epithelial cells (347, 350, 351). Case reports have shown conflicting results with the use of sildenafil citrate, inducing clinically significant changes in a paediatric patient (352) while failing to prove effective in two adults (353).

In recent years a new drug has been designed and tested in vivo (V2R-KO mouse model) proving a successful reduction in polyuria up to 50%. This specific compound named as NDI-5033 is an activator of AMPK (adenosine monophosphate–activated protein kinase) whose key function is the phosphorylation of AQP2, setting the path for its translocation to the apical membrane (354).

2.1.8.2.3. Other alternatives

Alternative AVP-independent strategies are the use of calcitonin, which has a vasopressin-like effect on AQP2 trafficking and urine-concentrating ability via cAMP-mediated mechanism (355). In vitro and animal studies have shown a potential positive effect on AQP2 abundance in the apical membrane in the renal collecting ducts by statins, in particular fluvastatin and simvastatin (356, 357). Analysis of 37 patients with hypercholesterolemia and normal kidney function revealed that simvastatin treatment for 12 weeks induced a rapid and sustained elevation of urinary AQP2 excretion and also in urinary osmolality. A similar effect has also been reported in patients on long-term simvastatin treatment (358).

Fluvastatin has also been studied in combination with secretin, a gastrointestinal peptide hormone coupled to Gs dependent cAMP formation with very positive results including a reduction in urine output by 90% (359).

Using a systemic high-throughput chemical screening procedure, Nomura and collaborators identified AG-490 (an EGF receptor and JAK-2 kinase inhibitor) as a compound that stimulates AQP2 exocytosis, induces AQP2 membrane accumulation, and stimulates urine concentration in an AVP-independent manner (360).

Amongst other drugs/compounds currently available for alternative conditions but with a potential benefit from using in NDI are: tamoxifen (a vasopressin-independent positive regulator of AQP2 in kidney collecting ducts (361)), aliskiren (a direct renin inhibitor which increases aquaporin-2 expression and attenuates lithium-induced nephrogenic diabetes insipidus (362)) and Hydrogen sulphide (upregulates renal AQP2 protein expression therefore promoting urine concentration (363)).

A particular pathway that has been targeted on various research projects is the one involving Protein Kinase A (PKA). As examples we have 1) the AKAPs-PKA (A-kinase anchoring proteins) where the use of its known disruptor FMP-API-1 has shown a prolonged PKA activation therefore an increased AQP2 activity independent of vasopressin (364) and 2) sPRR-His (soluble prorenin receptor agonist) which activates cAMP-PKA pathways palliating effects of V2R antagonists (365).

Finally, specific genes have been reported as influencing renal tubular water handling and could become therapeutical targets in the future. Amongst them, *EPAC1* when repressed in mice can manifest clinically as NDI with a deficient corticomedullary osmotic gradient and weaker collecting duct junctions (366). *WNT5A* when activated enhances intracellular calcium signalling, as a result it has shown to attenuate the aquaretic effect of V2R antagonists (such as tolvaptan) in mice (367). Lastly, activation of *NRF2* has been proved to be protective against Lithium-induced NDI (368).

Despite promising results *in vitro* studies and in animal models, none of these compounds/pathways have yet been translated into approved therapies of NDI.

2.1.8.2.4. Therapeutic Strategies for Treatment of Autosomal NDI

As it is the case with V2R, a majority of AQP2 mutants causing autosomal recessive NDI are secondary to missense mutations leading to aberrant folding of AQP2 in the endoplasmic reticulum. Thus, one of the therapeutic targets that researchers have been particularly focused on, has been the discovery of molecules with the potential to restore a functional AQP2.

Some of these potential therapies are described here:

I. Glycerol. In vitro studies in CHO (Chinese Hamster Ovary) and MDCK (Madin-Darby Canine Kidney) cells have proven that glycerol, as a chemical chaperon to AQP2, can significantly restore its exportation from the endoplasmic reticulum (258).
II. 17-Allylamin Geldanamycin (17-AAG). In vivo experiments with AQP2-T126M knock-in mice, treated with an Hsp90 inhibitor such as 17-AAG, have shown a partial restoration of cellular AQP2 processing. This effect had a clinically significant impact with subsequent improvement in the urinary concentrating ability (259). The precise mechanism how this process occurs is yet not known, in addition Hsp90 inhibition can potentially cause serious adverse effects (369). At the present, studies addressing safety and applicability of Hsp90 or other chaperone inhibitors are lacking and more evidence is required to elucidate their therapeutic potential.

III. Rolipram. Based on the principal that AQP2 translocation in response to V2R activation is mediated by increased cAMP production, Sohara and collaborators designed an experiment using an in vivo model for NDI (dominant negative *AQP2* pathogenic mutation knock-in mouse) to which a phosphodiesterase-4 inhibitor was administrated, rolipram (370). Their data showed that Rolipram was an effective PDE4 inhibitor in collecting duct cells, increased cAMP levels, and its administration favoured the functional translocation of AQP2 channels to the apical membrane in opposition to the basolateral localisation seen in the non-treated animals. Interestingly other PDE inhibitors such as milrinone and sildenafil failed to get the same result, despite PDE3 and PDE5 are known to be expressed in the collecting ducts. Unfortunately when administered to two human patients with NDI, rolipram failed to ameliorate polyuria (371). Whether other PDE inhibitors could potentially be an alternative therapy is yet to be seen.

2.2. PROJECT 2. TREATMENT AND LONG-TERM OUTCOME IN PRIMARY NEPHROGENIC DIABETES INSIPIDUS

2.2.1. INTRODUCTION AND OBJECTIVES

As a very rare condition with an estimated prevalence of < 1:100.000, gathering and presenting informative data for a better understanding of nephrogenic diabetes insipidus has been challenging. As with several other rare tubulopathies, there are efforts from rare disease networks such as ERKNet, ESPN and ERA/EDTA in Europe to overcome these difficulties and gather data on these rare conditions, including primary nephrogenic diabetes insipidus (233, 372). These efforts have contributed to an expansion of knowledge not only about the phenotype but also the genotype, revealing many novel variants in the previously described causative genes.

Despite the above-mentioned work, there are still many clinical questions that remain unanswered regarding primary nephrogenic diabetes insipidus:

1. Epidemiological data here as well is still scarce.
2. What proportion of patients may have an earlier diagnosis vs delayed for example in late childhood/ adulthood. What factors could potentially be influencing age at diagnosis and what impact the age at diagnosis could have in their long-term outcomes.
3. How does NDI impact nutrition and growth both during childhood (for example requirement of feeding support) but also in adulthood? Are current guidelines addressing all these important factors?
4. Could pathogenic mutations in different genes have a significantly different effect in outcomes?
5. Previous short reports did not have enough power to dig deeply into the NDI consequences on kidney function, could we describe after this project a more accurate paradigm of CKD in patients with primary NDI?
6. What is the pharmacological burden in primary NDI and what factors are associated to a larger/shorter use of different drugs?

7. While flow uropathy has been previously described in patients with primary NDI, could we more accurately report the prevalence of this condition and its specific complications?
8. Mental health disorders have been linked to primary NDI for many years, but apart from ADHD, little is known about other conditions and how frequently are they manifesting in this population.
9. As a chronic disease and particularly as previously linked to intellectual disabilities, what could be the effect of primary NDI on patients' life from an educational and labour perspective.

While reflecting on these highly relevant questions and also on the potential impact on patient care and their quality of life if these could be answered successfully, I recalled my previous and successful collaboration with ERA-EDTA (European Renal Association – European Dialysis and Transplant Association), ESPN (European Society for Paediatric Nephrology) Working Groups for inherited Kidney Diseases and the European Rare Kidney Disease Network (ERKnet). This past experience highlighted the importance of large-scale collaboration with international networks particularly when researching on rare diseases such as in this case. Hence, I designed another international collaborative project for the study of primary nephrogenic diabetes insipidus with the support of world experts on this topic. Further details on how this project was developed are explained in the methods section of this chapter.

In summary, and with the help of the multiple collaborators of this project, the group did report on clinical and genetic features in a multinational extensive cohort of 315 patients with inherited NDI. This report has provided highly informative data on the clinical management of affected patients including extensive genetic information, data on growth, obesity, kidney function, therapeutic management, mental health and urological co-morbidities but also very important on alternative factors affecting patient's quality of life such as education and employment.

2.2.2. PATIENTS AND METHODS

2.2.2.1. Design of the study and data collection

Initially a panel of experts in NDI organized several meetings to agree on the proposed questions and to design a strategy for data collection. Once completed, clinicians and investigators from around the globe were contacted via email through the membership of the European Reference Network for Rare Kidney Diseases (ERKNet), the European Renal Association – European Dialysis and Transplant Association (ERA-EDTA) and the European Society for Paediatric Nephrology (ESPN) through their respective working groups for inherited kidney diseases. This email included a summary of the strategy and aims of the project and an invitation to provide data on their patients with a clinical diagnosis of inherited NDI. The survey was online and a link to access it was included in the email. The survey was open from the 26th of June to the 31st of August 2019.

Table 2.3. Questions from the questionnaire

Renal Unit details	**Tube feeding**
Treating physician	Nasogastric +/- Gastrostomy
Email address	**Treatment (name and dose)**
Centre – City	Thiazide diuretic
Country	NSAIDs
Demographic data	Potassium sparing diuretic
Patient ID	**Comorbidities**
Gender (F/M)	Flow Uropathy
Ethnicity	Ureterohydronephrosis
Age at diagnosis (years)	Bladder dysfunction
Genetic information	Mental Health Issues
Gene	ADHD
Mutation details	Others
Auxology	Persistent Nocturnal Enuresis
Current age (years)	**Social and Education**
Current height (cm)	Educational degree
Current weight (kg)	Living arrangements
Laboratory	Employment status
Serum creatinine (µmol/L)	

A total of 27 questions were asked about demographics, auxology, treatment, kidney function, comorbidities, such as hydronephrosis, bladder dysfunction and mental health problems. A list of all questions is provided in table 2.3.

In cases of missing information or if provided data points were noted to be outliers, corresponding clinicians were contacted via email for completion and/or verification of data. Data were deemed adequate for analysis if <5 items were missing, and the information provided was confirmed by the responsible clinician.

The threshold to consider an individual as adult was 18.0 years, this cut-off value was generally used during analysis except for the estimation of the glomerular filtration rate (eGFR); due to the “Schwartz” formula been recommended for use up to the age of 20 years (135).

2.2.2.2. Genotype-phenotype analysis

For genotype-phenotype analysis, the entire cohort was classified in four major groups according to the underline genetic diagnose: 1) Negative: no causative variants identified in *AVPR2* and *AQP2*; 2) Untested: genetic testing not performed; or causative variant(s) had been identified in either 3) *AVPR2* or 4) *AQP2*. Further analysis was performed between missense and predicted loss of function (pLoF, which includes nonsense, frame shift and splice site) variants.

Genetic testing results were provided by the contributing clinicians according to their best knowledge, and in some patients testing may have been performed on a research basis only without confirmation in a clinical laboratory. Mutation details, as provided, were reviewed by a clinical geneticist (D.I.) to ensure that they accorded to the ACMG (American College of Medical Genetics and Genomics) standard (129).

2.2.2.3. Weight and Height

Data on patients’ height was normalised and presented as SDS (Standard Deviation Score). In childhood, conversion was done according to references form the World Health Organization (373); in adulthood refence came from the US 2000 CDC growth charts (according to National Health and Nutrition Survey (NHANES) data) (132).

Height SDS was defined as normal when its value was ≥ -2.00. Paediatric weight data was normalised and reported as SDS (Standard Deviation Score), again as per the WHO growth charts' reference.

The body mass index (BMI) was calculated and defined as underweight (<18.5), normal (18.5-24.9), overweight (25.0-29.9) or obese (≥30.0 kg/m^2), according to standard convention (374). Information from weight and height was extracted at the time of patient's last clinical appointment. Prevalence data on Obesity of children and adults from this cohort was compared to large European reference populations (Feel4Diabetes study and EU28-eurostat) (19).

2.2.2.4. Kidney function

The eGFR in adults (>20 years old) was calculated using the modification of diet in renal disease (MDRD) formula [175 × Serum Creatinine$^{-1.154}$ × age$^{-0.203}$ × 1.212 (if patient is black) × 0.742 (if female)] without correction for ethnicity (133, 135).

The MDRD formula uses mg/dL for creatinine quantification, considering this study measured creatinine in µmol/L, the reported results were divided by a factor = 88.4 for conversion to mg/dL. For children (2-20 years), we used the modified "Schwartz" formula (135).

Prevalence of CKD was calculated as per KDIGO Guidelines recommendations, CKD was classified in stages 1-5 and defined in this population by either a history of chronic tubular disorder and/or abnormal radiological imaging of the kidney; no data on urinary sediment abnormalities, proteinuria or histopathology was collected in this project (137). Data on kidney function from the large NHANES III cohort (138) was used as population reference for analysis of CKD prevalence, specifically information from patients aged 20-60 years (N=76). As there were only 7 patients >60 years, meaningful comparison for that group was not possible.

2.2.2.5. Psychosocial information

When analysing data on mental health, the references used were: an estimate of a 5% worldwide prevalence of ADHD, and European epidemiological data on

educational attainment and employment extracted from the EuroStat database (375-377). Mental Health disorders were classified according to DSM-5 criteria (378).

2.2.2.6. Gross Domestic Product (GDP) per capita ($)

GDP per capita was based data on from The World Bank (379). For analysis, we defined a low and high subgroup based on a 15.000$/year cut-off, as it roughly divided the cohort in half.

2.2.2.7. Statistics

Analysis was done in IBM SPSS Statistics for windows version 24.0 (Armonk, NY: IBM Corp.). Kolmogorov-Smirnov test was performed to assess normality of the data. Data following a normal distribution was expressed as mean (± Standard Deviation [SD]) and non-normally distributed data as median (range or Interquartile Range [IQR]). Statistical significance for categorical binary variables was analysed through the Pearson Chi-square test. The Student-t as a parametric test, was employed when comparing means between two groups with normal distribution of data; when doing so with three or more different groups the choice was the one-way ANOVA test. When studying medians, two different non-parametric tests were used: 1) Mann-Whitney U-test for binary and 2) Kruskal-Wallis test for other nominal variables. A p-value of < 0.05 was used to define a statistical association between certain variables as significant.

2.2.3. RESULTS

2.2.3.1. Demographic and genetic data

A total of 315 cases (from 22 countries, table 2.4) were available for final analysis.

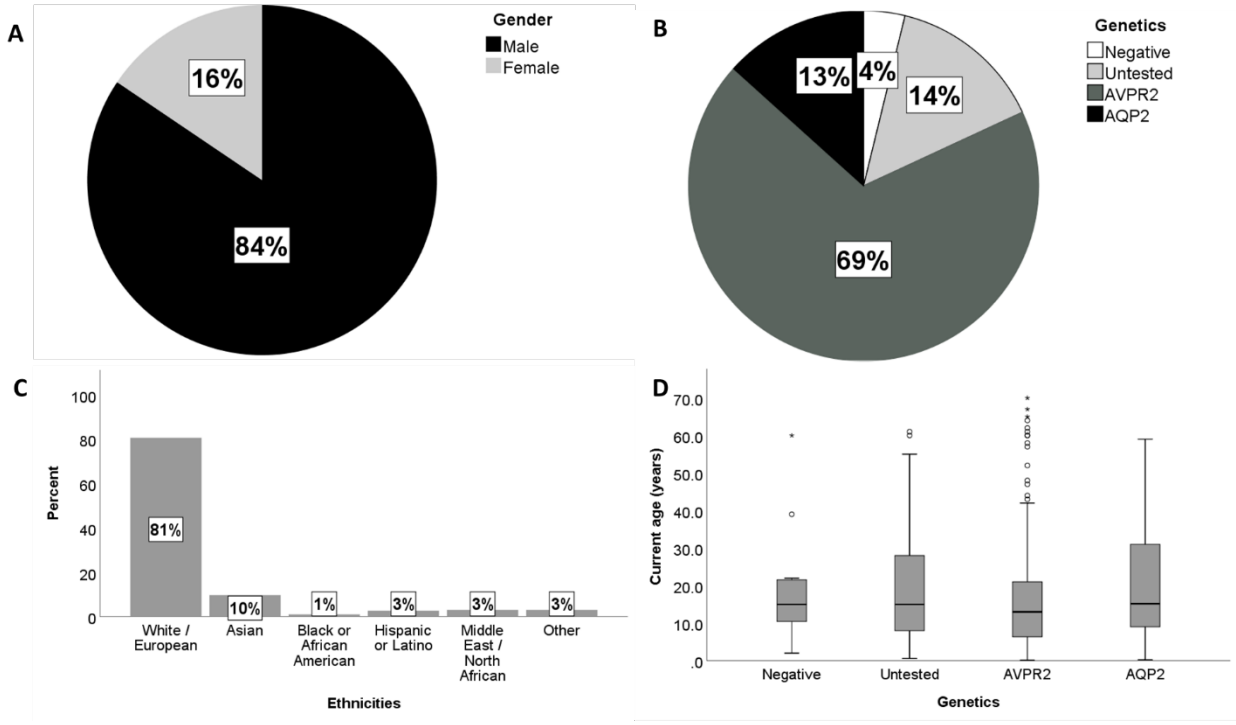
Table 2.4. Countries contribution in number of patients

Country	Number of patients	Country	Number of patients
UK	69	Greece	5
Italy	37	Switzerland	5
France	36	Australia	4
Spain	32	Greece	3
Netherlands	25	Iran	3
Belgium	16	Romania	3
Russia	12	Sweden	3
Turkey	12	UAE	3
Canada	11	Lithuania	2
Germany	9	Poland	2
India	8	Cyprus	1
Ireland	7	Macedonia	1
Scotland	7		

Gender distribution was unequal with 266 (84%) patients being males while only 49 (16%) were females (Figure 2.7a). Genetic analysis (screening for the two well-known classical genes associated to NDI) had been performed in 270 cases (86%). From this group, 258 (96%) did have a confirmed genetic diagnose (*AVPR2*=216 and *AQP2*=42). The remaining 45 individuals were not genetically tested (Figure 2.7b). Analysis of the different ethnicities revealed a high proportion of white/European (81%), followed by Asian (10%) (Figure 2.7c). A total of 25 genotypes (in 58 patients) were reported at least twice by the same clinician, suggesting a familial relationship. At last clinical appointment, the median age (range) of the patients was 14.0 (0.1-70) years (Figure 2.7d), and a total of 110 patients (35%) were adults (≥18 years old).

A large proportion (58%, N=179) presented in the first year of life, yet 6% were diagnosed during adulthood (of these, 79% had a confirmed genetic diagnosis).

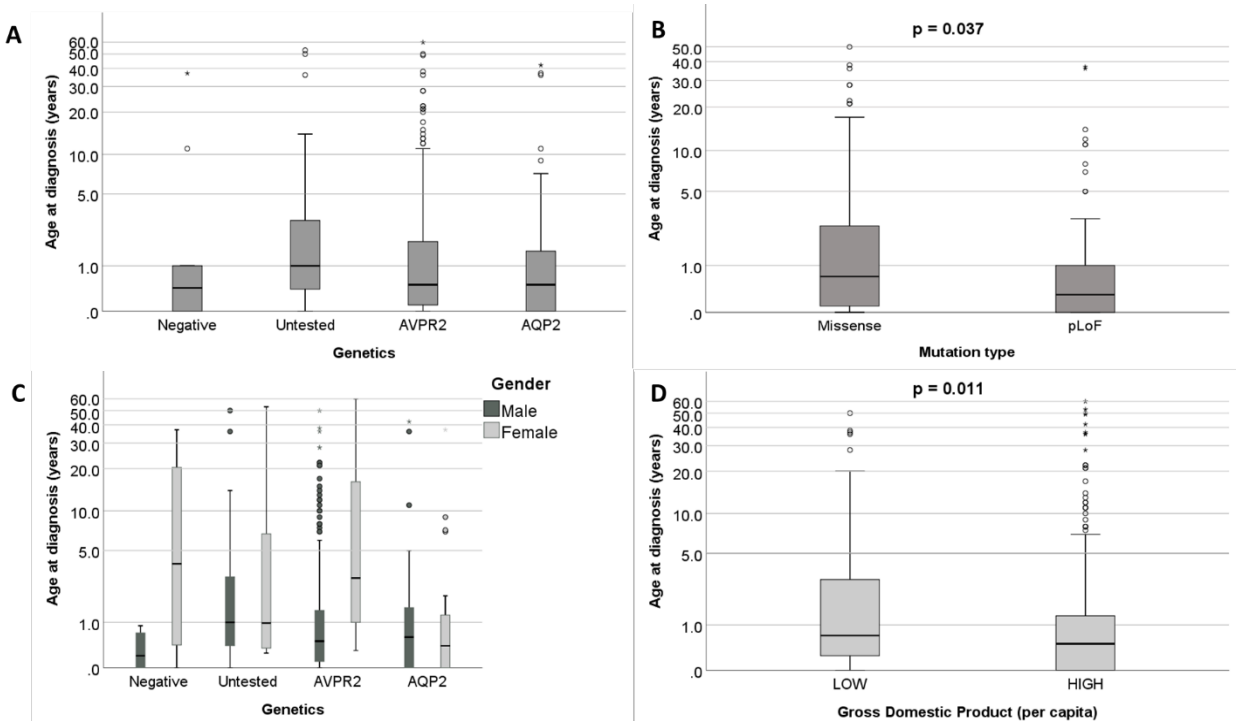
Figure 2.7. Demographic aspect of the NDI Cohort



(N=315, unless otherwise stated): **A)** Gender distribution, male >> female; **B)** genetic groups (for details see text); **C)** reported ethnicity (N = 300) and **D)** boxplot graph detailing age at last follow-up according to genetic group (Negative (N=12), Untested (N=45), AVPR2 (N=216) and AQP2 (N=42).

On presentation median age (IQR) was 0.6 (0.1-2.0) years. There was no significant difference among the four different genetic groups regarding age of presentation (Figure 2.8a). Analysis by variant type (Figure 2.8b) showed an earlier diagnosis in the group with predicted loss of function (pLoF) compared to missense variants (0.3 [0.0-1.0] versus 0.7 [0.1-2.] years; p = 0.037). On presentation, male patients with pathogenic variants in *AVPR2* were significantly younger than females (0.5 [0.1-1.5] versus 3.1 [1.0-18.3] years; *p*=0.01) (Figure 2.8c). The GDP per capita of the countries was found to be inversely correlated with age at diagnosis, meaning that in countries classified as High GDP patients were identified earlier than those with low DGP per capita (0.5 [0.0-1.4] versus 0.7 [0.3-3.0] years; p=0.011) (Figure 2.8d).

Figure 2.8. Genetic groups and GDP vs age at diagnosis



A) Distribution of data comparing different genetic groups according to age at presentation/diagnosis, 90% of the cases did present under 10 years of age. **B)** Patients from the group of mutations with predicted Loss of Function (pLoF) did have a significant earlier presentation 0.3 (0.0-1.0) years compared to Missense group 0.7 (0.1-2.7) years. **C)** Distribution of cases according to gender and genetic group did show an earlier presentation in males both in the Negative and the AVPR2 groups however due to small size (N =10) of the first one only statistically significant difference was observed in the *AVPR2* group (199 male and 12 female). The delayed and smaller representation of the female individuals very likely is due to the X-linked recessive inheritance of the *AVPR2* gen with fewer and milder symptomatic women. **D)** Diagnose of individuals with primary NDI happened earlier in countries with higher (N=186; >15.000$/year) Gross Domestic Product per capita 0.5 (0.0-1.4) years compared to lower 0.7 (0.3-3.0) years (N=121). Log_{10} scaled y axis.

2.2.3.2. Weight and height

Table 2.5. Auxology analysis according to genetic group

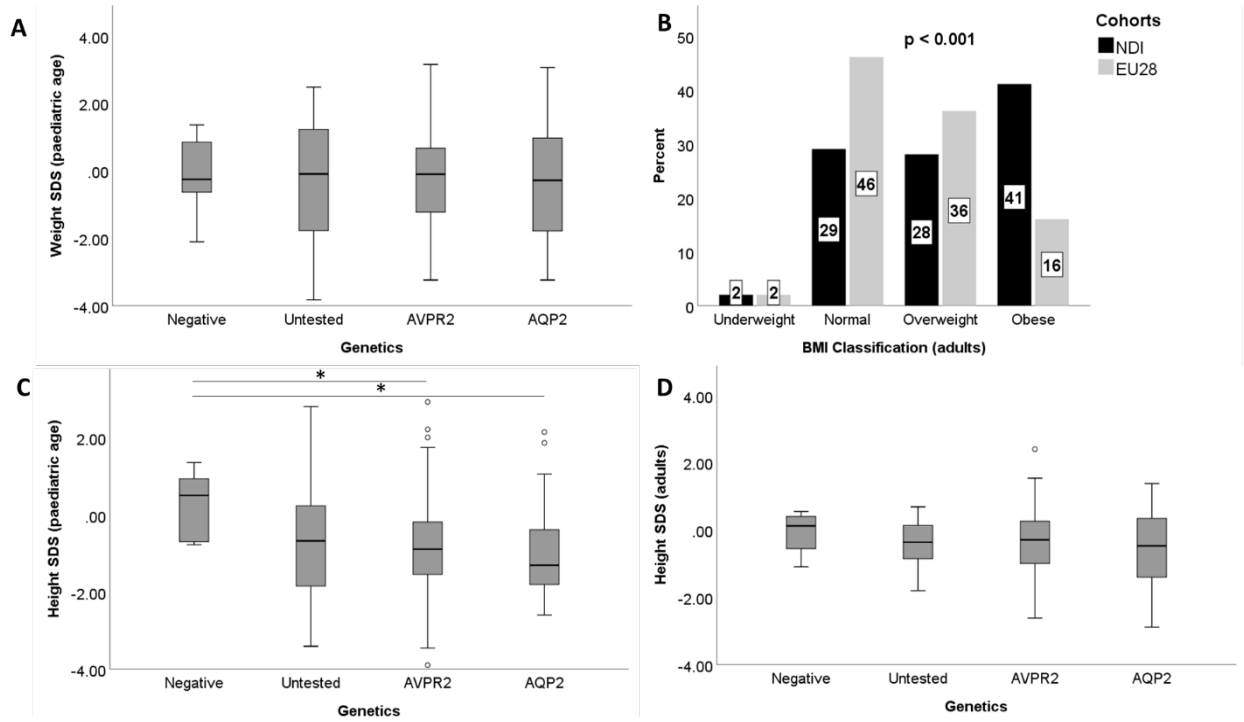
Genetic Group (N)	Weight SDS	Height SDS
Negative		
Children (7)	-0.26 (-0.77 – 1.01)	0.50 (-0.75 – 1.73)**
Adults (5)	0.06 (-0.51 – 0.89)*	0.12 (-0.83 – 0.47)
Untested		
Children (25)	-0.1 (-1.98 – 1.39)	-0.68 (-1.98 – 0.30)
Adults (18)	1.0 (0.05 – 1.52)	-0.37 (-0.93 – 0.16)
AVPR2		
Children (148)	-0.11 (-1.24 – 0.68)	-0.90 (-1.56 to -0.19)**
Adults (67)	1.36 (0.49 – 1.79)*	-0.30 (-1.07 – 0.30)
AQP2		
Children (25)	-0.29 (-1.83 – 0.96)	-1.31 (-1.83 to -0.27)**
Adults (17)	1.32 (0.8 – 1.89)*	-0.48 (-1.41 – 0.54)

Reported weight and height SDS for both paediatric and adult patients were analysed for the 4 genetic groups (table 2.5). There was no significant difference for weight SDS between the paediatric groups (Figure 2.9a) unlike in adults, specifically when the Negative group was compared to both *AVPR2* and *AQP2* (* $p<0.05$).

This difference in adults' weight was also reflected on their BMI, with an increased BMI (Mean±SD) in patients with confirmed variants (29.3±6.2) compared to those with undefined genetic diagnosis (26.0±5.5) (p = 0.03). Indeed, further comparison with adult EU-28 reference population revealed that the proportion of obese adult patients in this NDI cohort was higher than expected (41% versus 16%; $p<0.001$) (Figure 2.9b).

Analysis on height showed opposite findings, while no significant difference was found between the genetic groups in adults (Figure2.9c), children from the Negative genetic group were found to have higher Height SDS compared to those with proved genetic diagnosis (** $p<0.05$) (Tablet 2.5, Figure 2.9c and Figure 2.9d). Overall, 12.6% of individuals from the entire cohort did have a low height (SDS<-2.0) at last follow up.

Figure 2.9. Weight, height and BMI vs genetic group



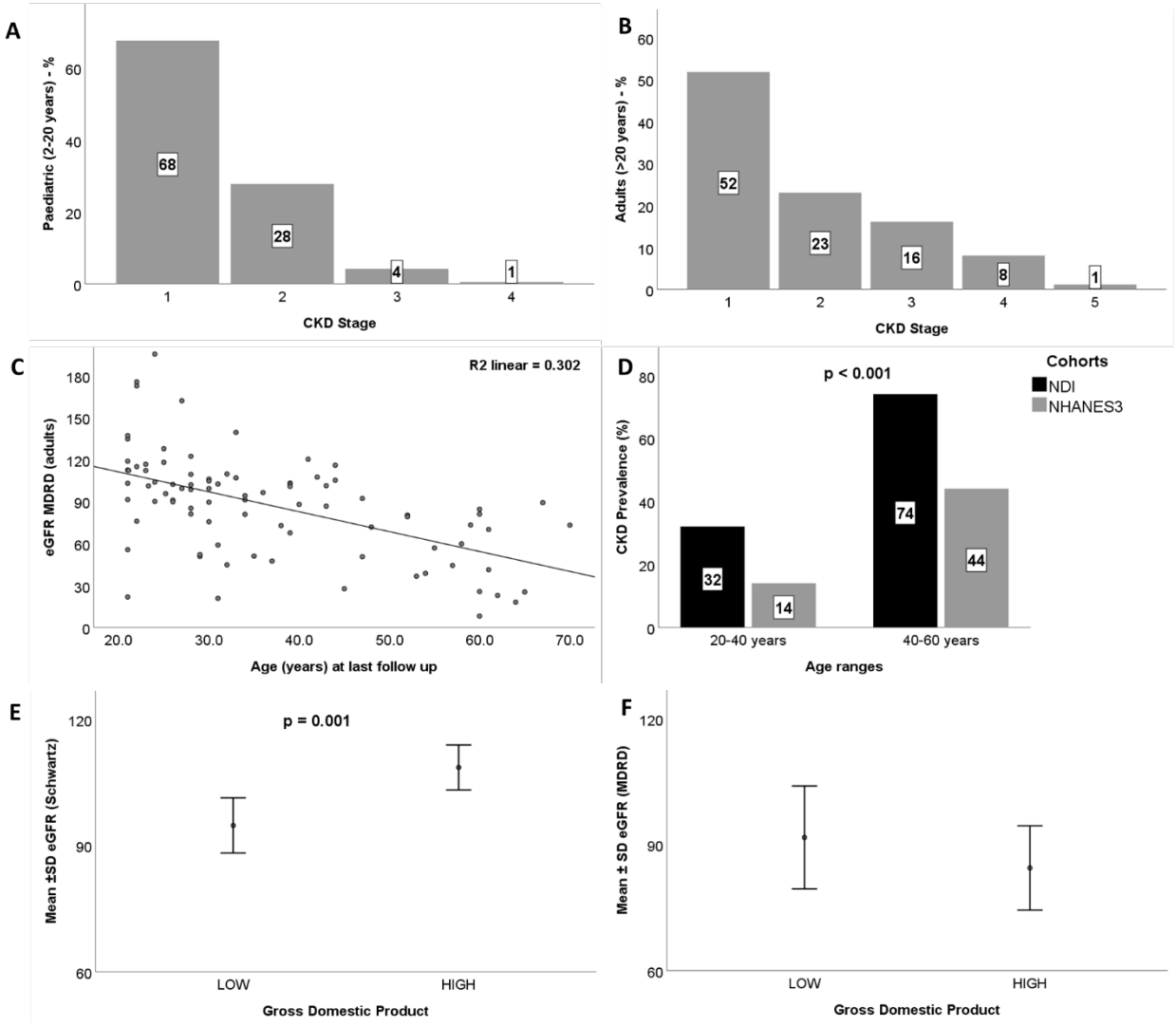
A) Boxplot representing weight SDS in paediatric patients according to their genetic group (N): Negative (7), Untested (25), AVPR2 (148) and AQP2 (25). Statistical analysis did not show significant difference. **B)** Classification of adult patients according to their BMI (Underweight <18.5, Normal 18.5-24.9, Overweight 25.0-29.9 and Obese ≥30.0 kg/m^2) showed an elevated prevalence of obesity in this NDI cohort, subsequent comparison against reference population showed significant difference p < 0.001). **C)** In the paediatric group 15% of the individuals were found to have a low height (under -2.0 SDS); genetic distribution (N) was Negative (7), Untested (25), AVPR2 (147) and AQP2 (24); and significant differences (*p < 0.05) in median (IQR) were noticed between individuals with pathogenic mutations in AVPR2 -0.9 (-1.6 to -0.2) and AQP2 -1.3 (-1.8 to -0.3) compared to the Negative group 0.5 (-0.8 to 1.0). Notice that although majority of cases are within the normal -2.0 to 2.0 SDS range, both median and IQR -0.9 (-1.7 to -0.1) are lower than expected for age. **D)** Adult height was not markedly short and did not differ between genetic groups (N): Negative (4), Untested (18), AVPR2 (63) AQP2 (14).

2.2.3.3. Kidney function

CKD prevalence stage≥2 (eGFR < 90ml/min/1.73m^2) in the paediatric group (2-20 years old) was 32%, with a large proportion (85%) of the cases being classified as stage 2 (Figure 2.10a). In adults, the mean (±SD) eGFR in last clinical visit was 87 (±36) ml/min/1.73m^2 with no significant differences between the different genetic groups: Negative 74 (±35), Untested 84 (±44), *AVPR2* 87 (±32) and *AQP2* 97 (±43) ml/min/1.73m^2; (p=0.8). From the adult group with available eGFR information (N=87), approximately half (48%) had CKD stage≥2 and interestingly one patient with end-stage kidney disease (ESKD) was reported (Figure 2.10b).

In the adult cohort the linear decline of kidney function was estimated at an average of 1.4 ml/min/1.73m^2/year, from an age of 20 years old and with a starting eGFR of 110ml/min/1.73m^2 (Figure 2.10c). Comparison of the prevalence of CKD stage≥2 between the individuals with age 20-60 and the reference population NHANES III showed a marked difference of 44% vs 26% respectively (p<0.001).

Figure 2.10. Kidney function



Shown are data for eGFR and corresponding CKD changes. **A)** CKD stage distribution in paediatric (2-20 years, N=199) and **B)** adult patients (N=87). Note that 5% of children and 25% of adults are in CKD stage ≥3. **C)** eGFR in adult patients (>20 years, N=87) against age at last follow up. Note the kidney function decline estimated at 1.4mL/min/1.73m2 per year with a starting eGFR of 110mL/min/1.73m2 at age 20 years. **D)** CKD stages in adult patients compared to a reference population (NHANES 3). Note the significantly (p < 0.001) higher prevalence of CKD in the NDI cohort in both age groups: 20-40 years (N=57) and 40-60 years (N=19). **E)** eGFR of paediatric and **F)** adult patients according to their countries' per capita GDP. Note that eGFR was significantly lower in children living in countries with low (<15.000$, N=77) compared to high GDP (>15.000$, N=122). This difference was no longer noted in adult patients.

The difference was especially pronounced at younger adult age (20-39 years) with a prevalence of 32% (N=57), compared to 14% in NHANES III (Figure 2.10d). Analysis of differences on kidney function in between cohorts of adult patients from low and high GDP per capita countries did not find any significant difference. However, in the paediatric age (2-20 years), those cases from countries with high GDP were found to have a higher mean (±SD) eGFR 110 (±29) versus 95 (±29) ml/min/1.73m^2 (p=0.001) (Figure 2.10e and Figure 2.10f).

2.2.3.4. Treatment

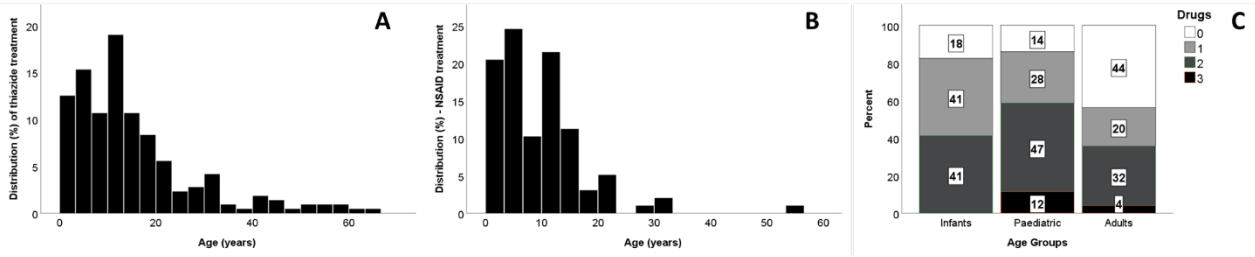
Reported data (N=315) showed that at last follow up the most frequently prescribed drugs were thiazides (67% of patients), followed by potassium-sparing diuretics (35%) and last were nonsteroidal anti-inflammatory drugs (NSAIDs) with 31%. A summary of prescription patterns among different age ranges is provided in table 2.5. Interestingly potassium-sparing diuretics were not used in the youngest patients and significantly (p = 0.001) less drugs are prescribed in adult patients compared to children.

Table 2.5. Medications prescribed according to age

Drug	AGE GROUP		
	Infants (N=17)	Paediatrics (N=200)	Adults (N=98)
Thiazide N(%)	12(71%)	153 (77%)	51 (52%)
NSAID N(%)	9(64%)	80 (40%)	9 (9%)
Potassium-sparing diuretics N(%)	0(0%)	79 (40%)	30 (31%)

Histograms representing prescription frequencies of thiazides and NSAIDs according to age are shown in Figure 2.11a and Figure 2.11b. Patients under the age of 20 years were more likely to be prescribed drugs (p<0.001). In the adult cohort 44% of patients were not prescribed either of these drugs unlike 15% of the children (Figure 2.11c). With regards to tube feeding for medium/long-term enteral nutrition/hydration, 18% (N=59) had a nasogastric tube (NGT) and 7% (N=23) a gastrostomy in place at some point during their life (N=247). For patients who required enteral tubes, the median (IQR) age in months for tube insertion was under 1 (<1 to 10), while for removal median (IQR) age was 2.0 (1.0-3.8) years.

Figure 2.11. Drug treatment summary

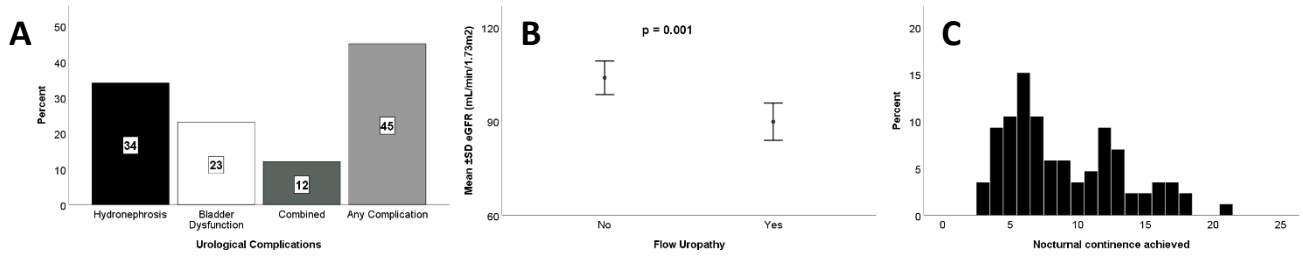


A) & **B)** Histograms with distribution according to age of thiazide diuretics and NSAIDs. Notice that both treatments are predominantly prescribed within the paediatric age, 80% of thiazide (N=216) was given under 20 years of age compared to 90% of NSAIDs (98) for same age group. **C)** After dividing the cohort in 3 subgroups: infants (under 2 years), paediatrics (2-18) and adults (>18 years) it was noticed a tendency to provide a medication free management more frequently in adults (44%) compared to children (15%). Age groups (N): Infant (17), Paediatric (200) Adults (98).

2.2.3.5. Flow Uropathy

The term "flow uropathy" was used for urinary tract abnormalities likely caused by the high urine flow, such as hydronephrosis and bladder dysfunction. Flow uropathy was reported in 45% of the patients. The prevalence of hydronephrosis, bladder dysfunction or both (N=266) were 34%, 23% and 12%, respectively (Figure 2.12a). A significant correlation (p = 0.001) was found between the presence of flow uropathy and a reduced eGFR at last clinic visit (90 versus 103 ml/min/1.73m^2) (Figure 2.12b). Interestingly ethnicity correlated with flow uropathy, with individuals from Asian background having a higher prevalence (20/27; 74%) than Europeans (83/207; 40%) (p<0.001).

Figure 2.12. Urological Comorbidities



A) Flow Uropathy was described in a high proportion of patients from this NDI cohort, in total 45% of the cases did have at least hydronephrosis or bladder dysfunction. **B)** Presence of Flow Uropathy was associated with worst kidney function (Mean 90 vs 103 mL/min/1.73m2). **C)** Histogram showing the distribution of age when nocturnal continence was achieved. Range = 3-21 years and median (IQR) = 8(6-12) years.

Other factors like gender, genetics or medication prescription were not significantly associated with an increased risk of flow uropathy. A large proportion (38% from N=250) of patients with age ≥6 years old was reported to suffer from primary nocturnal enuresis (N=250) and the median (IQR) age at achieving nocturnal continence was 8 (6-12) years (Figure 2.12c). No significant correlation between enuresis and flow uropathy was seen.

2.2.3.6. Mental health

A high prevalence of mental health disorders was reported, for the entire cohort this was 36% (41% in adults), a value significantly higher ($p<0.001$) to what has been reported in the general European population (25%) (380). The most common disorder was ADHD (Table 2.6), reported in 16% of the cases, and again this was significantly ($p<0.001$) higher than in the general population (5%) (Figure 2.13a).

Among demographic variables that correlated positively ($p<0.05$) with ADHD were male gender, living in a low GDP country and European ethnicity; in contrast, there was no significant correlation with genetic group, type of variant, flow uropathy or drug therapy requirement. Following ADHD, the second most frequent mental health disorder was intellectual disability, reported in 9%; for this, there was no significant association with gender, ethnicity, age at diagnosis or GDP.

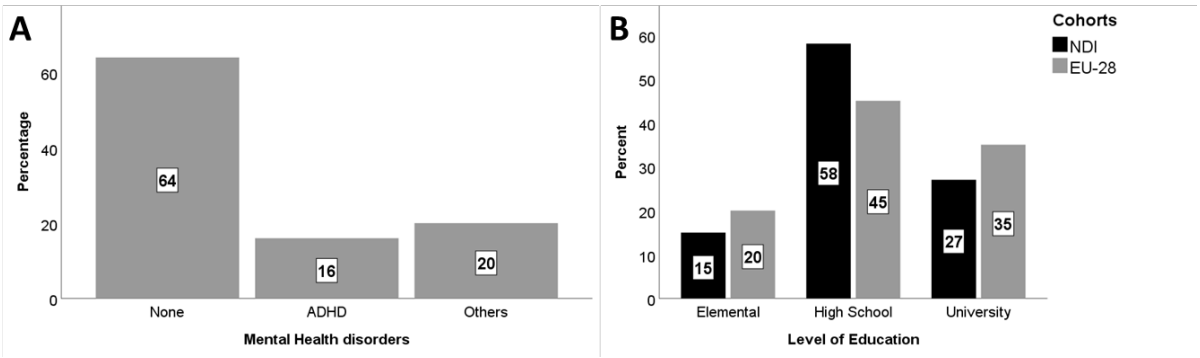
Table 2.6. List of mental health disorders in the NDI cohort

Mental Health Disorders	**N**
Attention deficit hyperactivity disorder (ADHD)	33
Intellectual disability	16
Eating disorder	6
Anxiety/Depression	5
Addictive disorder	4
Language disorder	3
Schizophrenia	2
Autism Spectrum Disorders (ASD)	2
Developmental coordination disorder	2
Personality disorder	2
Gender dysphoria	1
Stress related disorder	1

2.2.3.7. Education, employment and living arrangements.

For those individuals (N=41) within the 25-54 years age range and belonging to one of the EU28 countries, the highest level of education achieved was primary (15%), secondary (58%) and tertiary (27%) (Figure 2.13b). When compared to average data from 28 European countries, this NDI cohort has a significantly (p=0.03) smaller proportion of patients achieving university graduation (EU-28: 35%). The rate of patients ≥25 years of age in full employment was 73%. In the age range of 25-55 years for which comparable general population data are available, there was no significant difference for full-time employment between this NDI cohort (78.4%) and EU28 (80.4%). 79% of the individuals with age 30 years and older were living independently (away from parental home).

Figure 2.13. Psychosocial data



A) Mental health disorders were highly prevalent in this NDI cohort with ADHD being the more frequent single diagnose. **B)** NDI patients from EU-28 countries did not achieve as many high degrees as it was expected by looking at their reference population.

2.2.4. DISCUSSION

This project informs on multiple aspects of primary nephrogenic diabetes insipidus, such as genetic diagnosis, phenotype, management and psychosocial aspects. Indeed, this work so far represents the largest NDI cohort reported in medical literature, spanning an age range up to 70 years (figure 2.7d) and including patients from 22 countries. One of the main aims of this study was precisely to provide robust and relevant data on long term prognosis in primary NDI and to do so there was a focus on looking at growth, kidney function, educational achievement, employment and living arrangements in adult patients, who are representing approximately a third of the total cases (N=110), again the largest adult series so far that has been reported.

2.2.4.1. Genetics

Most of the included patients (86%) had genetic testing for the two known disease genes (*AQP2* and *AVPR2*) (Figure 2.7b), and the diagnostic yield was high at 96%, a proportion that fits with previous studies siting between 90-95% (233, 310, 311). Similarly to previous publications this project reported a predominance of male patients (84%), consistent with the X-linked recessive inheritance pattern of *AVPR2*, the most frequent gene associated with primary NDI. In this cohort, 16% of individuals with pathogenic mutations had autosomal recessive NDI, somewhat higher than in previous publications (10%) (186, 310). This higher prevalence of AR inheritance could potentially be explained by a larger inclusion of patients with a consanguineous background, a fact that may be supported by a large proportion of homozygosity for *AQP2* variants (69%). Indeed, when analysis was limited to patients reported from European centres, the prevalence of *AQP2*-associated NDI was lower at 12% and thus similar to previous reports.

2.2.4.2. Age at diagnosis

The majority of patients (58%) were diagnosed during the first year of life (Figure 2.8) presenting with the typical manifestations of dehydration, vomiting and failure to thrive (186). Nevertheless, 6% were diagnosed in adulthood, the eldest at age of 60 years. This likely reflects the wide spectrum of severity in this condition.

Some of the factors that may contribute to a late presentation, particularly during adulthood are:

1) 32% (N=6) of patients diagnosed in adult age were women with confirmed (N=3) or potential (untested or no identified causative variant) *AVPR2* mutations, likely reflecting skewed X-inactivation with some residual receptor expression (381).
2) Some missense variants may not fully impair function of the expressed protein causing a milder phenotype such as partial NDI (248, 382, 383). This is supported by the finding that predicted pLoF variants were correlated with an earlier age at diagnosis compared to missense variants (Figure 2.8b).
3) In addition, differences in access to health care services may also affect the age at diagnosis. Those patients who reside in countries with low GDP had a higher age at diagnosis compared to those in high GDP countries (Figure 2.8d).

2.2.4.3. Weight and Height

Long-term data on growth was satisfactory: while childhood height SDS was below the average of the reference population, this was still within the normal range (>-2.0 SDS) and consistent with catch-up growth after presentation (Figure 2.9c) (298). This positive outcome was confirmed in adult cases (Figure 2.9d) where height SDS was very similar to the reference population.

Weight at last appointment was within the normal range for children included in this cohort suggesting that current medical treatment and specialised dietetic input occasionally with tube feeding support is sufficient to normalise weight.

An unexpected finding was the significantly increased prevalence of obesity in adult patients with NDI (41% vs 16%) compared to reference population (EU-28) (Figure 2.9b). As this was a retrospective study, the reasons for this can only be speculated about.

It is possible that advice by dieticians to maximise their caloric intake by increasing proportion of lipids and carbohydrates in their diet to normalise weight (186) may

establish dietary habits which in adult age cause obesity, such as a high intake of sugar-containing drinks.

While further confirmation of tyhis finding in other cohorts is needed, adult physicians may consider ongoing specialised dietetic management of their patients with NDI but now with the goal of avoiding excessive caloric intake. Particularly concerning in this population is the risk of developing diabetes mellitus for various reasons: firstly due to the observed increased prevalence of obesity, secondly due to extra risks added by the long term use of thiazides and lastly, these patients may be at risk of delayed presentation considering that one of the cardinal signs of diabetes is polyuria which is already present in all patients with NDI.

2.2.4.4. Kidney function

The prevalence of CKD in this large cohort of patients with NDI is particularly striking, about a third in younger patients (under 20 years) and a half in the adult cohort (Figure 2.10). This is significantly higher than in the NHANES III study. Similar comparisons are more challenging in paediatric age as unfortunately no large-scale epidemiological data of CKD in this age period are available. Some registry projects may suggest that the average prevalence of CKD in the general paediatric population is around 70 per million of the age-related population (<0.01%) (158, 159). If this is the case, it becomes obvious then that this cohort has a much higher prevalence.

Possible causes for this higher proportion of individuals with CKD are: flow uropathy (this group showed lower mean eGFR), recurrent acute on chronic kidney injury secondary to episodes of dehydration, nephrotoxicity secondary to prolonged use of NSAID and potentially also the direct/indirect effect of long term exposure to high AVP levels secondary to its kidney resistance (384). On a positive note, ESKD continues to be uncommon in NDI, and it was reported in only one patient from this cohort.

2.2.4.5. Drug treatment

Medicines used for the treatment of NDI aim to reduce urine output and ultimately improve patients' quality of life. The classically used drugs are NSAIDs, thiazide diuretics and potassium-sparing diuretics like amiloride (186). Current evidence reported in the literature regarding therapeutic management in NDI is quite limited.

Some of the more recent and larger studies have suggested that the efficiency of drug therapy seems to wane with increasing age, reflected by the finding that a large proportion of cases have stopped drug treatment completely after school age (310, 311). However, in part this may also reflect concerns over side effects from long-term treatment (310).

Data from this project confirms that at younger ages, the proportion of patients taking medicines is higher (80% of paediatric patients compared to 54% in adults). Typically, thiazides in combination with NSAIDs are the drugs of choice to start in young patients. After infancy, potassium-sparing diuretics are increasingly used, likely to compensate for the hypokalaemia secondary to thiazides. Finally, in adulthood, thiazides and especially NSAIDS start to be weaned off, with the latter being given to less than 10% of the patients in this age cohort. It is not very clear what the reasons are for adults to have a reduced efficacy on oral drugs; however, different factors such as reduced osmotic load in the diet and a potentially increased accommodation of large urine volumes may provide patients with a significant reduction in the frequency of micturition per day, making the polyuria more manageable.

2.2.4.6. Urological Complications

Primary nocturnal enuresis is one of the factors that more negatively impact on paediatric patient's quality of life, due to the associated social stigma and potential bullying in school (311). While children usually achieve nocturnal continence at the latest by 6 years (385), in this project the median age was delayed till 8 years. This is consistent with a previous study that showed a prevalence of 44% at age 6 years (310).

The large polyuria in NDI is associated with multiple complications in the urinary tract such as bladder enlargement and dysfunction, plus uretero-hydronephrosis (307, 311). The prevalence of flow uropathy in this study was close to 50% (similar to a previous publication) and it was associated with lower eGFR, suggesting that flow uropathy may have a deleterious effect on kidney function (310). Yet, whether this truly reflects direct kidney damage from the flow uropathy, or whether patients with this complication have just more severe disease with potentially more episodes of dehydration and/or

higher exposure to NSAIDs treatment, cannot be concluded from this retrospective study.

2.2.4.7. Mental Health

The relation of NDI with neurodevelopmental and psychological disorders has been well documented for more than a century (169). Intellectual disability and ADHD are among the most commonly reported issues (305). Although it is believed that recurrent and severe episodes of dehydration may be the most common cause of neurological damage in these patients, research studies with animal models have also suggested an importance role of AVP in brain development (386).

Large scale reports on mental health disorders and NDI are non-existent, with the few published studies being limited to case reports and series, the largest including 17 cases (305). This study from a single centre reported that almost half of patients met criteria for a diagnosis of ADHD (305); in a separate cohort, a diagnosis of ADHD was noted in only 12.5%, but in that study patients were not formally tested (311). Both reported a large proportion (72-80%) of their patients were diagnosed within the first 1.5 years of age.

The prevalence of mental health disorders in this cohort has been found at 16%. There is however a possibility of underestimation, as the survey did not mandate for a formal neuropsychological assessment of the cases. What is evident, is the fact that this reported prevalence is clearly higher than the one expected in the general population (5%) (375). The prevalence was equal for X-linked and recessive NDI, suggesting that the underlying gene does not directly contribute and It is possible that other factors such as brain injury from repeated severe dehydration or difficulties concentrating due to severe thirst and polyuria with constant need to go to the toilet, are more relevant (305).

The second most prevalent neurodevelopmental disorder identified was intellectual disability, reported in approximately 10% of those patients with available report. Early reports of NDI had highlighted complications of severe mental impairment and intracranial calcifications: in one study, 3 out of 17 patients had an intelligence quotient ≥1SD below the norm and there are further reports of patients with severe intellectual

disability (298, 300, 305, 387). In those reports, it was considered a complication from repeated episodes of severe dehydration potentially preventable with adequate management (186). Although it is reassuring that the frequency of intellectual disability in this study is lower compared to previous reports, it does remain a considerable concern. Meaningful statistical analysis to identify potential risk factors associated with intellectual disability was not possible in this project due to reduced numbers. Lastly, this project did not capture specific details on grade of disability or formal intelligence assessments, hence further assessment of this specific issue was not possible.

2.2.4.8. Education, employment and living arrangements.

This project is the first one in the literature to look at academical and employment outcomes in patients with NDI. Regarding education, data showed that while primary and secondary education achievements were similar between the included patients and the European average, a smaller proportion of individuals with NDI completed tertiary education (University). Whether this is particularly related to NDI remains unknown, it simply may reflect the decreased educational attainment in children and young persons with chronic health conditions, including CKD (388-390). Reassuringly, individuals from the age range of 25-55-year-old were in full-time employment in the same proportion than the general population. It was also positive to report that most of the adult patients were living independently (away from their parents' home).

2.2.5. LIMITATIONS

This project represents the largest and most comprehensive cohort so far presented in the medical literature, However, there are still certain limitations, particularly in relation to its design as a retrospective cross-sectional study with data mostly captured at patients' last follow-up. There always must be a balance between feasibility and the comprehensiveness of the data collected, otherwise, in large studies like this, contributing clinicians may find it very challenging and time consuming to provide all requested data and the rate of participation could be substantially reduced.

Although this study has successfully addressed a substantial number of questions about primary NDI, the lack of longitudinal data did not make it possible to answer other relevant ones such as aetiology of CKD (including its association with use of NSAID). This highlights the importance of establishing a comprehensive international registry for prospective data collection.

Furthermore, it is possible that some patients with an incorrect diagnose of primary NDI were included in the final cohort. Despite a thorough screening of clinician's reported data (including pathogenicity of the reported genetic variants) some information at diagnosis point was not included in the survey, for example the maximal urine osmolality after DDAVP, in addition not all patients had confirmation by genetic testing. On the other hand it is also possible that others with partial NDI (without genetic testing) and maximal urinary osmolality ascending beyond 600 mosm/kg may have been wrongly identified as having central DI, therefore excluded from the study (248, 382, 383). It is thus conceivable that a small number of patients in this study may have been misdiagnosed as NDI. However, as a very large proportion (82%) of cases had genetic confirmation, misdiagnosis seems unlikely to have influenced these results. Lastly, reference population databases like NHANES and EU-28 may not fully match with this NDI cohort, something to take into consideration when citing this project for prognostic purposes in clinical practice.

Last, but not least, this cohort included predominantly white individuals, thus it is important to highlight that results and conclusions are potentially biased and may not be equally informative for individuals from a different ethnic background.

2.2.6. CONCLUSIONS

This extensive study (the largest in medical literature) provides informative data on primary NDI at different levels: socio-demographic, genetics, clinical prognosis, management, etc. Some of the more relevant and novel points derived from this work are:

1. Many adult patients included (N=110), within a wide age spectrum (18-70 years old), provides highly valuable information regarding prognosis and long-term outcomes of this rare condition.
2. Although more than half of the patients were diagnosed within the first year of age, a non-negligible 6% did present during adulthood with a majority having a confirmed genetic diagnose. Primary NDI should not be considered exclusively a severe disorder with presentation limited to early years and genetic confirmation should be offered to any patient with a suspected diagnose regardless of their age.
3. Although classically known as an X-linked recessive disorder, women can still fully manifest it and indeed they made up 16% of the entire cohort with a quarter presenting in the first year of life including patients with *AVPR2* mutations.
4. Genotype and in particularly type of mutation (pLoF vs missense) can inform on clinical manifestations, another reason to suggest genetic testing when suspected.
5. A countries affluence (as measured by GDP) is associated with a significant impact of patient care.
6. Overall outcomes with regards to growth are encouraging with adults achieving a Height SDS similar to the general population.
7. This project is the first one to raise concerns on obesity prevalence in adults with primary NDI and it is suggested that dietetic assessment continues also during adulthood.
8. Patients with primary NDI are at higher risk to develop CKD already since childhood, and the exact contributing factors are not fully understood.
9. This is the first project to present data on drug therapy for both children and adults. There is a remarkable reduction of medicines' use in adult practice and contributing factors are not fully known.

10. Almost half of the patients are reported to have flow uropathy and this correlates with poorer kidney function. It is important that patients with primary NDI are adequately screened, followed up and managed for this complication.
11. A significant delay on achieving nocturnal urinary continence during childhood has been documented, Considering the negative socio-economic impact that enuresis has on patients and their families, this study encourages clinicians to provide support and follow this appropriately.
12. Mental health disorders are more prevalent in patients with NDI than in the general population and these are not only limited to ADHD and intellectual disability as it was suggested in previous publications. Eating disorders such as hyperphagia are also observed and may also correlate with the high burden of obesity documented here.
13. Data on adult academic and employment achievements is similar to the general population, undoubtedly a very positive finding.

CHAPTER 3: CLINICAL AND MOLECULAR INVESTIGATIONS OF A COHORT OF CHILDREN WITH UROLITHIASIS

3.1 INTRODUCTION:

3.1.1. Definition and epidemiology

Urolithiasis also known as urinary stone disease is defined as the presence of crystalline calculi within the urinary tract (147). In the adult population, for instance, urolithiasis represents the third most common cause of urological disease only preceded by urinary tract infections and prostate conditions (391). It has an incidence of approximately 1.5% and a prevalence of 5.2% in general population. Differences according to gender have also been described, with a risk of developing at least one episode of 12% in men and 5% in women (392, 393).

In children prevalence and incidence are lower than in adults, however, they have been progressively increasing over the last 20-30 years (394). A study performed in Minnesota (USA) assessing paediatric cases between 1984-1990 and 2003-2008 described a doubled incidence from 7.2 to 14.5 per 100000 person/year (395). Yet, another study from the US showed a threefold increase in the incidence of symptomatic urolithiasis in paediatric patients, resulting in hospital admission in the period from 1999 to 2008 (396). Such substantial changes in the epidemiology of urolithiasis over a short period of time are likely the consequence of mainly environmental factors. Among them increased obesity rates and diet changes with high content in salt and animal protein have been described (395).

Other epidemiologic factors relevant for paediatric urolithiasis are:

- Age: the prevalence of urinary stone disease is lower in children than in adults. Within the paediatric group we can find differences in relation to age ranges, thus teenagers are having a higher incidence than younger children (397).
- Gender: in infants the incidence is higher in males while in teenagers it is higher in females, most likely reflecting also the incidence of urinary tract infections (398).

- Family history: particularly relevant in the paediatric age, in whom either monogenic and/or polygenic traits are more prevalent than in adults. Multiple studies describe the presence of 1st and/or 2nd degree relatives affected in 30-65% of the cases (395, 399, 400). It is expected that individuals from the same family will be exposed to the same environmental conditions, such as similar diet and geographic particularities. However, when analysis is adjusted for these confounding factors, there are still significant differences between groups according to family history. In fact, studies based on patients with onset during adulthood show a lower presence of family history (12-16%) compared to paediatrics (401, 402).
- Ethnicity: studies in USA have showed an increased incidence in African American children compared to Caucasian (403). In Australia for example young aboriginal individuals are found to have a particular risk for urate stones (404).
- Geography: in parallel to what is observed in adult population, the incidence of paediatric urolithiasis varies worldwide. Studies from Iceland and Near and Far East (eg: Turkey, Taiwan and Thailand) have described the highest proportion of children with urolithiasis (394, 405).

3.1.2. Aetiology

The aetiology of urolithiasis is notably diverse and commonly classified into 3 major groups: metabolic, infection and idiopathic.

Compared to adults, children have a higher incidence of primary metabolic disorders, and therefore an increased risk for stone recurrence. In fact, while the risk of recurrence is high within the paediatric age (20-50%), the majority of patients with repetitive episodes (50-70%) also have an underlying metabolic abnormality (406, 407). There are basically two mechanisms how metabolic abnormalities induce stone formation (394):

1. Solute excess: increased urinary concentration of calcium, oxalate, cystine or urate with/without decreased urinary volume.
2. Reduced presence of anti-lithogenic factors, such as magnesium and citrate.

The metabolic disorders can in general be classified in 5 major groups including: hypercalciuria (the most frequent), hyperoxaluria, cystinuria, disorders of purine metabolism and combined conditions such as hypocitraturia-hypercalciuria (seen in distal rental tubular acidosis). Specific primary diseases associated with these conditions are listed in table 3.1 (modified from Habbig et al.)(408).

Apart from primary disorders, these metabolic abnormalities can be associated with other factors (394):

- Hypercalciuria: prolonged immobilization (increased bone resorption), medications (excess vitamin D, loop diuretics and glucocorticoids among others) typically in preterm babies, chronic metabolic acidosis and hypercalcaemia.
- Hyperoxaluria: fat malabsorption, very common in chronic gastrointestinal diseases like Crohn's disease, Cystic Fibrosis, pancreatic insufficiency, and short bowel syndrome. Fatty acids, while in high concentration within the intestinal lumen, can act as a calcium chelator leading to increased free oxalate and therefore enhancing its enteral absorption.
- Hyperuricosuria: lymphoproliferative disorders especially in children.
- Hypocitraturia: chronic metabolic acidosis increases citrate reabsorption in the proximal renal tubule.

Table 3.1. Primary disorders associated with metabolic predisposition to urolithiasis

Metabolic disorder	Condition	Gene	Inheritance
HYPERCALCIURIA			
	AD hypocalcemic hypercalciuria	*CASR*	AD
	Familial isolated hyperparathyroidism	*MEN1, CDC73 & CASR*	AD
	Absorptive hypercalciuria	*SAC*	AD
	Resorptive hypercalciuria	*VDR*	AD
	Bartter Syndrome (BS):		
	Type 1	*SLC12A1*	AR
	Type 2	*KCNJ*	AR
	Type 3	*CLCNKB*	AR
	Type 4	*BSND*	AR
	Type 5	*CASR*	AD
	Dent's Disease:		
	Type 1	*CLCN5*	XR
	Type 2	*OCRL1*	XR
	Lowe's syndrome	*OCRL1*	XR
	Urolithiasis, osteopetrosis and persistent hypophosphatemia	*NPT2a*	AD
	Hereditary hypophosphatemic rickets with hypercalciuria	*NPT2c*	AR
	FHHNC	*CLDN16/CLDN19*	AR
	Liddle's syndrome	*SCNN1B*	AD
	Gordon's syndrome	*WNK1/WNK4*	AD
HYPEROXALURIA			
	Primary Hyperoxaluria Type 1	*AGXT*	AR
	Primary Hyperoxaluria Type 2	*GRHPR*	AR
	Primary Hyperoxaluria Type 3	*DHDPSL*	AR
CYSTINURIA			
	Type 1/A	*SLC3A1*	AR
	Type 2/B	*SLC7A9*	AD (ip)
PURINE DISORDERS			
	Hyperuricosuria		
	Lesch–Nyhan syndrome	*HPRT*	XR
	Glycogenosis type 1a	*G6PC*	AR
	Hypouricosuria		
	APRT deficiency	*APRT*	AR
	Xanthinuria	*XDH*	AR
COMBINED			
	Renal Tubular Acidosis		
	Type 1	*ATP6V1B1, ATP6V0A4, SLC4A1* & *FOXI-1*	AR/AD
	Type 2	*SLC4A4*	AR
	Type 3	*CA2*	AR

Infection is the second most frequent aetiology in children. Estimations of incidence vary according to geography (lower in western developed countries) and have decreased during the past few decades (406), as a reflection of different factors including: easier access to antibiotic therapy plus early diagnosis and treatment of urinary tract anomalies (160). *E. coli* is the most common bacteria causing UTI (urinary tract infection) in patients with urolithiasis (409, 410), as it is in normal population. However, the classic infective stone, made of a mineral called struvite (magnesium ammonium phosphate) is associated with the presence of many other bacteria, particularly those with urease-positive activity.

This group of bacteria includes *Proteus* sp, *Klebsiella* sp, *Pseudomonas* sp and *Enterococcus* sp among others. The process of struvite generation is based initially on the lysis of Urea (a molecule with antibacterial activity) into ammonium and bicarbonate. On one hand ammonium (NH_4^+) will react with PO_4^{3-} (and Mg^{2+}) creating struvite, on the other hand bicarbonate will increase urine pH enhancing the supersaturation of struvite (411). Precipitation of struvite creates a mineral nucleus which posteriorly combines with an organic material produced by the bacteria (biofilm). Biofilm has two main functions here: one is to enhance mineral crystallisation and the second one is to become a surface barrier, isolating and protecting the bacteria from urinary soluble bactericides including antibiotics (412).

Infective stones are particularly frequent in patients with concomitant urinary tract abnormalities, especially those which generate urinary stasis such as pyelo-ureteral junction obstruction/stenosis (PUJO), cloacal malformations or augmented bladders (406).

3.1.3. Clinical Manifestations

Compared to adults a relatively high percentage of children are asymptomatic on presentation (15-40%) and the stones are found incidentally during an abdominal imaging procedure for other purposes (147, 413, 414). In those who present with symptoms, colicky abdominal pain is the most common reason for consultation, especially in older children, while infants and younger patients usually manifest abdominal discomfort and/or irritability (405, 415, 416). Age does not only correlate

with the characteristics of the pain, also the prevalence, with pain being more frequent in adolescents than infants (60% vs 20%) (417).

Gross haematuria is present in 30–50 % of cases while microhaematuria is seen in most affected children (405, 418). Approximately 10% of the patients do present with symptoms related to irritation of the urinary tract such as: dysuria, urgency, voiding problems, or urine retention (caused by lower urinary tract stones). These symptoms can be misinterpreted as a urinary tract infection by clinicians, although it is common to have a concomitant urinary tract infection with the stone (415).

3.1.4. Diagnosis

The diagnosis of urolithiasis and its aetiology in children has three main components: a suggestive clinical presentation (abdominal pain, haematuria and/or dysuria), a metabolic/infective screening (including serum and urinary tests) and imaging (ultrasound and abdominal computed tomography CT).

- Metabolic/infective assessment: ideally every paediatric patient should complete a full metabolic and infective screening including blood and urine tests, also stone composition analysis when available. The aim in the acute phase is to provide emergency care (if required) of complications such as: urosepsis, urinary tract obstruction with secondary acute kidney injury and pain. Subsequent routine investigations can help to identify underlying risk factors for stone formation; this will guide the medical/interventional management in order to prevent recurrence (413).
- Radiological evaluation:
    - Abdomen ultrasound: due to absence of radiation and good sensitivity in detection of renal and ureteral stones, ultrasound is the recommended primary imaging modality for suspected nephrolithiasis (419). However, it does have some limitations to find small calculi (< 5 mm), particularly if located in mid-ureters and also to distinguish between nephrolithiasis or calcified plaques within papillae or calyces (413).
    - Abdomen CT: as in adults, non-contrast helical CT is the most sensitive modality to diagnose renal or ureteral stones. It can help to detect lower-

mid non obstructive ureteral stones and very small stones of approximately 1-2mm which tend to avoid detection by ultrasound (413). Still, there is radiation exposure although with modern low-dose protocols this is less than from an abdominal x-ray.

- Abdomen plain x-ray: is a very low sensitive (60%) test with added radiation burden when compared to ultrasound. It should only have a place in scenarios where ultrasound/CT are not available.

3.1.5. Treatment

The management of urolithiasis is divided in two phases: a first or acute one focussing on pain control, treatment of urine infection, release of obstruction and facilitation of stone passage (occasionally requiring surgical stone removal); and a posterior one based on stone recurrence prevention (420).

- Supportive care: including adequate hydration, control of nausea/vomiting with antiemetics, pain control with narcotics and/or nonsteroidal anti-inflammatory medications and antibiotics for urinary tract infection when present. Finally, but not exempt of controversy, medical expulsive therapy can be used in distal ureteric stones (unlicensed use in children and limited evidence in adults) (420, 421). Medical expulsive therapy uses alpha-adrenergic blocker drugs (such as tamsulosin or doxazosin), facilitating spontaneous passage by inducing lower ureteric sphincter relaxation.
- Surgical Stone Removal: due to a high spontaneous passage rate of small calculi, potential complications and technical difficulties in children, an observational period with adequate supportive care should be offered prior to surgical removal procedures when possible. Exceptions to this approach are: urinary tract obstruction (particularly in infective scenarios), unremitting severe pain and symptomatic stones that failed to pass after 2-4 weeks of conservative therapy (420). Most common procedures are summarised in table 3.2 (modified from Granberg CF, and Baker LA) (422).

Table 3.2. Description of stone removal procedures

	ESWL	Ureteroscopy	PCNL
Indication	Simple renal/ureteral stone < 1-2cm	Simple renal/ureteral stone < 1-2cm	Staghorn / multiple large stones
Removal rate	44-95%	50-100%	70-90%
Ureteral stent required	Very low	High	High
Nephrostomy tube required	None	None	High
Complications	0-18%	0-8%	0-30%
Days off from routine activities	Up to 7	Up to 14	Up to 20

- Prevention of recurrent disease: children are having an increased risk of recurrent urolithiasis compared to adults, especially those with structural urinary tract abnormalities, recurrent urine infections and metabolic abnormalities predisposing to stone formation. Infective urolithiasis, in a context of structural abnormalities, requires a more aggressive approach aiming for bacterial and stone clearance and in some cases corrective surgery. Acute treatment is based on antibiotic therapy and decompression of the urinary tract when obstructed. Second step includes long term antibiotic prophylaxis and stone removal procedure, which usually implies either an invasive urological procedure or corrective surgery. On the other hand, metabolic conditions, especially primary metabolic disorders, do not have a cure and patients keep a long-term risk for stone formation, frequently requiring added medications apart from diet modifications. A summary of medical treatment options for metabolic disorders with predisposition for urolithiasis has been presented in table 3.3 (modified from Edvarsson, Vidar, 2016) (160).

Table 3.3. Treatment summary for metabolic urolithiasis

Metabolic abnormality	First-line treatment	Second-line treatment
Hypercalciuria	Reduce dietary sodium	Alkali supplement
	Normal calcium diet	Thiazide diuretics
Hyperoxaluria	Reduced oxalate diet (enteric oxaluria)	Short interference RNA (PH 1-2)
	Alkali supplement	Liver transplant (PH1)
	Pyridoxine (PH-1)	
Hypocitraturia	Alkali supplement	
Hyperuricosuria	Alkali supplement	Allopurinol
Cystinuria	Hyperhydration	Thiola
	Alkali supplement	D-Penicillamine
	Reduce dietary sodium	

3.2. METHODS:

A retrospective electronic clinical records review was performed (including demographics, biochemistry and radiological data) from children attending the specialised monthly renal stone clinic at Great Ormond Street Hospital (GOSH, London UK) during a year period from June 2017 to June 2018.

Ethical approval was not required for retrospective analysis of anonymised patient information but for DNA extraction and storage parents were requested to sign a specific consent form. In the dedicated stone clinic, all children were reviewed directly by a consultant paediatric nephrologist (Dr. William Van't Hoff, Dr Wesley Hayes, Prof. Detlef Bockenhauer or Prof. Robert Kleta), consultant paediatric urologist (Mrs Naima Smeulders) or by a senior fellow in paediatric nephrology/urology under the supervision of the above consultants.

All patients received a complete clinical history including details such as age, gender, perinatal history, paternal consanguinity, and family history of renal stone disease. Weight, height and their respective centiles within the first 3 months of presentation were calculated according to UK-WHO growth data (373, 423).

A full metabolic screen, if obtained, included: serum urea, creatinine, electrolytes (calcium, magnesium and inorganic phosphate), tCO_2, alkaline phosphatase, albumin and urate; in case of abnormal calcium/phosphate results, 25-OH vitamin D_3 and iPTH (intact parathyroid hormone) were also studied. Analysed urine is from non-fasted midmorning spot samples and/or 24 hours total volume collection when possible (fully continent patients). Urinary investigations included microscopy and culture, calcium, urate, oxalate, cystine and creatinine. Citraturia is not routinely investigated in all the patients and is requested under clinical discretion.

Stone fragments, if available, were sent for infrared-spectroscopy, the presence of any mineral (if mixed combination was present) within each fragment was arbitrary set as significant when greater than 10%.

Stone burden was assessed in every patient by urinary tract ultrasound and in selected cases also by low dose abdominal CT (computed tomography) or MRU (magnetic resonance urography). Multidisciplinary team meetings were organised monthly to discuss the most challenging diagnostic and therapeutic decisions.

Patients did receive a diagnosis of idiopathic, infective or metabolic urolithiasis when satisfying at least one of the following criteria:

3.2.1. Infective

Patients were classified as having infection related urinary stones when there was a documented history of recurrent infections or at least one proven urinary tract infection (UTI) associated with struvite (triple phosphate) stones.

3.2.2. Idiopathic

No metabolic abnormalities detected, neither history of recurrent urinary tract infections. Some patients in the idiopathic group could have other known risk factors such as immobility, prematurity or structural abnormalities. However, during assessment the metabolic and infective screening resulted negative and therefore they did not meet the criteria to be classified within these two groups. In particular, hypercalciuria has been described in prematurity and patients with immobility, on the

other hand, structural abnormalities have been linked to recurrent urinary tract infections (160). Whether these patients had a transient urine metabolic disorder, and/or they are having a genetic predisposition with a mild phenotype sensitive to environmental changes (such as diet and fluid intake) was not possible to be clarified during the clinical assessment.

3.2.3. Metabolic

3.2.3.1. Hypercalciuria

Defined as a spot urinary calcium/creatinine (UCa/UCr) ratio above the age specific upper normal reference value (<1 year: 2.2 mmol/mmol; 1–2 years: 1.5 mmol/mmol; 2–3 years: 1.4 mmol/mmol; 3–5 years: 1.1 mmol/mmol; 5–7 years: 0.8 mmol/mmol; 7–17 years 0.7 mmol/mmol) (139). When the child was able to complete a 24 hour urine collection this measure was preferred over the single spot sample and the upper limit for any age was placed at 0.1mmol/kg/day (160). A 24-hour urine collection was considered adequate if the creatinine excretion was 0.1–0.2 mmol/kg/day. Transient hypercalciuria is commonly see within the paediatric age so patients required at least two high results before they were considered to have pathological hypercalciuria.

3.2.3.2. Hyperoxaluria

Defined as a spot urinary oxalate/creatinine (UOx/UCr) ratio above the age specific upper normal reference value (<1 year up-to 98 µmol/mmol; 1–4 years 72 µmol/mmol; 5–12 years 71 µmol/mmol; above 12 years 38 µmol/mmol) (139). Confirmation required a second abnormally high ratio in a spot sample or when possible a 24-hour urinary collection in an acidified container (upper normal limit of normality = 460 µmol/day/1.73m^2) (424). When hyperoxaluria was confirmed, other oxalate related metabolites were measured to inform the diagnose (such as glycolate, L-glycerate and 4-hydroxy-2-oxoglutarate).

Genetic analysis was also available for these patients and provided confirmation of the three recognised forms: PH1-*AGXT*, PH2-*GRHPR* and PH3-*HOGA1* (160).

Hyperoxaluria in children can also be secondary to gastrointestinal disorders that induce fat malabsorption such us: short bowel syndrome, cystic fibrosis and chronic diarrhoea (425). This is also known as secondary/enteric hyperoxaluria. Patients with primary hyperoxaluria type 1 (PH-1) received a trial of oral pyridoxine (426) and also may require liver/combined kidney-liver transplantation before entering in CKD stage 4 (427).

At the time of data collection, a newly developed si-RNA drug named ALN-GO1 (428) was on a phase II clinical trial specifically for patients with PH-1. ALN-GO1 is currently available within the NHS for treatment of patients with moderate/severe PH-1, under the commercial name of Lumasiran. A second si-RNA drug called Nedosiran is currently under consideration for NICE approval (429). None of the patients with primary hyperoxaluria in this cohort were treated with Nedosiran.

3.2.3.3. Cystinuria

Testing for cystinuria was performed via ion exchange chromatography of urine amino acids, upper normal limit of urinary cystine/creatinine ratio in mmol/mmol was established as 20 µmol/mmol creatinine (however this can be higher (up to 100 µmol/mmol) in infants younger than 1 year of life (430). Typically, also another 3 dibasic aminoacids (arginine, lysine and ornithine) are elevated in the urine as well, as these amino acids share a common transport modus with cystine. Standard therapy in cystinuria includes hyperhydration and urine alkalinisation (431) but in some occasions treatment is escalated due to recurrent lithiasis; these patients receive oral treatment with either D-penicillamine or Tiopronin (thiola) (432, 433).

3.2.3.4. Abnormal adenine/purine metabolism

Urate/creatinine ratios in urine higher than normal upper limit according to age (<7 days 1.96 mmol/mmol; 7 days to 2 years 1.53 mmol/mmol; 2–6 years 1.35 mmol/mmol; 6–10 years 0.85 mmol/mmol and 10–18 years 0.67 mmol/mmol) (139). Patients were diagnosed with APRT (Adenine Phospho-Ribosyl Transferase) deficiency based on stone composition (DHA or 2,8-dihydroxyadenine) and subsequent genetic confirmation.

3.2.3.5. Hypocitraturia

Not routinely measured in all patients. Patients were diagnosed with hypocitraturia when the urine creatinine ratio was below the lower normal range according to gender (0.11 mmol/mmol for females and 0.04 mmol/mmol for males) (139).

3.2.4. Kidney function at end of follow up

The eGFR (estimated glomerular filtration rate) according to Schwartz modified formula (135) was calculated in those patients with recorded serum creatinine (μmol/L) and height at the same follow up appointment.

3.2.5. Statistics

Data were analysed for normality using the Kolmogorov-Smirnov test. Normal data are presented in mean and standard deviation while non-normal or skewed data are presented in median and interquartile range. Significance was assessed by Student-t for normal data, Mann-Whitney U Test for non-parametric data and Chi-Square Test for categorical variables. A p value < 0.05 was considered significant during the analysis. IBM SPSS Statistics Version 24 for Windows was used for statistical analysis.

3.3. RESULTS:

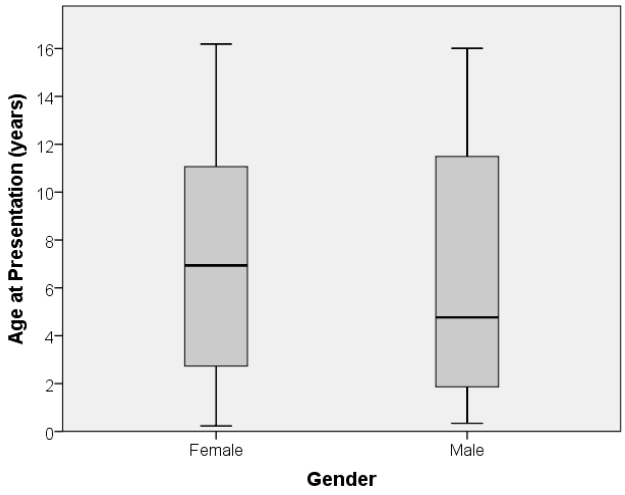
3.3.1. Demographics

During the year period from June 2017 to June 2018 there were 144 attendees in the stone clinic. Renal stone disease was not confirmed in 3 of them, thus, the final cohort included 141 patients. Gender distribution showed a male/female ratio of 1.6; 61% were male (N=86) compared to 39% females (N=55). Patients were followed up in the renal stone clinic for a median (range) of 2.4 (0.0-16.0) years.

3.3.2. Presentation

Median (range) age at presentation was 5.5 (0.2-16.2) years. Male patients had an earlier onset with a median (IQR) of 4.8 (1.8-11.5) years compared to female ones with median (IQR) of 6.9 (2.6-11.1) years. This gender difference was not statistically significant ($p > 0.05$) (Figure 3.1).

Figure 3.1. Age at presentation according to gender

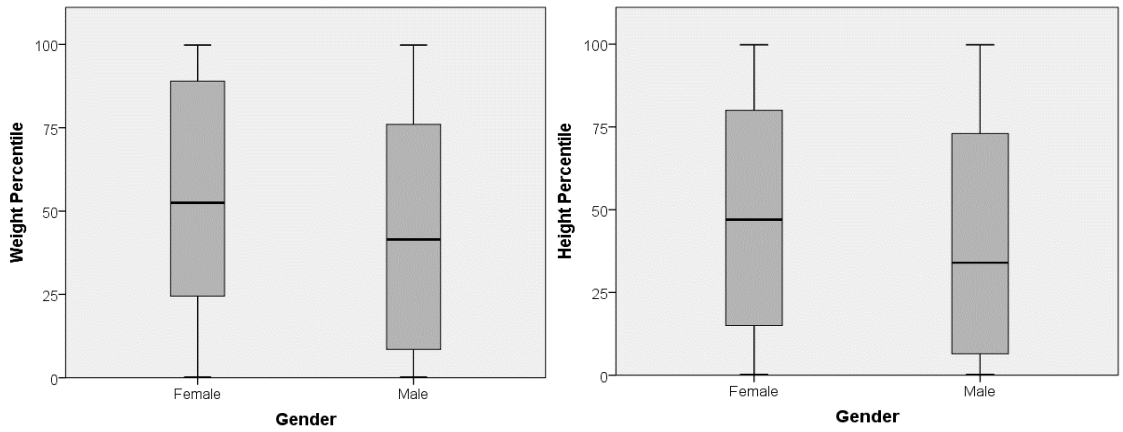


Family history was recorded in all patients. 48/141 (34%) had either a first, a second degree relative or both affected. Co-sanguinity (N=119) was described in 15 (13%) patients. All of them had a proved metabolic predisposition to renal stone formation compared to 58% in children with non-consanguineous parents ($p = 0.002$).

Weight at presentation (N=136) had a median (IQR) of 45th (11th-80th) centile. For males the median (IQR) was 42nd (8th-77th) vs 53rd (24th-90th) in females ($p > 0.05$) (Figure 3.2). In addition, a second analysis was performed according to the three

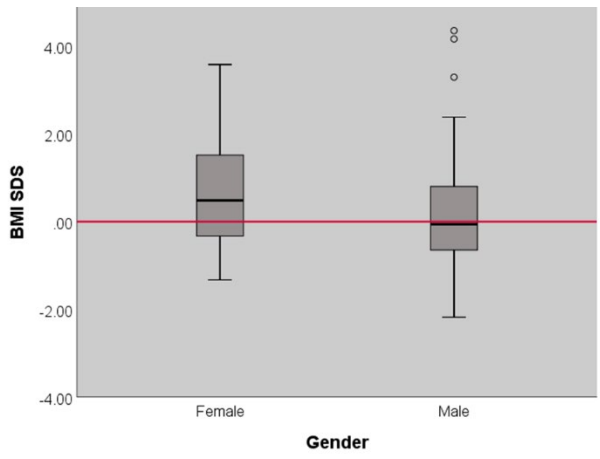
classical groups (infective, metabolic and idiopathic). Weight centiles did not differ significantly between groups. For the total cohort the median (IQR) for height (N=120) was 41st (9th-78th) centile. Differences between the two genders were not significant with male group on the 34th (6th-74th) vs females 47th (15th-84th) centiles ($p > 0.05$) (Figure 3.2).

Figure 3.2. Weight and height centiles on presentation



BMI SDS was also not significantly different between gender groups with total mean 0.18 (± 1.64); female 0.39 (±1.34) vs male 0.05 (±1.79) (Figure 3.3). In both groups the prevalence of obesity was 6%.

Figure 3.3. BMI SDS on presentation



Hypertension on presentation (N=138) was detected in 6(4%) cases. From the entire cohort only 2 patients did have AKI on presentation, both had a normal eGFR at the end of follow up (96 and 106 ml/min/1.73m^2). Clinical features on presentation were:

- Abdominal pain 63(45%)
- UTI 43(31%)
- Macroscopic (visible) haematuria 38(27%)
- Microscopic haematuria 21(15%)
- AKI 2(1%)
- Familial screening 7(5%)
- Incidental 30(21%)

3.3.3. Infective Stones

A total of 32 (23%) cases were diagnosed as having infective stones. From those, the identified causative bacteria were:

- *E. coli* in 18 cases (56%)
- *P. mirabilis* in 11 cases (34%)
- *P. aeruginosa* in 8 cases (25%)
- Others in 6 cases (19%)
- *Enterococcus* sp. in 5 cases (16%)
- *Klebsiella* sp. in 2 cases (6%)
- *S. epidermidis* in 1 case (3%)
- Unknown in 4 cases (13%)

Infection related cases were analysed in relation to the presence or absence of structural abnormalities of the urinary tract. From the 32 patients, 12 (38%) had an abnormal urinary tract and in 20 (62%) it was normal. When the isolated bacteria were analysed taking in account the presence of urinary tract abnormalities only *P. aeruginosa* showed a significant association ($p = 0.03$). *P. aeruginosa* was found in the urine from 50% (6/12) of the patients with abnormal urinary tract vs 10% (2/20) in the group with normal urinary tract. The calculated odds ratio for this association was equal to 9.0 (CI 95% 1.4-57.1).

3.3.4. Idiopathic

In 44 patients (31%) it was not possible to find an abnormal result during the metabolic/infective assessment, therefore those cases were classified as idiopathic.

In 17 of these patients (39% from the entire idiopathic group) there was a positive history for: prematurity, immobility and/or structural abnormalities; all of them known risk factors for urolithiasis. Within this group of 17 patients with negative metabolic/infective screening, the distribution was as follow: 11 (65%) did have structural abnormalities, 5 (29%) had prolonged immobility, 5 (29%) were preterm with a range of gestational age from 25-31 weeks and in 5 (29%) there was a combination of these risk factors.

These 3 risk factors also had a global impact on the entire cohort as follow:

- immobility (N=141): 13 (9%) patients (7 did not have hypercalciuria)
- prematurity (N=120): 25 (21%) patients with a median (range) of 33 (25-36) weeks of gestation
- structural abnormalities (N=141): 33/141 (23%). When comparing this group with the rest of the cohort, a significant association (p = 0.025) was found with infective stones. 38% of patients with abnormal urinary tract had infective stones vs 19% in the rest (normal structure).

3.3.5. Serum screening for metabolic predisposition

As part of the metabolic screening patients had a serum biochemistry including serum tCO_2 (total CO_2, equivalent to serum bicarbonate, N=109) and total calcium (N=130). Results for both tests are summarized as follow:

- bicarbonate (mmol/L): median (IQR) 24.0 (22.0-25.0)
- total Calcium (mmol/L): mean (SD) 2.44 (±0.1)

From the entire cohort 81/141 (57%) patients had a confirmed metabolic disorder, and from those, a total of 16 (20%) had a combined infective-metabolic stone. The prevalence of the different causes is described in table 3.4 ([a]not routinely tested in clinic, [b]Bartter syndrome type 2 and [c]hyperoxaluria + hypercalciuria/hypocitraturia).

Table 3.4. Distribution of metabolic risk factors for stone formation

Metabolic condition	**N(%)**
Hypercalciuria	43(53%)
Cystinuria	15(19%)
Primary hyperoxaluria type 1 (PH-1)	6(7%)
Abnormal purine metabolism	4(5%)
PH-2	3(4%)
PH-3	2(2%)
PH no 1-3	2(2%)
Enteric hyperoxaluria	2(2%)
Abnormal purine metabolism	4(5%)
Combined urinary metabolic disorder[c]	2(2%)
Hypocitraturia[a]	1(1%)
Tubulopathies[b]	1(1%)

3.3.6. Stone analysis

The IR-spectroscopy analysis on the stones was performed in 88 patients (62%), revealing the presence of the following minerals:

- calcium oxalate: 48 cases (55%)
- calcium phosphate: 37 cases (42%)
- cystine: 10 cases (11%)
- purine: 3 cases (3%)
- struvite (“triple phosphate”): 15 cases (17%).

Calcium salts (oxalate and phosphate) were the most common identified from the analysed stones 99/128 (77%), cystine was only isolated in patients suffering of cystinuria (10/10). Phosphate salts (calcium phosphate and triple phosphate) were the most frequently identified in infective stones with 34/38 times (89%).

3.3.7. Staghorn Stones

Staghorn stones were found in 16/141 cases (11%). Sub analysis for these patients showed:

- 56% (9/16) were having an underline metabolic condition.
- 63% (10/16) were associated with UTI, while the proportion of infective stones in the patients with no staghorn stones was 18% (p value < 0.001).

The odds ratio of staghorn stones to be associated with urine infection was 7.8 (CI 95% = 2.6-23.7).

- 4/16 (25%) did have a structural abnormality of the urinary tract.
- 1/16 (6%) presented initially with AKI.

The eGFR at end of follow up in patients with diagnosed staghorn stone was available in 11 cases from which the mean (±SD) was 97 (±26) ml/min/1.73m^2. 36% (4/11) of the patients were having CKD (stage 2/3) at the end of the follow up (eGFR 52-78 ml/min/1.73m^2).

3.3.8. Kidney function at end of follow up

In 86 patients there was enough information available (height and serum creatinine) to estimate the renal function through Schwartz formula. The estimated GFR for these patients had a mean (±SD) of 104 (±23) ml/min/1.73m^2. In order to stablish the prevalence of CKD within our cohort a second analysis was performed by filtering out the patients younger than 2 years old (N=2), CKD was defined according to KDOQI Guidelines (434) as eGFR < 90ml/min/1.73m^2. From a total of 84 patients, 19 (23%) were found to have CKD. From the entire cohort (older than 2 years, N=137) this represented the 14%.

3.3.9. Treatment

Increased water intake (including hyperhydration in patients with cystinuria and primary hyperoxaluria) and a low sodium diet is part of the standard recommendation in the stone clinic. Other treatment options include urine alkalinisation with potassium citrate, a pyridoxine (vitamin B6) trial in patients with PH-1, d-penicillamine or tiopronin for cystinuria, thiazide diuretics for hypercalciuria not responding to standard recommendations, and antibiotic prophylaxis in cases of infective stones (at least until patients have been 1 year free of urinary tract infections). The distribution of the treatments within the metabolic group has been summarised in table 3.5.

Table 3.5. Offered treatment according to specific disorder

Treatment	Underlying metabolic abnormalities				
	Hypercalciuria (n=44)	PH-1 (n=6)	PH-2, PH-3 and others[a] (n=11)	Cystinuria (n=15)	Purine disorder (n=4)
Potassium Citrate	21	5	8	15	0
Thiazides	23	0	0	0	0
D-Penicillamine	0	0	0	1	0
Tiopronin	0	0	0	6	0
Pyridoxine	0	3	0	0	0
Allopurinol	0	0	0	0	3
Liver Transplant	0	1	0	0	0
ALN-GO1[b]	0	1	0	0	0

[a] *Including enteric hyperoxaluria*

[b] *Part of an active research study.*

3.3.10. Stone distribution

Stones were predominantly found within the upper urinary tract (117/141 patients = 83%). The exact distribution in the urinary tract is summarised as follow (percentages sum is greater than 100% due to the concomitant presence in several patients of bilateral and/or multifocal stones):

- Right kidney: 80/141 (57%)
- Left kidney: 81/141 (57%)
- Bilateral (both kidneys): 44/141 (31%).
- Right Ureter: 25/141 (18%)
- Left Ureter: 17/141 (12%)
- Bladder and urethra: 10/141 (7%)
- 35/141 (25%) patients did spontaneously passed stones at least one time.

The risk of developing bilateral renal stones was significantly increased in the metabolic group with 32/81 cases (40%) compared to the non-metabolic group 12/60 (20%) (p value = 0.013). The odds ratio for the association of bilateral urolithiasis and a metabolic condition was 2.6 (95% CI: 1.2-5.7). Patients who develop bilateral kidney stones did not have a significantly different eGFR at the end of follow up (105±26 vs 104±21 ml/min/1.73m^2).

3.3.11. Stone removal procedures

A total of 108 (77%) patients from the whole cohort underwent at least one urological procedure. The mean (range) of total procedures was 1.8 (1-10). Out of these 108 patients, 68 (63%) received ESWL, 41 (38%) PCNL/PCCL, 38 (35%) ureteroscopy, and 16 (15%) an open surgical procedure (pyeloplasty or nephrectomy).

The proportion of patients who received a stone removal procedure within the metabolic group was 64/81 (79%) vs 44/60 (73%) from the non-metabolic groups ($p > 0.05$). When the infective group was assessed, 31/32 (97%) required a removal procedure while the proportion in the non-infective was 77/109 (71%), this difference was statistically significant ($p=0.002$).

Complete stone clearance at end of follow up was achieved in more than half of the patients (77/141; 55%). In 44 (31%) of the cases some fragments remained within the urinary tract (partial clearance), and finally in 20 (14%) patients the stones persisted unchanged. Stone clearance at end of follow up also correlated with diagnostic group. In the metabolic cohort, only 42% were completely stone free compared to non-metabolic with 72% ($p = 0.002$). Opposite findings were found in the idiopathic group, 70% vs 47% (0.034). For infective stones there was no significant difference ($p=0.145$).

3.4. DISCUSSION:

3.4.1. Demographics

Here it is assembled a large cohort of paediatric patients seen in a dedicated renal stone clinic during one-year period in GOSH, a quaternary hospital. The aim is to present a detailed phenotyping of paediatric patients with urolithiasis and to identify correlations between outcomes and various risk factors that could potentially help to better understand the multifactorial aetiology of childhood urolithiasis and provide us with relevant questions for future research projects. Details regarding both clinical and laboratory data were included. A high number of patients (N=141) with proven urolithiasis were reviewed during this period, providing informative date on epidemiology, aetiology, clinical presentation, treatment and long-term kidney function for children with urinary stone disease in a Western Developed Country. Despite being a single year review, the number of patients included is one of the highest published up to date (395, 414, 416, 417, 435).

As commonly seen in adult population (147, 160, 397, 435), this study has found a higher prevalence in males compared to females. But unlike in adults, in children this relation is thought to be caused by a higher prevalence of congenital urinary tract abnormalities, especially in young male children (397). The analysis of this cohort did not show a significant difference for gender and prevalence, most likely due to a limited size population.

3.4.2. Presentation

In our cohort boys had a higher prevalence than girls, also presented earlier, approximately 2 years (4.8 vs 6.9 in girls). The younger age in boys may reflect a theoretical higher prevalence of urine infections associated with congenital structural abnormalities in the young male group. Boys tend to present UTIs in the first decade of life while girls do it in the second (398). The association in this cohort was not significant on statistical analysis, most likely in relation to the size of the studied cohort. However, this is a well-known phenomenon described not only in children but also in adults (392, 403, 414).

Family background is an important topic to discuss when taking the clinical history of children with urolithiasis. The existence of first and/or second-degree relatives affected was reported by 34% of the patients. Practically same prevalence (33%) was found in an Icelandic paediatric study (405) and others in adults have shown values in the range of 25-65% (160). Consanguinity was documented in 13% (from the 119 patients with recorded genetic tree) and all of them were from the metabolic group (primary hyperoxaluria, cystinuria, purine metabolism disorder or idiopathic hypercalciuria). This was consistent with the expected presence of autosomal recessive inherited disorders.

Obesity is one of the most common known risk factors for urinary stones in adults (160), however in children this is less clear (414, 416, 435). BMI SDS calculation for both boys and girls did show an estimated prevalence of 6% of obesity in this cohort, this is a markedly lower result compared to recent epidemiological studies based on UK paediatric population which estimates prevalences between 10-17% (436). Weight centiles were estimated according to age and gender UK-WHO criteria (373, 423) and neither male/female (median 42^{nd} / 53^{rd}) were significantly high. While weight centiles did not differ from normal population, height centiles on the other hand were lower in both boys (34^{th}) and girls (47^{th}). A discrete failure to thrive, as we described, has also been previously reported (435). This is thought to be associated to other comorbidities (recurrent UTIs, episodes of renal colic with poor intake and vomits and frequent hospital admissions).

Urolithiasis in children frequently presents with atypical or unspecific signs and symptoms, especially in the younger ones where the only clinical manifestation might be a non-colicky abdominal pain (147) or the stone is found incidentally (15-20%) by an abdominal ultrasound scan (413). Indeed, in our group 21% of the cases did have an incidental diagnose. Almost half (45%) of the patients presented with abdominal pain, this is consistent with other epidemiological studies where this symptom was noticed in 50-75% of the cases (414, 417), especially in older children.

UTI was the second most documented clinical feature on presentation, 31% of the cases. A higher prevalence in younger children was appreciated when patients were subclassified as under and over 9 years (38% vs 19%, $p = 0.008$). Previous reports

have also described a similar relation between UTI and young age (417). More than a quarter of the patients with abnormal urinary metabolic screening also had urinary tract infections. This highlights the importance of a complete metabolic evaluation in every child presenting with urinary tract infection and urolithiasis.

Macroscopic haematuria was present in roughly a quarter (27%) of this cohort, being associated half of the times with abdominal pain (14%). The incidence of haematuria described in the literature fluctuates from 30-55% (414, 417, 435), confirming our findings.

Only 2 patients (1%) presented with AKI. One of them had cystinuria and bilateral staghorn stones at onset while the other had a severe stone burden with bilateral renal and ureteral obstructive stones. AKI is not common on presentation and in both children and adults represents less than 1% (437).

3.4.3. Infective stones

From the entire cohort 23% of the patients did have a diagnose of infection related urolithiasis. Classically infective stones are associated with urease-positive bacteria (like *Proteus* sp.) and therefore made of triple phosphate (also called struvite or magnesium ammonium phosphate) (160). However, in this group *E. coli* was the most frequent isolated bacteria, which is the most common uropathogen in all paediatric ages (438). The two other most frequent bacteria identified have indeed urease-positive activity: *Proteus mirabilis* and *Pseudomonas aeruginosa*.

The association between urinary tract abnormalities, urine infections and urolithiasis is well known and have been discussed in this manuscript. The analysis showed a significant correlation between the isolation of *P. aeruginosa* in urine cultures of patients with urolithiasis and the presence of urological malformations (439). Therefore, it is suggested that paediatric patients with urinary tract infections caused by *P. aeruginosa* UTIs will need further screening including abdominal ultrasound.

3.4.4. Idiopathic stones

From the entire cohort a total of 44 patients (31%) did not meet the criteria for inclusion in the metabolic or the infective groups, therefore were classified as idiopathic. However, in 17 of them (39%) it was found at least another risk factor for urolithiasis including immobility, prematurity and abnormal urinary tract (435). The first two of these factors are associated with hypercalciuria; immobility due to increased bone resorption and prematurity due to preterm hypophosphataemic rickets, metabolic acidosis and drugs (such as loop diuretics and steroids). Very likely these patients had a transient hypercalciuria and cleared it by the time the studies were performed; however they had to be classified as idiopathic due to negative metabolic screening. Patients with abnormal urine drainage are well known to have urine stasis and, in some cases, increased rates of urinary metabolic abnormalities (160, 406, 408).

3.4.5. Metabolic group

More than half (57%) of all the patients from this cohort were found to have a metabolic predisposition for stone formation. Hypercalciuria (53%), cystinuria (19%) and PH (15%) were the three most common disorders. Similar distributions have been described in previous reports, with hypercalciuria (mainly idiopathic) being the most common and fluctuating between 50 to 80% (405, 417, 435). The high prevalence of rare conditions such as cystinuria and primary hyperoxaluria is likely reflecting the specialised nature of this paediatric kidney stone clinic which takes place in a quaternary hospital.

Moreover, 16 (20%) of the patients with at least one metabolic risk factor have developed infective stones. This combined infective-metabolic pattern has been discussed in other publications (440, 441). In these articles it has been suggested that bacterial infections are not always secondary to urine flow obstruction, also having a pathogenic role in metabolic stones.

The rest of the metabolic group included disorders with low prevalence in the general population and therefore their distribution was also scarce. One exception was hypocitraturia with only 3%, this is just reflecting the clinical practice of this clinic, as urinary citrate is only requested under clinician’s discretion and not routinely in all the

patients. Its prevalence in other epidemiological studies fluctuates from 10% to 70% (160, 397).

3.4.6. Stone analysis

The analysis of the stone composition is essential for both the diagnosis and the therapeutic approach. This is particularly relevant in metabolic cases, such as primary hyperoxaluria (predominance of monohydrate calcium oxalate), cystinuria (cystine) and purine metabolism disorders (urate and 2,8 dihydroxyadenine). However, not always this analysis is possible, such is the case of patients who have spontaneously passed their stones (and not collected them) or patients who have undergone extracorporeal lithotripsy.

In this group, stones were analysed in 88 patients (66%). This revealed a clear predominance of calcium-based minerals (77%) over phosphate (struvite) and cystine. Similar findings have also been confirmed in previous studies where calcium has been present in up to 98% of the analysed stones and in addition phosphate rich stones were typically found in the infective group (416, 417). Cystine, as expected, was the predominant mineral in 100% of the stones identified in patients with cystinuria.

3.4.7. Staghorn stones and kidney function at the end of follow up

Staghorn stones are large branching stones filling the renal pelvis and the calyces. They can be complete or partial depending on the level of occupancy of the collecting system and are generally associated with urinary tract infections and/or structural defects of the urinary tract (49-68%). This paradigm has been substantially changing for the last decades, as staghorn stones tend now to manifest more in patients with underline metabolic abnormalities and less frequently in those with only recurrent infections (392, 442, 443).

Here, a total of 16 patients (11%) developed staghorn stones, and from these 63% were having a concurrent urinary tract infection, reflecting similar results as previous epidemiological studies. Interestingly 56% of the 16 cases did also have an underline metabolic risk for stone formation. This highlights the importance of a complete metabolic screening, including those who presented with infective stones (444).

Considering that Schwartz formula requires height and serum creatinine for eGFR calculation and patients with good clinical progress do not have regular blood tests, only a limited number of patients could have their kidney function estimated at end of follow up. Thus, CKD prevalence within this selected group was found to be high at 23% (but less at 13% if compared to the entire population). Even if we assume the lowest prevalence of 13% for CKD stage ≥ 2, this value is still significantly higher than average paediatric population (< 0.01%) (158, 159). There is a well-known relation between urolithiasis and CKD progression. In adulthood cardiovascular risk factors are the most relevant while in children the association is mainly with urine infections (445).

Staghorn stones are associated with both acute and chronic kidney injury for multiple reasons such as urinary tract obstruction and recurrent urine infections. Thus, one of the treatment priorities is to achieve a complete stone clearance in the urinary tract (442).

In this cohort only 1 patient (6%) presented with severe AKI. From those with available data about eGFR at the end of follow up (N=11), 36% (N=4) were having chronic kidney disease stage ≥ 2 (eGFR < 90 ml/min/1.73m^2): three had progressive deterioration and one had static eGFR. These results remark the importance of an early intervention in patients with staghorn stones. They are also relatively alarming taking in account that adult literature (446) reports progressive loss of renal function in approximately 14% of patients with staghorn stones.

3.4.8. Stone distribution

Stones were predominantly found within the upper urinary tract (kidney, pelvis and proximal ureter), exactly in 83% of the patients. This distribution has been previously described and is one of the reasons why younger children present initially with atypical features (vague abdominal pain rather than colicky lower abdominal pain) (447). Bilateral presentation was observed in 31% of the patients and this has been associated also with a higher risk of having an underlying metabolic condition (435).

3.4.9. Treatment

3.4.9.1. Prevention of recurrent disease

The different types of treatment offered to the patients, and their distribution are summarised in table 3.5. They basically reflect our current practice, which is based on previously published scientific evidence and international guidelines and formularies. In summary, all the patients undertake a relatively high fluid intake and low salt diet. Potassium citrate is used for urine alkalisation in patients with hypercalciuria, hypocitraturia, hyperoxaluria and cystinuria, with different urine pH target depending on the underline disorder.

Thiazide diuretics are prescribed in patients with hypercalciuria and recurrent urolithiasis despite well-established fluid intake and low sodium diet. Pyridoxine can achieve good results in some patients with primary hyperoxaluria type 1. Tiopronin and D-penicillamine are indicated in patients with cystinuria and recurrent stone disease not responding to standard treatment (hyperhydration and urine alkalisation).

Allopurinol is indicated in patients with APRT deficiency and hyperuricemia. One of the patients with PH-1 did have a successful liver transplant hence does not require specific urolithiasis treatment anymore and another one was participating in a research trial (Illuminate-A).

3.4.9.2. Stone removal procedures

The majority of small ureteric stones (< 5 mm) will pass spontaneously, even in small children (447). Therefore, a conservative management is generally advised unless symptoms are not self-resolved or particular risk factors are added. There are essentially four main interventional procedures performed by the urology team: ESWL also known as “Extracorporeal Shockwave Lithotripsy”; PCNL or PCCL (standing for percutaneous nephrolithotomy/cystolithotomy); ureteroscopy (stone extraction +/- double J stent insertion) and very rarely open surgical procedures (such as nephrolithotomy, cystolithotomy and nephrectomy) (391).

The indications for each procedure depend on different clinical and molecular aspects and are well established (in general) in different international clinical guidelines (448-

450). Our proportion of patients undergoing each procedure reflects the general practice specified in those guidelines, with exceptions according to specific scenarios. This cohort experienced a higher proportion of treatment by ESWL, compared to PCNL and ureteroscopies (63% vs 35-38%). Reasons for this preference are: less invasive technique (shorter admissions and fewer complications) (451), predominance of upper urinary tract involvement, stone size unlikely to be higher than 10-20cm in children and smaller proportion of struvite stones in this cohort.

Almost the entire infective cohort (97%) required a stone removal procedure, compared to the rest of the cases (71%), reflecting the importance of stone clearance in this specific group. Failure to clear the stone burden is associated with recurrent UTIs and severe outcomes such as CKD and progression to End Stage Renal Disease in addition to urosepsis and potential death (452).

Achievement of complete stone clearance in children is more difficult than in adults. Some of the reasons include: a higher proportion of abnormalities within the urinary tract, metabolic conditions like PH or cystinuria, a bigger proportion of upper urinary tract stones and a more frequent indication of ESWL. This might explain why the proportion of our patients with complete clearance was limited to barely half of the cohort (55%), when the specific success rate of each procedure is ESWL 44-95%, PCNL 70-90%% and ureteroscopy 50-100% (422). In the metabolic group achieving complete clearance by the time of last follow up appointment was indeed significantly reduced compared to the rest of the cohort. Although the rates reported in this cohort fit with other studies, the high level of medical complexity addressed in this centre may have reduced considerably the clearance success rates.

3.5. LIMITATIONS AND FUTURE WORK

This study is based on a cohort of patients referred (regionally and nationally) to the specialised renal stone clinic at GOSH and is not a population study, therefore epidemiological estimations about paediatric urolithiasis cannot be provided with high accuracy.

Although complete metabolic screening is aimed for all the reviewed patients, urinary citrate was not routinely investigated in some of the individuals, making hypocitraturia more likely to be underestimated. Complete data on infection diagnosis (urine culture) is lacking in some cases as initial episodes may have been reviewed in local hospital and not fully reported in referral documentation. Some of the patients included in the idiopathic group may have had an unremarkable screening during consultation in GOSH despite the presence of a significant metabolic abnormality on initial presentation at their local centre, particularly if marked lifestyle changes applied.

Data on kidney function at end of follow up is markedly limited as patients without significant complications may not need routine blood tests, hence accurate estimation of CKD prevalence in the entire cohort may be underestimated.

3.6. CONCLUSIONS:

In conclusion here it is presented one of the largest cohort of children with urolithiasis in a Western developed country. Indeed, extensive phenotypical information has been documented for all the patients; from epidemiology, aetiology, clinical presentation, medical and surgical treatment till long term outcomes including stone clearance and kidney function.

This project reported on higher severity in boys regarding larger prevalence and earlier age at presentation. Also highlighted are specific differences between children and adults, such as atypical signs on presentation and higher than expected underweight distribution contrary to classical associated obesity in adults. Regarding aetiology, infection remains a large contributing factor, *E. coli* is the most common bacterial cause while *Pseudomonas spp* is likely to suggest the presence of other comorbidities. Underlying metabolic abnormalities are by far the most common cause of lithiasis in children and they can be present in high proportion, also in patients with infections, hence every paediatric patient should complete full urinary metabolic screening. High stone burden, particularly if bilateral may point towards an underlying metabolic disorder, also staghorn calculi may do the same.

The prevalence of CKD is significantly higher than in general population, particularly in patients with staghorn stones, highlighting the importance of early diagnose and adequate management to reduce risk of recurrence.

Finally, one third of the patients does not have yet a proven cause to develop renal stones (idiopathic), half of the patients within the metabolic group have idiopathic hypercalciuria and moreover above a third of the whole cohort are reported to have a positive family history for urolithiasis. Genetic variances are very likely to be in relation with a significant number of these cases. A recruiting process for DNA extraction was taking place setting the ground for comprehensive genetic studies, such as GWAS, in paediatric urolithiasis.

CHAPTER 4: STUDY OF A PAEDIATRIC CYSTINURIA COHORT.

4.1. INTRODUCTION:

Cystinuria is an inherited disorder of amino acid transport, clinically defined by excessive urinary losses of the dibasic amino acids cystine, ornithine, lysine and arginine (COLA). This epithelial transport defect is present not only in the proximal tubule of the kidney but also in the small intestine (453, 454).

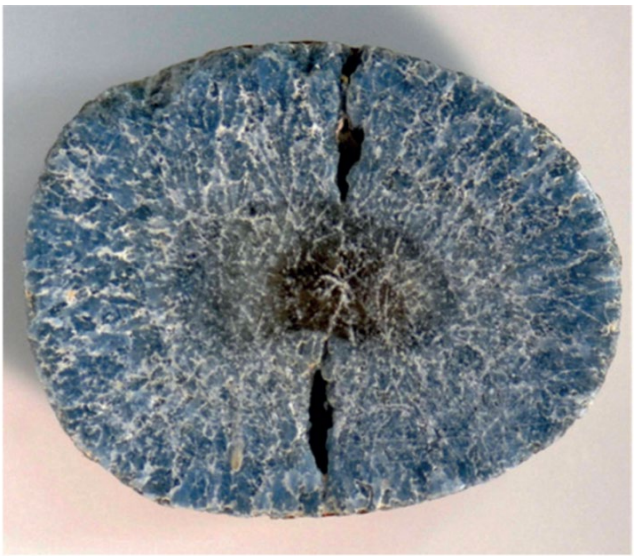
Cystine is a homo-dipeptide formed by the combination of two molecules of cysteine and has very low solubility in the urine under physiological conditions, and hence a high tendency to precipitate forming crystals and stones. Interestingly, the other three dibasic amino acids (ornithine, lysine and arginine) are more soluble and do not form urinary tract stones; this is the reason why the disease has been classically named in relation to the high urinary cystine excretion.

Cystine urinary stones are linked to a large degree of morbidity including obstruction of the urinary tract, recurrent pain (renal colic), urinary tract infections, hypertension and impaired kidney function (CKD). In some of the most severe cases, it can lead to a nephrectomy and rarely to renal replacement therapy and transplantation. At intestinal level, the impairment of cystine transport does not cause any symptoms, in contrast to the multiple complications described in the urinary tract.

The first scientific description of a cystine stone goes as far back as 1810 when the English chemist and physicist Thomas H Wollaston published his results in the Philosophical Transactions of the Royal Society of London. The first stones described by Wollaston were initially made of an unknown substance he decided to name “cystic oxide”; based on its chemical properties and typical distribution within the urinary tract (bladder = cyst in greek) (455). Not only did Wollaston describe the chemical specificities of the cystine stones, he also reported on the microscopic hexagonal morphology of the cystine crystals, a finding that remains nowadays highly specific for

cystinuria (456). A sample of this first described cystine stone is shown in Figure 4.1.

Figure 4.1. Fragment of a cystine stone



First described cystine stone by Wollaston (1810). Image extracted from Thomas et al (2014). and courtesy of Gordon Museum, King's College London.

Two decades later, further analysis of "cystic oxide" stones was done by the Swedish chemist J.J. Berzelius who suggested changing its name to cystine, and since then the disease has been known as cystinuria (457). A century later, in 1908, E. Garrod and W.H. Hurtley classified cystinuria as an inherited metabolic disorder of cystine handling (458). The exact pathogenic mechanism of impaired renal tubular reabsorption of these dibasic amino acids was still unknown at that time and would not be acknowledged until forty years later (in the 1950s by Dent and Rose). Finally, the underlying genetic architecture was revealed in 1994 (*SLC3A1*; M.J. Calonge) and 1999 (*SLC7A9*; L. Fediubadalo) (459-461).

4.1.1. Epidemiology:

Cystinuria is estimated to have a world-wide prevalence of 1:7000, with significant differences between ethnic groups. For example, the highest prevalence is reported at 1:2,500 in the Libyan Jewish population, while in Sweden the prevalence is as low as 1:100,000 and in the USA it is estimated to be about 1:15,000 (431, 454, 462, 463).

Cystinuria is known to have an incomplete penetrance, meaning that a considerable proportion (up to 50%) of homozygous individuals can remain asymptomatic

throughout their life. Based on this premise it has been suggested that a more meaningful way to assess its epidemiology is by focusing the analysis on stone forming cohorts (431).

From a clinician's perspective, cystinuria is relatively uncommon in adult practice, accounting for just 1–2% of all kidney stone cases, yet more prevalent in paediatric cohorts with an average of 6–8% (435, 464, 465). The largest UK paediatric cohort reported a 7% prevalence (435), with other paediatric groups in the literature reaching proportions as high as 25% (453).

In paediatrics, symptomatic stones can present as early as infancy. There is a very high variability in age at presentation, but the median age tends to be during the second decade of life, hence less than half of stone forming cases may not be identified till adulthood (431). This fact highlights the importance of completing urine metabolic screenings in patients with suspected underlying monogenic stone disease; particularly those with a positive family history, those with recurrent stone formation, or with bilateral stones (seen in up to 75% of cystinuric stone formers), as these are all risk factors for developing CKD. Cystinuria is associated with a significantly higher clinical burden compared to other stone forming conditions, with patients generally requiring numerous stone removal procedures despite adequate medical treatment (466).

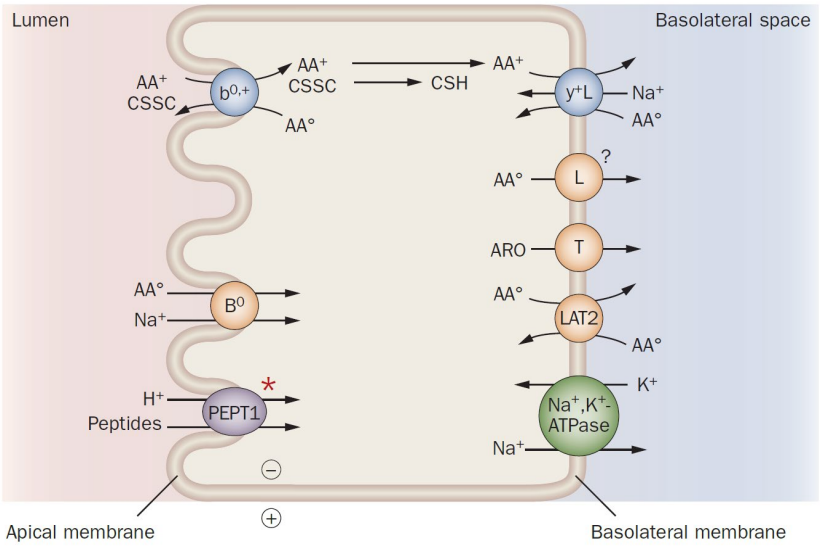
As with stone formation in general, male cases tend to have a more severe phenotype with earlier presentation but also higher recurrency of stone formation (3 years vs 5 years in females) (463).

4.1.2. Physiopathology

In the gastrointestinal tract but also in the proximal renal tubule, reabsorption of cystine is mediated by a specific transmembrane transporter. This amino acid transporter is located in the luminal membrane and has a heterodimeric conformation with two different subunits connected by a di-sulphide bridge (rBAT, encoded by *SLC3A1* and $B^{0,+}AT$, encoded by *SLC7A9*, see below). Following transluminal reabsorption, the dipeptide cystine is hydrolyzed into two cysteines, which then exit through the basolateral membrane via a separate system (y^+L) (figure 4.2) (464).

Under physiological conditions, the fractional excretion of dibasic amino acids is approximately 1%. In cystinuria, cystine excretion may be equivalent to the glomerular filtration rate (GFR). The other three dibasic amino acids can be reabsorbed by alternative transporters to the $B^{0,+}$ system; this explains why the fractional excretion of lysine and ornithine varies between 30–80% of the GFR with arginine having the lowest values (465).

Figure 4.2. Dibasic amino acid transport



Trafficking of cystine and dibasic amino acids in epithelial cells of the renal proximal tubule or small intestine. Extracted from Chillaron et al. (2010)

The classic urinary biochemical profile of cystinuria with raised cystine and other dibasic amino acids suggested to Dent and Harris the presence of a single transport mechanism shared by these four amino acids (467). Subsequent reports on isolated cystinuria and isolated dibasic aminoaciduria have raised the possible presence of other specific epithelial transport systems in humans (461, 468, 469).

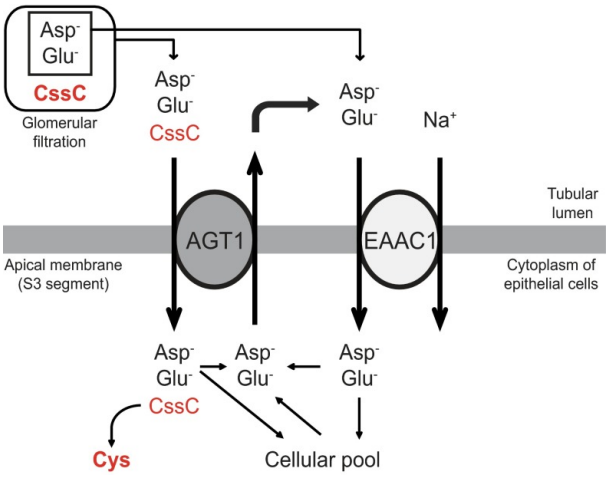
Studies in rats using isolated cortical tubules and border brush membrane vesicles (BBMV) have proposed up to three brush-border membrane bound carrier systems (470).

The renal proximal tubule is classically divided into three segments S1-S3. Until very recently only the high affinity (low-Km) system of the proximal tubule ($B^{0,+}$) was identified

at cellular/molecular level. The $B^{0,+}$ AT subunit has been found highly expressed in the S1 segment, while on the contrary the rBAT is mainly expressed in the distal segment S3 (471). This location-related discrepancy raises questions about the exclusive role of the complex AT-rBAT in the reabsorption of cystine in the proximal tubule.

In recent years a second system has been proposed, particularly relevant in the S3 segment where it has been postulated as partner subunit of rBAT (472). AGT1, as it is currently known, is codified by the *SLC7A13* gene and evidence provided by mice and proteo-liposome studies suggests that it does have a role in renal tubular cystine reabsorption. The AGT1-rBAT complex expressed in the apical membrane is thought to exchange intracellular aspartate and glutamate with intraluminal cystine. Also, in the apical membrane a sodium-dependent acidic amino acid transporter (EAAC1) is co-localized with AGT1, where its main function is the reabsorption of the two amino acids excreted by AGT1-rBAT (figure 4.3) (472).

Figure 4.3. Cystine transport in S3 segment of proximal tubule



Proposed functional coupling of AGT1 with EAAC1. Extracted from Nagamori et al. (2016)

While the description of this alternative system has been received with certain excitement, patient data has not yet confirmed its relevance. In a cohort of 17 patients with no known mutations in either of the known classical genes (*SLC3A1/SLC7A9*), none of the analyzed individuals was found to have pathogenic variants in *SLC7A13* (473). A more recent study, has reported on three individuals with suspected pathogenic variants in *SLC7A13* (p.Asn45Lys & p.Leu270Phe) that negatively interfere with the efficient membrane localization of rBAT (474). If confirmed, this pattern would fit with a digenic inheritance.

The basolateral membrane has two specific transport systems: one for dibasic amino acids, also known as system y^+L, and one for cysteine (resulting from intracellular cystine hydrolyzation). Currently, the specific basolateral transporter for cysteine is not recognized. The basolateral membrane y^+L transporter for dibasic amino acids, present in both the proximal renal tubule and also the small bowel, is impaired in lysinuric protein intolerance, which is another autosomal recessive (*SLC7A7*) condition characterized by a severe multisystemic disorder with no specific kidney stone formation risk (453).

4.1.3. Genetics of cystinuria

Classic cystinuria was initially described as an autosomal-recessive inherited disorder (467). Further reports revealed a significant phenotypic and genetic heterogeneity (475). Indeed, some individuals who were suspected to be simple heterozygotes (parents and children of known patients) were found to have an elevated urinary cystine excretion, questioning the exclusive recessive inheritance. An early phenotypic classification based on urinary cystine and dibasic amino acids excretion established at least three different groups of heterozygous individuals (476):

I. Carriers with n urinary normal amino acid excretion in all cases; this was the most frequently identified group.
II. Those presenting with elevated urinary levels of cystine and dibasic amino acids, occasionally as high as those of homozygous individuals.
III. Those showing intermediate excretion rates between I and II, varying from borderline normal levels to moderately elevated ones.

Initially this triple group of heterozygotes was thought to be explained by the presence of three different alleles in a single gene: mild, moderate, and severe. However, later evidence revealed that type I and III were related to two different loci (477). In fact, in those individuals who were expected to combine both type I and III alleles, urinary levels of cystine were lower compared to those homozygous for I/I. If type I was expected to be a mild allele it should not be the case that individuals carrying two mildly pathogenic alleles had higher levels of cystine excretion compared to someone who combines a mild and a moderate allele.

Considering the possibility of at least two different genes involved in the physiopathology of cystinuria, the condition was reclassified, this time in type I and non-type I, which made more sense in clinical practice considering the great overlap in cystine excretion patterns between the previous groups of heterozygous type II and III. As per the new classification: Type I Cystinuria was still considered to be a disease with an autosomal recessive inheritance pattern (heterozygous individuals had normal cystine urinary excretion). Non type I heterozygous carriers on the other hand could have different levels of dibasic aminoaciduria, implying that inheritance would be autosomal dominant with an incomplete penetrance (453).

Years later, advances in molecular genetics finally allowed the scientific community to identify the specific genes behind phenotypes related to type I and non-type I cystinuria. In 1994, initial linkage analysis on a large number of families with cystinuria described three genetic markers on chromosome 2p, with the first pathogenic mutations identified the same year by Calonge and collaborators (460, 478). The specific gene localized there was *SLC3A1* (OMIM #104614), which encodes for a subunit (rBAT) of a dibasic amino acid transporter that is expressed in the apical membrane of proximal tubular epithelial cells (and in the small bowel).

Currently more than 200 different mutations in *SLC3A1* associated to cystinuria have been reported (HGMD 2022) mainly thanks to a multinational collaboration - the International Cystinuria Consortium (ICC) – which has collected data from an extensive number of families. The most common mutations described by the ICC, both in general and also in specific ethnic groups have been (453):

- Met467Thr (26% of cases). Particularly frequent (>40%) in Germany, Sweden and Czech Republic.
- Thr216Met (12% of cases). Up to 70% in Turkey, the Balkans and Greece.
- Glu298_Asp539dup (5% of cases), higher frequency in Germany and Italy.
- Arg270X (4% of cases). Identified in almost ¾ of Ashkenazi Jews.

Type I cystinuria has also been described in cases with large chromosomic

rearrangements. Other genes in close distance to *SLC3A1* are *PREPL*, *PPM1B*, and *CAMKMT*. These rearrangements may produce the so called Hypotonia-cystinuria syndrome (HCS, Phenotype MIM #606407), a condition with a very heterogenous phenotype including neonatal hypotonia, cystine urolithiasis, growth hormone deficiency, minor facial dysmorphism and initial failure to thrive followed by hyperphagia and rapid weight gain in late childhood. The most severe cases present with severe lactic acidosis and neonatal seizures. Atypical HCS and 2p21 deletion syndrome have also been reported in relation to these rearrangements and they all have in common an autosomal recessive inheritance pattern. A large deletion (78.5 kb) from exon 2 in *SLC3A1* to exon 13 in PREPL is typically described in Belgian patients (479-482).

In 1997, two independent groups reported for the first time on non-type I cystinuria being linked to a locus on chromosome 19q13.1 (483, 484). Following these preliminary results and thanks again to collaborations within the ICC, *SLC7A9* was identified as a causative gene related to non-type I cystinuria (459). *SLC7A9* encodes the 487 amino acid protein $b^{0,+}$AT that belongs to the family of light subunits of amino acid transporters.

Under the ICC umbrella, *SLC7A9* mutations were reported initially in individuals from Spain, Italy, North America, and in cystinuric Libyan Jewish population (459, 471). Currently more than 140 mutations have been reported (HGMD 2022) as pathogenic. Among them, the most frequently recognized (% in general cohorts) are (453):

- Gly105Arg (21% in white individuals).
- Pro482Leu (13%). Identified in 88% of Japanese cohorts with non-type I cystinuria.
- Phe161X (7%). 29% in Spain (founder effect from Asturias in the North-Western region)
- Arg333Trp (6%). Mainly described in white and Japanese patients.

Japanese and Israeli Jewish groups are significantly less diverse compared to other populations in regards to *SLC7A9* allelic variation (485).

Extensive group genetic analysis has identified pathogenic mutations in either *SLC3A1*

and/or *SLC7A9* genes in more than 85% of the patients but still there is a non-negligible percentage of cystinuric patients whose genetic diagnose remains uncertain (453). There are at least 3 possible explanations for this:

1. Most likely, mutations are present in non-coding/regulatory regions of the genome, hence remain unknown.
2. The causative genes are not the 2 classically described. International studies looking at two candidates, such as *SLC7A10* and *SLC1A5* (486, 487) could not prove them as causative.
3. Lastly, the potential effect of hypomorphic variants, currently not known as pathogenic but perhaps in combination with other causative alleles could explain stone forming cystinuria in a considerable proportion of genetically yet unidentified patients (488, 489).

Although the Type I/Non-type I classification of cystinuria has shown a relatively high degree of accuracy, in clinical practice we can observe certain exceptions, particularly since the two causative genes have been identified:

1. Around 15% of heterozygous carriers for *SLC7A9* have a type I like phenotype
2. Carriers of a complex, in-frame duplication of *SLC3A1* (also known as dup5e9) resulting in p.Glu298_Asp539dup will have raised urinary cystine levels, hence their phenotype is compatible with non-type I (485).

Ultimately, in recent years this has led the ICC to develop a new classification focused on a simplified genotype (453, 485):

- Type A (38%). Patients with pathogenic variants in *SLC3A1*.
- Type B (47%). Patients with pathogenic variants in *SLC7A9*.
- Combined type AB (14%). These individuals, although likely to have raised urinary levels of cystine, have not been so far proved to form stones, meaning that inheritance is not digenic in this disease. However, relatives from these families may potentially have a genotype AAB or ABB in which case one should indeed expect a high risk for stone formation (485).

4.1.4. Clinical Features

Signs of cystinuria may be present as early as during antenatal visits. Sonographic evidence of foetal colonic hyper-echogenicity before 36 weeks of gestation has been reported to have a high positive predictive value of almost 89% for cystinuria (490, 491). Patients with cystinuria will have high urinary excretion but also an impaired intestinal reabsorption of cystine. Regular swallowing of amniotic fluid during gestation can saturate an already limited intestinal uptake, leading to a high concentration of cystine in the intestinal lumen which may crystallize, resulting in echogenic foci visible during antenatal ultrasounds (492, 493).

A high variability in the genotype-phenotype correlation of cystinuria is also reflected in its multiple ways of presenting:

1. Classical clinical features of urolithiasis such as renal colic, haematuria and/or recurrent urinary tract infections (UTIs)
2. As an incidental finding when urinary amino acids are requested for screening (neurometabolic investigations) or radiology testing (abdominal/spine x-ray, abdomen ultrasound/CT)
3. Familial screening. This is generally a group with a better prognosis, as early diagnose and initiation of therapy may prevent significant stone formation (494).

Considering that the most severe cases tend to recur with very high frequency, it is not uncommon for them to also develop early chronic kidney disease, hypertension and on occasionally to undergo single nephrectomy during childhood (454, 464). A majority of adult cystinuric patients from previously published studies had a degree of CKD with eGFR < 90ml/min/1.73m^2. Usually most of them will be in Stage 2, with two large studies reporting a prevalence of 17.8% and 26.8% for eGFR <60ml/min/1.73m^2 and only anecdotic cases reaching end stage kidney disease (ESKD) (492, 495). Some of the main risk factors linked to moderate or severe degrees of CKD are previous nephrectomy, congenital anomalies of the urinary tract and renal hypoplasia (494, 496). Hypertension has been described in cohorts of patients with cystinuria in up to half of cases, particularly in association to male gender, older age and CKD (492, 495).

Data from the ICC has reported that approximately 6% of individuals with confirmed pathogenic variants on either *SLC3A1/SLC7A9* may not develop kidney stones. Again, significant heterogenicity is observed in regards to age at presentation, but in general up to 60% of cases would have their first symptomatic stone before 20 years of age with 28% of the males presenting before the age of three years (453).

Although both known genes are not sex-linked (as in other cases of urolithiasis except for Dent's disease (497)), male individuals tend to have a more severe course. Dietary patterns are likely one of the main contributing factors, but potentially others, such as hormones, may play a significant role considering that medical/surgical castration is an effective therapeutic option indicated in a subgroup of dogs and cats with recurrent cystine stone formation (498).

In very rare occasions, patients may also present with significant dysmorphic and neurological features as part of the HCS (480).

4.1.5. Diagnosis

Cystinuria can be diagnosed by (494):

1. Stone composition analysis.
2. Direct microscopic observation of cystine crystals in the urinary sediment.
3. Detection of an abnormal urinary excretion of cystine and dibasic amino acids (COLA).
4. Genetic testing.

4.1.5.1. Stone Composition analysis

Stone composition analysis by infrared spectroscopy and/or x-ray diffraction has proved to be very efficient compared to other methods (such as wet chemical analysis) (499). Stone analysis is not only directly diagnostic but may also guide medical management in patients with recurrent calculi formation and no response to routine medical therapy. In the context of hypercalciuria and/or recurrent urinary tract infections, cystinuric patients can develop stones predominantly made of calcium phosphate, indeed in 20-40% of the cases, other urinary metabolic abnormalities are detected during routine follow up (500, 501).

Most cystine stones are easily recognized by their appearance (Figure 4.4); in general there are two main morphologies: one is the classic and more frequent yellowish colour with poorly or radiating organized structure, the second one has an external layer of cream/yellow colour and smooth appearance with a nucleus similar to the first morphology described, the latter can be the result of over-alkalinization of the urine as part of medical treatment and/or recurrent urinary tract infections (502).

4.1.5.2. Urine microscopy

Direct observation of urine samples with a microscope can reveal the presence of the characteristic flat hexagonal crystals (Figure 4.4), typical from patients with cystinuria and supersaturated urine. They have a high specificity and can be detected in up to 2/3 of the patients (494). Acidification of the urine sample can help to improve sensitivity (160).

Figure 4.4. Cystine stones and crystals

Macroscopy: yellow-brown colour with a granular surface and radial structure. This morphology may change with alkali therapy as calcium phosphate is incorporated and cystine crystal are reduced in size. Microscopy (urine): characteristic large hexagonal crystals with lamellar appearance. Extracted from Servais et al. (2020)

4.1.5.3. Cyanide-nitroprusside test

It is a relatively quick and cheap bedside screening test classically used in the past The test works by splitting cystine into cysteine mediated by the cyanide; cysteine can then react with the nitroprusside, turning the urine purple in color. It can detect levels of cystine/creatinine as low as 40 µmol/mmol. Unfortunately, this level of sensitivity is not good enough as a screening technique, which, together with its use of unstable and hazardous chemical reactants, has reduced its use in clinical practice. It is also important to take into consideration that false positives can be obtained when acetone or homocysteine are present (494).

4.1.5.4. Urinary quantification of cystine/dibasic aminoacids

In the outpatient setting, the most used screening test for cystinuria is the quantification of dibasic amino acids in relation to creatinine in isolated urine sample. A 24-hour collection could also potentially be used for screening, but in clinical practice this is more frequently reserved for monitoring, treatment guidance and estimation of stone formation risk (table 4.1) (485, 494, 503). Both ion exchange chromatography and liquid chromatography–tandem mass spectrometry are reliable methods for urinary cystine analysis.

Table 4.1. References for urinary cystine excretion according to genotype

Variable	Urinary Cystine (mmol/day)	Urinary cystine/creatinine (µmol/mmol*)
Reference	0.13	<1month: <39 < 1 year: <25 >1 year: <17
AA / BB / AAB / ABB / AAA / BBB	>1.7	>150
A0**	<0.4	2-18
B0 / AB	0.17-1.7	16-152

*Conversion factor: µmol/mmol = 2.1 x mg/g = 0.113 x µmol/g. **With exception of Exon dup5-9, a distinct variation in *SLC3A1* having an AD negative effect with high urinary cystine excretion and stone formation (504).

When interpreting urinary results compatible with cystinuria it is important to consider also in the differential: general aminoaciduria (Renal Fanconi syndrome), organic acidaemia but also tubular immaturity, particularly in infants where transient cystinuria is physiological and potentially misleading. Hence, positive urine results are always to be repeated after the age of 2-3 years, particularly in those with no stone formation or without genetic confirmation (464, 505).

4.1.5.5. Urinary cystine capacity

Currently, routine monitoring of patients with cystinuria is unfortunately unable to provide a sufficiently accurate prediction for the risk of stone formation. It is not uncommon that during follow up, patients with adequate compliance on treatment, including recommended dilution targets (<1mmol/L) of urinary cystine, may continue to have recurrent and frequent stone formation. Furthermore, some of the common

methods of cystine quantification in urine suffer from a significant risk of inaccuracy (433).

Two decades ago, a new method known as cystine capacity was developed in order to overcome previous inaccuracies with testing. In essence this assay consists of adding solid cystine to the urine sample and measuring how much it dissolves and how much precipitates in excess (506). While recent trials have shown significant differences in test results regarding risk for stone formation, stone growth and passage of previous stones, this has only been meaningful at a statistical level and it is not very clear whether it will have as high an impact in clinical practice as initially expected (507).

4.1.5.6. S-methyl-L-ergothioneine to L-ergothioneine ratio in urine.

Ergothioneine is a natural antioxidant and also a novel urinary biomarker identified in a mouse model of *SLC7A9* which has shown correlation with a higher stone formation risk around the time of testing (508). Whether this animal research will be translated into routine human medical practice is still to be determined.

4.1.5.7. Radiological imaging

While imaging patients with cystine stones it is important to take into consideration the following (500):

1. Pure cystine stones are typically radiolucent, hence it would not be appropriate to use plain X-rays for screening.
2. Ultrasound urinary tract. It is most commonly the first radiological diagnostic test to perform, in particular for children and pregnant women where avoidance of radiation exposure is particularly important. Large accuracy for stones >4mm in size. Its safety profile also makes it ideal for routine monitoring in a condition such as cystinuria where stone formation is quite recurrent.
3. Non contrast CT KUB (with low dose radiation) is becoming the gold standard in radiology for the diagnosis of urolithiasis, particularly in adults. New techniques allow the use of lower amounts of radiation although the dose is still considerable. It also allows the measurement of stone density (Hounsfield Units, HU); however, whether this has a meaningful impact on the selection of a particular urological treatment is still debatable and lacking in evidence. It

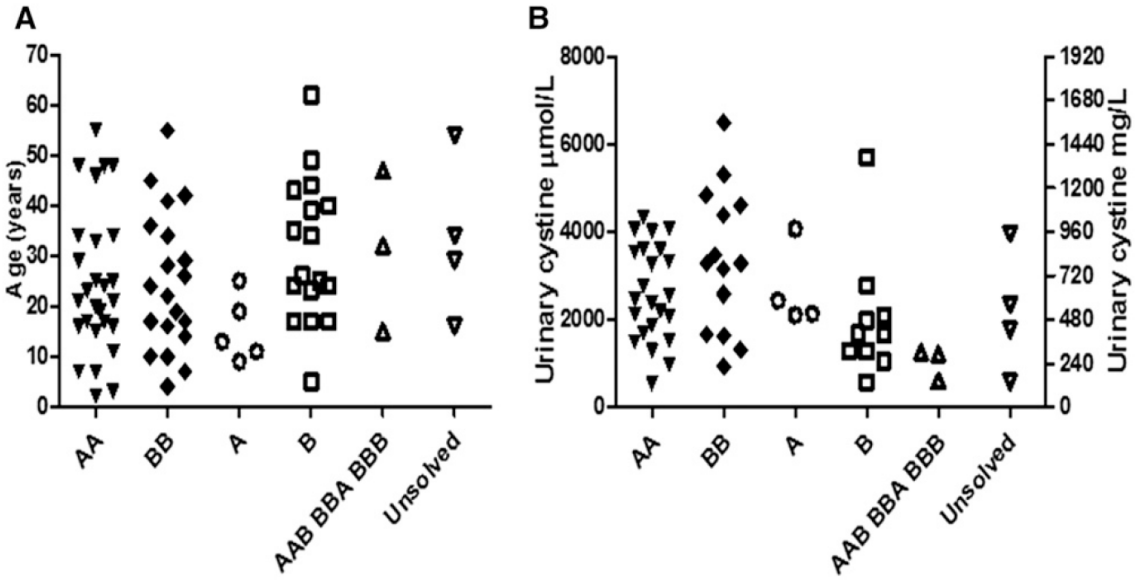
may be able to help to predict stone composition as cystine stones typically have a lower HU compared to other stones (509).

4.1.5.8. Genetic testing

Genetic confirmation can be established by sequencing of the known genes or alternatively using specific gene panels (virtual or real) for nephrocalcinosis/ nephrolithiasis. This is not, however, routinely performed in clinical practice, and is generally limited to research projects or occasionally to genetic counselling when required.

There are multiple reasons why this is the case: first, no specific phenotype/genotype correlation is yet to be shown (Figure 4.5) despite various studies (504). Second, there are multiple other more cost-effective diagnostic methods such as stone composition analysis and/or direct urinary cystine measurement. Furthermore, cystinuria has only been reported as a primary disorder with no alternative causes described; hence genetic confirmation will change neither diagnosis nor management. Lastly, considering the possibility of identifying variants of uncertain significance, the interpretation of pathogenicity needs careful assessment and always adequate correlation with the clinical scenario (160).

Figure 4.5. Cystinuria phenotype-genotype correlation

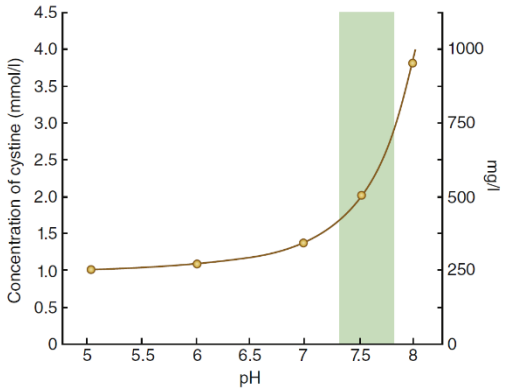


Lack of correlation between phenotype traits: age at presentation (**A**) and urinary cystine excretion (**B**) with reported genotypes. Taken from Rhodes et al, 2005.

4.1.6. Treatment

Crystallization of cystine in urine generally happens either in a supersaturation state which mainly occurs when cystine concentration rises beyond 1000µmol/L (250mg/L), or when urine pH remains predominantly low (acidic or under 6.5). Conversely, its solubility increases if urinary pH rises to 7.0 or higher (Figure 4.6) (494).

Figure 4.6. Cystine solubility vs urine pH



Cystine solubility according to urinary pH. Extracted from Servais et al. 2020.

Based on these principles, conservative stone management of patients with cystinuria is mainly based on oral hyperhydration and urine alkalinization. Other important factors also include dietary changes (reduced intake of salt and protein of animal origin) and the use of drugs that aim to make cysteine compounds more soluble than the dipeptide cystine. Finally, if all of the above treatments are unsuccessful stone removal procedures may be indicated (extracorporeal shockwave lithotripsy (ESWL), ureteroscopy, PCNL) and less frequently nephrectomy (453).

4.1.6.1. High fluid intake:

This is at the core of treatment management and the most essential component of it, as is the case with all metabolic stone formation disorders. It has been estimated that adequate concordance with the recommended fluid intake can be effective in stone prevention in up to 70% of cases (510). By increasing fluid intake either orally or through enteral tubes such as nasogastric or gastrostomies (particularly in younger patients with more severe disease), patients can increase urine output and cystine solubility.

Adult patients with cystinuria can typically excrete about 2-3 mmols of cystine per day, hence when aiming for 24 h urine concentrations below 1 mmol/L some individuals may require a minimum intake of more than 3 L. The general recommendation in paediatrics is to calculate based on body surface area and routine hyperhydration protocols (2-3 L/m^2/day); however as soon as 24-hour urine collection becomes feasible it should become the standard measurement for estimation of adequate fluid intake (that is, the higher the concentration beyond 1 mmol/L, the larger the intake volume required).

24-hour collections for patients with kidney stones are routinely obtained in outpatient settings. They are generally taken around appointments, which implies that measurements are done once/twice per year and may not fully reflect patient's routines. Around appointments, individuals may increase their average water intake, optimize their dietary habits and/or improve compliance with medications, therefore their general stone formation risk may be underestimated. An alternative monitoring method for adequacy of fluid intake is the measurement of sediment gravity (SG) at home by using dipsticks, targeting values under 1005 in early morning samples (494). Self-monitoring at home can potentially empower patients to take a more active role in their clinical care, improving treatment compliance and subsequent clinical outcomes (reduced stone formation and/or need for stone removal procedures) (494, 511, 512).

Ideally fluid intake should be spread throughout the day. However, children are not routinely encouraged to interrupt sleeping times for an extra drink. Unfortunately, sleeping time is a particular sensitive period as urine output tends to drop and urinary acidity tends to increase, both contributing factors to cystine supersaturation. Alternatively, children are advised to take extra water at bedtime and whenever they spontaneously wake up in the middle of the night. Medication doses can also be adjusted (prescribing higher doses of alkali or CBTD) at night and this has been shown to be of help (454, 464, 494).

4.1.6.2. Urine alkalinization

Supplementing oral alkali (with citrate or bicarbonate salts) is another key element of the medical management, always in addition to hyperhydration.

Considering that urine pH<7.0 increases cystine supersaturation, the usual therapeutic target tends to be around 7.5 (7.0-8.0) (494). Measuring urine pH first thing in the morning is recommended to monitor the effect of the overnight alkali dose; but this is not practical in an outpatient setting where samples are typically obtained several hours after the patient has woken. Home monitoring is an alternative also in this case.

Average doses per weight of the patient are approximately 1-2 mmol/kg/day of alkali equivalent (1mmol of citrate is equal to 3 mmol/mEq of bicarbonate), with usual adult doses of 60-90 mEq/day (513-515). In general, potassium containing salts are preferred for alkali supplementation over sodium formulations. Sodium intake is known to increase calcium and cystine excretion hence to be an extra risk factor for stone formation in cystinuria. However, there is a lack of clinical evidence supporting this rationale, either in general stone formers or in cystinuric patients. Sodium bicarbonate is equally as effective as potassium citrate for urinary alkalinization and for increasing citrate excretion (122, 465, 494).

In clinical practice it is common to observe values of urine pH beyond 8.0 in cystinuria patients. Over-alkalinization could potentially increase the risk of calcium-phosphate crystallization and stone formation (either apatite exclusively or more commonly in combination with cystine). This is particularly the case in individuals with recurrent urinary tract infections and/or hypercalciuria. Reducing or stopping alkali supplementation in these cases should be done with caution alongside regular monitoring of urinary calcium excretion (494). In cases of persistent hypercalciuria and increased stone formation rates, further dietetic review and use of thiazide diuretics could be considered (500, 516).

4.1.6.3. Dietary recommendations

Elevated urinary cystine can be related to a high intake of sodium or cysteine (highly present in protein from animal sources). Hence the dietary strategy in patients with cystinuria is based on restricting the intake of salt and following a low animal protein diet, also known as “low methionine diet” (due to methionine being a metabolic precursor of cysteine). Available evidence on the clinical benefits of a low methionine/animal protein diet is limited, with no clinical trials and only case reports

in the literature suggesting a potential benefit on reducing cystine excretion. At least one case did report a complete resolution of cystinuria (517, 518).

Diets low in methionine should not provide less than daily physiological requirements (1200-1400 mg methionine) (519). Their use in children is controversial due to protein restriction and potential interference with growth, hence, it is not routinely recommended in this group of patients (494). Dedicated clinics for the management of patients with cystinuria may not only combine expertise from nephrologists and urologists but also from specialized dieticians who will supervise patients to lower animal protein (to approximately 60% of total protein intake) and maintain optimal nutrition (517). Decreasing intake of animal protein is not only beneficial through the reduction of cystine intake, but also reduces urine acidification which as previously mentioned has a beneficial effect on this condition (453).

Salt restriction may also play a role in the dietary management of cystinuria, and this is based on fact that cystine and sodium urinary excretion are correlated to some extent. Indeed, a reduction in sodium intake to 3.5 g/day may be able to reduce cystine excretion by an extra 650 µmol, a modest change that in some patients may be sufficient to keep urinary cystine under saturation levels (520-522). Considering that in Western countries like the UK, daily salt intake approximates to 8.5 grams (3.4 grams of sodium), a reduction such as above will be targeting at least a standard daily recommendation of <6 g/day of salt. Particularly important is moderation of processed food which can provide up to 75% of all salt daily intake (494). Further recommendations include regular monitoring of food labels, avoiding adding extra salt to their food and ensuring fresh food is eaten regularly.

Finally, previous reports have suggested a beneficial effect of L-Glutamine supplementation in reducing urinary cystine excretion, but this has only been described in a context of high salt intake (521). The exact mechanism involved in this hypocystinuric effect of glutamine remains uncertain.

4.1.6.4. Cystine-binding thiol drugs (CBTDs).

CBTDs are the second line medication agents for the reduction of cystine excretion. The most used CBTDs in current clinical practice are d-penicillamine (initially

described in 1963) and tiopronin (also known as alfa-mercapto propionyl glycine, first reported in 1975) (523, 524). CBTDs are sulfhydryl molecules that facilitate the splitting of cystine into 2 cysteine moieties which then form a mixed disulfide with them; this new complex is on average up to 50 times more soluble than cystine, resulting in reduced risk of stone formation (494).

Both d-penicillamine and tiopronin are effective in reducing stone formation but can also be responsible for several concerning side effects. In general they are reserved for the most challenging cases when adequate compliance with diet, fluids and alkali has been unsuccessful (494).

D-penicillamine is usually given in adults at an average dose of 1–2 g/24 h (20-30 mg/kg in children). Some of its well-known side effects (on occasions affecting up to 50 % of patients) are rashes (including pemphigus), fever, arthralgia, nephrotoxicity (secondary nephrotic syndrome and rapidly progressive glomerulonephritis), pancytopenia, and loss of taste (525). D-penicillamine is also used in the treatment of Wilson's Disease as it can also act as a copper and zinc chelating agent. Low copper levels can cause hypo/ageusia (loss of taste) (465). It can also cause Vitamin B6 (pyridoxine) deficiency which may need supplementation. Most of these adverse effects will correct themselves after drug discontinuation (510).

Tiopronin (alfa-mercapto propionyl glycine or MPG) is another compound that can facilitate a disulfide exchange reaction (cleave cystine and mix with cysteine). Clinically it is as effective as penicillamine, however its adverse effect profile is safer, hence it has become the CBTD of choice. The average dose in children is 15-20 mg/kg/day divided in 2-3 doses. In adults, it is usually given as 1-2 g/day with maximum doses of 3 g/day. A recent study has found no significant improvement in the urinary cystine capacity beyond the standard dose of 1 g daily (526)

Monitoring for patients on penicillamine/tiopronin includes regular full blood counts, and urine protein measurements. For patients on penicillamine, serum levels of trace elements and vitamin B6 should also be monitored. Because of the above-mentioned potential side effects, the duration of treatment should be evaluated case by case (494).

Considering that chelating agents are mainly eliminated through the urine, their use in patients with chronic kidney disease is a concern. In theory, reduced kidney function with preserved urine output should also reduce cystine excretion and thereby stone formation risk. The risk of drug accumulation is increased, therefore attention to dose reduction should be paid in patients with stage 2 CKD with strong consideration given to stopping CBTD in patients with eGFR < 60 ml/min/1.73 m^2 (494).

An alternative treatment with a specific angiotensin-converting enzyme inhibitor called captopril exists. Captopril (which also has a sulfhydryl group) can create highly soluble compounds with cysteine. Although captopril has been reported in several studies as effective in reducing cystine excretion, its urinary concentration at standard doses is not large enough to achieve an optimal level of cystine chelation. The required doses to achieve a meaningful clinical response are much higher than the average 50-150 mg/day used in routine clinical practice or ACE inhibition, leading to concerns regarding adverse effects. Due to its unproven clinical efficacy, captopril is not routinely recommended in cystinuric patients but can be considered in those patients who also need antihypertensive treatment. (527-531).

4.1.6.5. Urology procedures:

ESWL, ureteroscopy (URS), and mini percutaneous nephrolithotomy (mini-PCNL) are the stone removal procedures most frequently performed in patients with cystinuria. In general, stones made of cystine are meant to have a harder consistence than calcium stones, meaning also a higher resistance to shockwave treatment than the later. Nevertheless, depending on the size of the stones (the smaller the better) and the operator's experience it can still be offered as a first line of treatment in most patients. It is also more frequently performed in children, while aiming to reduce the potential risks associated with endoscopic and other surgical procedures (494)

Uretero-renoscopy (URS) is usually indicated for ureteric stones and also in other locations where a large size (10-20mm) is less likely to respond to ESWL but is not large enough to directly require an open surgical intervention (517).

Finally, mini percutaneous nephrolithotomy (mini-PCNL) is usually recommended as a miniaturized keyhole operation which is associated with reduced trauma to the kidneys in comparison with classic PCNL. While classic PCNL uses larger access sheaths of up to 30 Fr, a mini-PCNL will use those as small as 13 Fr. A mini-PCNL may be the procedure of choice in cases of larger stones (less likely to respond to ESWL or URS) and in those with congenital anomalies of the urinary tract (494, 517).

It is common practice to endoscopically insert double J ureteral stents prior to or after definitive stone removal procedures when trying to resolve or prevent an obstruction of the urinary tract system. Close monitoring should be maintained of those patients after leaving ureteral stents due to the high risk of both encrustation, which can start as early as 2 weeks post insertion, and also infection (494).

4.2. METHODS:

A retrospective electronic clinical records review was performed (including demographics, biochemistry, radiological and surgical data) from children followed up by the multidisciplinary kidney stone service at Great Ormond Street Hospital NHS Foundation Trust (London, UK) during a time-period of 22 years between June 1996 and April 2019.

Ethical approval was not required for retrospective analysis of anonymised patient information. In the dedicated stone clinic, all children were reviewed directly by a consultant paediatric nephrologist (Dr. William Van't Hoff, Dr Wesley Hayes, or Prof. Detlef Bockenhauer), by a consultant paediatric urologist (Mrs Naima Smeulders) or by a senior fellow in paediatric nephrology/urology under the supervision of the above consultants. Cases considered for possible surgical approach were discussed monthly in an MDT with the radiology consultant team to ensure conservative management was always optimised and stone removal procedures were decided under consensus.

All patients underwent a complete clinical history including details such as age, gender, perinatal history, parental consanguinity, and family history of kidney stone disease. Weight, height and their respective centiles within the first 3 months of presentation were calculated according to UK-WHO growth data (423, 532).

Every patient underwent a full "stone metabolic screen" including serum urea, creatinine, electrolytes (calcium, magnesium and inorganic phosphate), tCO_2, alkaline phosphatase, albumin and urate. In cases of abnormal calcium/phosphate balance, 25-OH vitamin D_3 and iPTH (intact parathyroid hormone) were also added. Analysed urine was from non-fasted mid-morning spot samples and/or a 24-hour total volume collection was performed when possible (for fully continent patients). Urinary testing included: microscopy and culture, calcium, urate, oxalate, amino acids and creatinine. Citraturia was not routinely investigated in all patients and was requested at the clinician's discretion. Urine pH was assessed in fresh samples from outpatient clinic via urine dipstick testing (the reportable range was 5.0-9.0).

Stone fragments, if available, were sent for infrared-spectroscopy, the presence of any mineral (if mixed combination was present) within each fragment was arbitrary set as significant when greater than 10%. The presence of cystine in composition analysis was diagnostic for cystinuria.

Stone burden was assessed in every patient by urinary tract ultrasound and in selected cases also by low dose abdominal CT (computed tomography).

For assessment of the kidney function at end of follow up**,** eGFR (estimated glomerular filtration rate) was calculated according to the Schwartz modified formula (135) in those patients with recorded serum creatinine (µmol/L) and height at the same time within last 12 months of follow up [36.5 x Height (cm) / serum creatinine (µmol/L)]. Patients with age < 2 years were excluded from eGFR calculation as full maturation of GFR is not normally achieved till the end of the second year of life (533). Chronic kidney disease classification (stage I-V) criteria was done according to the international KDIGO guidelines (137).

Specific testing for cystinuria was performed via ion exchange chromatography of urine amino acids. The upper normal limit of urinary cystine/creatinine ratio was defined as 20 µmol/mmol creatinine after infancy (however this can be higher - up to 100 µmol/mmol under 1 month and 40 µmol/mmol between 1 month and 1 year) (430). Excretion of the other three dibasic amino acids (ornithine, lysine and arginine) was noticed on screening, but exact quantities were not documented as this has no clinical relevance for routine monitoring/management. Twenty-four-hour urine cystine excretion and concentration (mmol/day and mmol/L) was obtained during routine outpatient monitoring to guide medical management. Urine pH detected by dipstick on consultation or alternatively monitored at home and reported by patients/families was documented to guide alkali therapy (target pH 7.0-8.0).

Regarding the conservative management, hyperhydration was defined in agreement with classical recommendations of >2 L/m^2/day. Due to compliance issues with such a high fluid intake an alternative threshold of 1.5 L/m2/day was also analysed in this

cohort. Fluid intake was documented as reported by patients during the last consultation. All patients received dietary advice on lowering salt intake but there were no routine recommendations on a low protein diet as per usual paediatric guidelines. Alkali supplementation was provided for every patient with potassium citrate either three times a day via liquid formulation or twice a day via moderate release tablets (Urocit-K©).

Cystine binding thiol drugs such as tiopronin and d-penicillamine were offered to patients with recurrent stone formation who did not respond to conservative management. Tiopronin was preferred over d-penicillamine (due to its safer adverse effect profile) and either drug was discontinued if patients developed severe adverse effects or were stable and stone free for a prolonged period of times (> 1 year).

Stone removal procedures offered in the unit were ESWL, mini-PCNL and Cysto-ureteroscopy.

4.2.1. Statistics

Data were analysed for normality using the Kolmogorov-Smirnov test. Normal data are presented in mean and standard deviation while non-normal or skewed data are presented in median and interquartile range (IQR). Significance was assessed by Student's t test for normal data, the Mann-Whitney U test for non-parametric data and the Chi-Square test for categorical variables. A p value < 0.05 was considered significant during the analysis. IBM SPSS Statistics Version 26 for Windows was used for the analysis.

4.3. RESULTS:

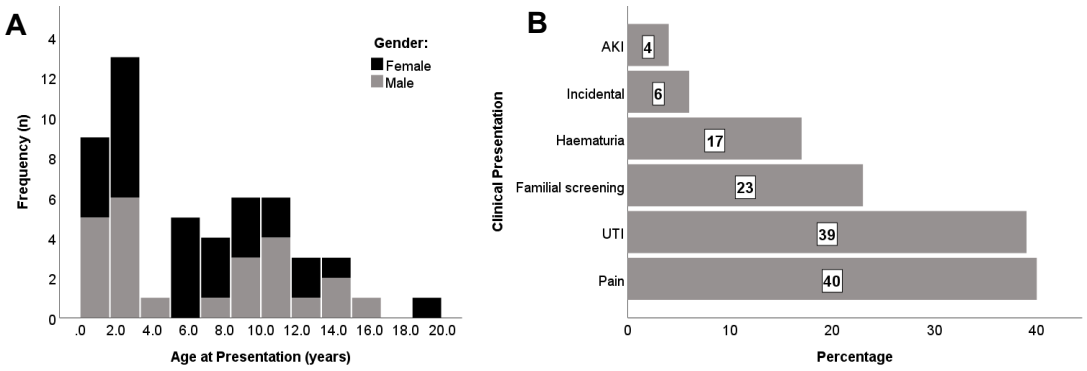
4.3.1. Demographics:

During the twenty-two years of practice reviewed in this study, a total of 52 patients with a diagnosis of cystinuria were followed up. From those, 43 were stone formers, while the other 9 remained stone-free throughout the follow-up period despite highly raised urinary cystine excretion. Of the 52 patients, 28 (54%) were female. There was a positive family history for cystinuria in 24 (46%) of the patients and of parental consanguinity in 10 (19%).

4.3.2. Presentation:

38 (73%) presented before 10 years of age, of which 6 (12%) presented in early infancy (under 1 year of age). Median (IQR: interquartile range) age of patients at presentation was 6.2 (1.9-10.3) years, with no significant difference between boys and girls. Mean (± Standard deviation, SD) for weight and height centiles on presentation were: 53(±31) kg and 46(±33) cm (figure 4.7).

Figure 4.7. Presentation in patients with cystinuria



A) Histogram representing age at presentation according to gender. **B)** Bar graph representing frequency of features leading to presentation.

Clinical features presented at diagnosis were (occasionally in combination):

- Pain 40% (n = 21)
- UTI 39% (n = 20)
- Haematuria 17% (n = 9)
- Acute Kidney Injury 4% (n =2)
- Familial screening (asymptomatic) 23% (n = 12)
- Incidental 6% (n =3)

Stone location at diagnosis were (n = 43 stone formers, some had multiple stones in different sites of the urinary tract):

- Bladder 10 (23%)
- Kidneys 30 (70%)
- Ureteric 10 (23%)

4.3.3. Comorbidities:

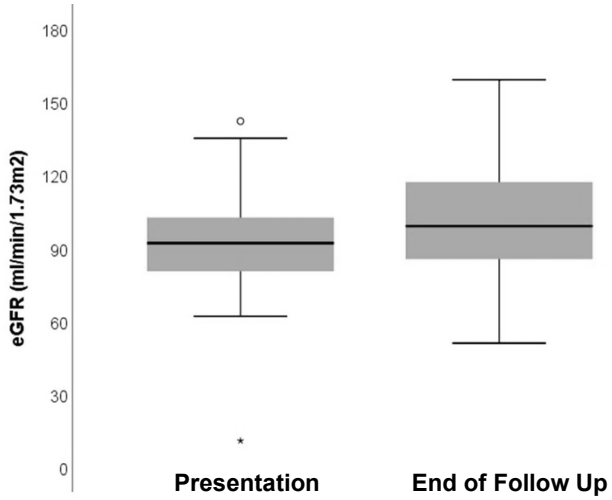
From the entire cohort, 4 individuals (8%) had a previous history of congenital abnormalities of the kidney and/or urinary tract (CAKUT), one had significantly reduced mobility (2%), and 10 (19%) had a history of recurrent UTIs, one of whom was not a stone former during the follow-up period. Hypertension was identified in 3 (6%) patients.

4.3.4. Kidney function:

On presentation, 9 cases were reported to have metabolic acidosis (17%), 16/40 (40%) were found to have an abnormal GFR (<90ml/min/1.73m2), 15 had CKD stage 2 (60-90 ml/min/1.73 m^2), and one patient presented with severe AKI (eGFR <15 ml/min/1.73 m^2) secondary to bilateral obstruction.

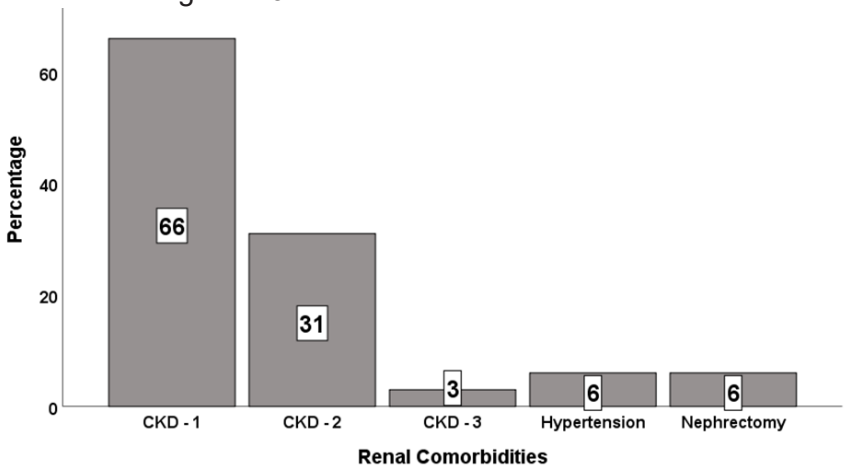
At the last documented visit, the median (IQR) age of this cohort in years was 14.8 (11.6-16.3) with a mean follow-up period of 8.6 years. The mean (±SD) eGFR reported at end of follow up was available for a total of 35 (67%) patients and was 102.4 (±23.4) ml/min/1.73 m^2 (Figure 4.8) with 11 cases (31%) having CKD 2 and 1 (3%) having CKD 3 (Figure 4.9). The patient who presented with severe AKI and bilateral obstruction had a last consultation at age 15.7 years with eGFR 88 mL/min/1.73 m^2).

Figure 4.8. eGFR at presentation vs last follow up



Boxplot representation of eGFR (Schwartz) at presentation (n = 40) and at last available follow up appointment (n = 35).

Figure 4.9. Distribution of comorbidities



4.3.5. Stone burden:

Stone composition was available for 40/43 individuals (93%). For 5 of these patients, stone composition was not completely cystine (<90%), including 2 patients where stone analysis did not show the presence of cystine (despite elevated urinary excretion of cystine) rather calcium phosphate instead (despite no history of recurrent UTIs and urinary pH within the therapeutic range (7.0-7.5)).

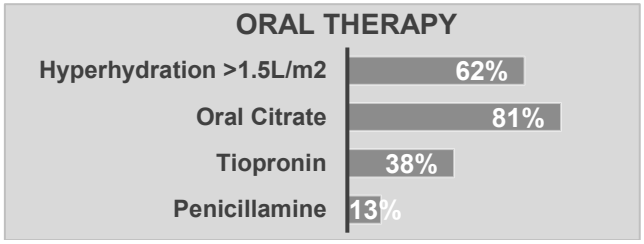
Bilateral stones were identified at some point during follow up in 19 (44%) of the cases, while in the bladder in 11 (26%) individuals. 14/43 (33%) patients developed at least one staghorn stone. During the follow-up period, stone formation rate per year was 0.11 (0.0-4.1).

4.3.6. Medical management:

Standard hyperhydration (>2.0L /m^2/day) was self-reported only by 14/52 (27%), a lower target (>1.5 L/m^2/day) by 32 (62%) (figure 4.10). Median (IQR) fluid intake in L/m^2/day was 1.6 (1.4-2.1). A low salt diet was reported to be followed by 28/51(55%) cases. No data was recorded on low animal protein/methionine diets. Antibiotic prophylaxis was prescribed at some point during the follow up in 9/52 cases (17%).

All 42 (81%) patients with documented alkali supplementation prescribed were receiving potassium citrate with a median dose of 0.5 (0.3-0.7) mEq/kg/day. Median urinary pH during outpatient consultations was 7.0 (7.0-8.0).

Figure 4.10. Medical management



Distribution of different oral therapies across the cohort of patients with a history of stone formation.

During the follow-up period in our centre, patients who had average urine pH ≥ 7.0 and average urine cystine < 1000 µmol/L were defined as individuals who achieved urine metabolic targets (n = 20). Surprisingly, those patients had significantly poorer outcomes compared to those who did only achieve one target or none (n =22)

- Total number of stone removal procedures [median (IQR)]: 3(2-9) vs 2(1-3) [p=0.049]
- Average procedures per year [median (IQR)]: 0.7(0.2-1.0) vs 0.2(0.1-0.5) [p=0.013]
- Rate of stone formation per year [median (IQR)]: 0.2(0-0.9) vs 0(0-0.2) [p=0.029)
- New stone formation (p=0.057) [median (IQR)]: 2(0.5-4) vs 0(0-1)

Treatment with cystine binding thiol drugs (CBTD) was documented in 24/52 patients (46%; 56% if only counting those with a history of stone formation). Those patients

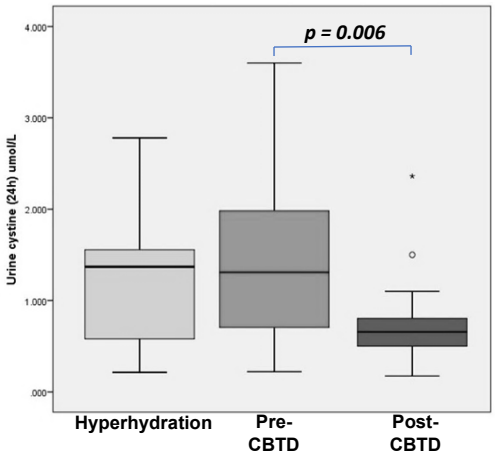
receiving CBTD had a total of 26 treatment periods for a median (IQR) duration of 34 (18-65) months. Median age at starting CBTD was 7.8 (4.3-12.9) years. In this group the proportion of urine metabolic targets achieved was higher 13/20 (65%) compared to those with no thiol drug treatment at 6/22 (37.5%) (p = 0.029).

Penicillamine was prescribed in 7/24 (29%) cases, the range of dose was 13.0-33.0 mg/kg/day. Adverse effects were reported in 4 of the individuals: one had hair loss, 2 had cutaneous rashes (one with severe rash immediately after starting treatment prompting a direct switch to tiopronin), and one had significant proteinuria and decreased count of white cells in blood prompting, again, a switch to tiopronin which was well tolerated. No patient had been offered captopril in this cohort.

Tiopronin was prescribed in 20 of the patients (83%) receiving CBTD. The treatment range was 5.5 – 25.0 mg/kg/day with a median (IQR) of 15.5 (10.2-18.5). The most common reported adverse effect was a rash (in 3/20 = 15%), while one of these cases also reported hair loss and tiredness. None of the patients stopped the tiopronin for safety reasons. All patients on tiopronin who eventually stopped it, only did so after a prolonged period (at least one year) of being stone free.

Mean urine cystine (±SD) was lower (p = 0.006) after CBTD was started at 0.67 (±0.31) mmol/L compared to levels of 1.69 (±1.40) mmol/L initially. Urinary cystine in this post-CBTD group was also lower than in patients not treated with CBTD, the mean (±SD) result for the later was 1.37 (0.6-1.6) mmol/L, with a statistically significant difference (p = 0.037) (figure 4.11).

Figure 4.11. Cystine excretion according to treatment



24h urinary cystine excretion in patients who have not taken CBTD (only hyperhydration) vs those who have taken them prior and after starting treatment.

4.3.7. Urological procedures:

38/43 (88%) patients underwent at least one stone removal procedure during follow up (FU). 13 (30%) patients had treatment with ESWL and the number of procedures per patient ranged from 1-9. Twenty-three (53%) patients had ureteroscopy with a number of procedures ranging from 1-6. Twenty-seven (63%) underwent mini-PCNL, range 1-10.

The median number of procedures per year per patient was 0.17 (0.07-0.35) with a minimum of 0 (spontaneous passed stones) and maximum of 4.17. Those patients treated with CBTD had a higher median (IQR) of stone removal procedures at 4 (2-8) vs those in the no-CBTD group at 1 (0-2), p value <0.001. Prior to CBTD therapy, the median (IQR) rate of removal procedure per year was 0.32 (0.21-0.59) compared to post CBTD therapy when it decreased to 0.17 (0.0-0.72), p value <0.001. Three (6%) cases underwent a single nephrectomy at some point during their follow up due to a poorly functioning kidney (< 10% on renal DMSA).

4.4. DISCUSSION:

The main motivation behind the development of this study is the sparsity of long-term data in paediatric patients with cystinuria and a lack of data relating to the efficacy and safety of cystine binding thiol drugs. The aim was to provide more evidence from routine clinical practice at a paediatric tertiary unit specialized in the treatment of children with cystinuria among other rare causes of urolithiasis. Although larger cohorts of individuals with cystinuria have been reported in the medical literature (as many as 442 cases in one study), paediatric cases when included were a minority and outcomes were only focused on CKD, hypertension and proteinuria (504, 517, 534-537)

4.4.1. Demographics:

As expected for an autosomal inherited disorder, the distribution from the entire cohort between male/female patients was equivalent 46/54%. Analysis of this cohort failed to show any significant difference in presentation or outcomes between the two gender groups. While in the asymptomatic or non-stone forming group there was a predominance of girls (n=6) over boys (n=3), this difference did not achieve statistical significance ($p > 0.05$). Nevertheless, this correlates with previous observations where females tend to have a less severe phenotype than males (463, 503).

Considering cystinuria is an inherited disorder with both recessive and dominant patterns according to the specific gene and penetrance it is expected that a proportion of the patients will have a positive family history. Indeed, familial screening supports early diagnosis and treatment in asymptomatic individuals potentially reducing the risks of stone formation. In our cohort up to 46% of the cases reported a positive family for cystinuria which is a similar value to other published studies (504, 538).

4.4.2. Presentation:

Data related to age at presentation in children is challenging to interpret when comparing against adult or mixed paediatric/adult cohorts in the literature. This cohort information will be biased in the sense that all medical records belong to patients during their childhood visiting a single tertiary unit, hence all of them presented in the

two first decades of life. Classically, the median age of presentation in cystinuria tends to be in the second decade of life (431); our dataset shows a median age during the first decade of life with ~75% presenting at <10 years of age. Unlike other monogenic stone forming conditions, patients with cystinuria do not tend to present with significant failure to thrive unless they have more complex conditions such as hypotonia-cystinuria syndrome (539). Our cohort did not include any patient with contiguous gene syndrome and indeed mean centiles for weight and height were close to the 50th.

On presentation, a large proportion of children had a history of renal colic and/or urinary tract infections (approximately 40% each) followed by haematuria. While renal colic has been indeed the most frequently reported symptom on presentation in the largest cohort of patients reviewed with cystinuria (492), it is relevant to highlight the high proportion of patients with a history of UTIs reported in our study compared to others, where it has been as low as 5% (534). The high proportion of patients presenting with UTI in this study could also be biased by different factors:

- Renal colic (abdominal pain with haematuria +/- leukocyturia) can commonly be mislabelled as urinary tract infections and empirical antibiotics used without obtaining urine cultures. Hence, the diagnosis was clinical and not microbiological.
- Presentation was most likely to a different hospital (our hospital provides quaternary care) hence full information was not available.

It is also important to discuss that a meaningful percentage of individuals were diagnosed following a routine familial screening, 23% of the entire cohort, a result considerably higher than other (predominantly adult) cohorts (534). Considering that paediatric units generally have a higher exposure to genetic conditions, it is possible that a higher awareness of these disorders may prompt paediatricians to request familial screening more regularly when following patients with cystinuria.

Patients with cystinuria may present with a higher stone burden compared to others with idiopathic uncomplicated stones. Another interesting finding from our cohort was that in 26% of the cases, stones were found in the bladder. Although this has been

described previously in a few single case reports it is not yet known as a common clinical feature of cystinuria (540, 541). Among other factors, bladder stones can in general be associated to recurrent urinary tract infections (542); indeed in our cohort 9/11 patients who developed cystolithiasis also had a history of previous UTIs. Interestingly, except for a single case where stone analysis revealed 100% triple phosphate, all other bladder stones were composed of 100% cystine. Staghorn stones were found in one third of the cases, a large proportion which is not dissimilar to previous reports in the UK (~20%) (504).

4.4.3. Kidney function:

On presentation, 17% of the cases had a serum bicarbonate level below the normal threshold (20.0 mmol/L in infants and 22.0 mmol/L in older patients), which was not associated on analysis with CKD (p = 1.0). Although data on serum bicarbonate at first visit was not as complete as urinary pH for a meaningful statistical analysis, the presence of metabolic acidosis in such a high proportion of individuals and its close relation with stone formation may highlight once again the importance of alkali supplementation in the management of cystinuria (543). A potential correlation between dietary habits (high animal protein intake, low consumption of fruits and vegetables…), low serum bicarbonate and onset of stone formation cannot be determined in this cohort, as no formal diet records where documented (544).

CKD is one of the most common clinical features in patients with cystinuria, as has been documented in multiple large studies (492, 495, 504, 534). While there is a known correlation between CKD and kidney stone formation, in general cystinuria seems to be one of the stone forming conditions that carries a higher risk due to its chronicity, association with recurrent urinary tract infections and obstructions plus the requirement of multiple stone removal procedures throughout the patient's life (466, 496, 545).

A large cohort of 442 patients from France, including 128 paediatric patients from which eGFR available data was recorded in 58 cases, reported that most individuals (82%) had a normal eGFR ≥ 90 ml/min/1.73 m^2, while 7% had stage 2 CKD and 5% stage 3 (492). It is important to note that this study, despite using the largest cohort

published to date, did not report on any patient going into end stage kidney disease. There are reports informing on individuals with advanced CKD, but the numbers of cases requiring a transplant or dialysis is quite limited (534) and they all happened during adulthood (however some as early as 22 years old).

Available data from our cohort on eGFR at presentation and end of follow up demonstrate stability of kidney function throughout their monitored period. While at the start CKD (eGFR <90) prevalence was 16/40 (40%), by the end it was 11/35 (31%), with a median follow up period of 8.6 years. It is notable that serum creatinine values were not available in all individuals, this was either due to the patient being stone free on presentation (in no need for blood tests), or blood test not being clinically indicated around the time this study marked end of follow up. This could potentially explain why the proportion of CKD at the end of follow up seems lower than at the beginning. Finally, a potential component of AKI on presentation could also have affected the initial eGFR results.

Other reports lower this prevalence to 17% at last visit in paediatric patients (492). It is also possible that our Centre has a selection bias when using data for analysis as it is a quaternary hospital that may not include all asymptomatic/non-stone former patients who have either not yet been diagnosed or, alternatively, are under surveillance in other units. Nevertheless, the high prevalence of reduced kidney function in our patients at a young age may suggest that cystinuria related comorbidities may be underestimated in the paediatric medical literature.

Hypertension was present in 3/52 (6%) of our paediatric patients. There was no correlation between CKD and high blood pressure, although the numbers were small. From these three patients, two had very mild reduced eGFR (80-90) while the other had completely normal kidney function with eGFR of 125 ml/min/1.73 m^2. This prevalence again was lower in a large French cohort (<1%) - only found in a girl with moderate CKD degree (eGFR 63 ml/min/1.73 m^2) (492).

In contrast to paediatric cohorts, hypertension can be as prevalent as 29-51% in adults with cystinuria, and this is particularly associated with advanced CKD, age and male

gender (492, 494, 495, 504, 517). Age has such an elevated correlation that while up to one third of patients under 40 years will have hypertension, the prevalence rises to two thirds in those above 40, reaching almost 90% in those 60 years or more of age (495). Considering the high correlation between hypertension and cystinuria, it is recommended that all patients with cystinuria should have their blood pressure monitored regularly during follow up appointments for early detection and management.

4.4.4. Stone Burden

It is routine practice in our centre to assess stone composition in cystinuric patients, hence more than 90% had their stones sent for analysis at least once during follow up. This analysis revealed that in 5/40 (12.5%) of cases the composition was not pure cystine.

On two occasions, stones were found not to contain detectable cystine in a minimally significant proportion (>10%) and this could potentially be in relation to added factors such as urinary tract infections, over-alkalinisation of the urine, coexistent urinary metabolic abnormality such as hypercalciuria or, according to some reports, treatment with ESWL (494).

Interestingly (and contrary to common belief) there is presently no evidence correlating over-alkalinisation with increased stone formation risk (546). It is not clear neither how ESWL treatment could correlate with an increased rate of calcium phosphate stone formation in patients with cystinuria. Different hypotheses include tubular damage and impaired urinary acidification capacity; yet, in the only publication addressing this question, the stone composition changes occurred prior to ESWL, hence the association is not backed by evidence (546).

Bilateral nephrolithiasis, manifested at least once during follow up, was identified in 19/43 (44%) of the individuals. This large prevalence is not very far from those reported in previous publications which have fluctuated between 60-70% and they both are markedly increased compared to other paediatric cohorts with 24% (435, 538, 547, 548). Indeed, bilateral stone disease in paediatric patients has been proposed as an indication to look for monogenic causes of urolithiasis in childhood

(435). From stone formers in our cohort, 14/43 (33%) of the patients developed at least one staghorn stone. This prevalence is remarkably elevated, highlighting the increased risk of comorbidities in patients with cystinuria, particularly those with impaired kidney function (504).

During the follow up period, median stone formation rate per year was 0.11 (0.0-4.1), in contrast with those who were not receiving preventative treatment (either because this was not previously offered or there was inadequate compliance) and had a yearly rate of 1.0, this later group also had a high stone recurrence of 45% after stone removal procedure (549).

There may be several reasons for the comparatively low yearly stone formation rate in our unit. First, this tertiary/quaternary unit has a long experience in the management of patients with cystinuria and offers all evidence-supported therapeutic options including CBTDs. Secondly, patients are followed up in periods no longer than 3-4 months and consultations include thorough discussions of all aspects of non-surgical management including strategies to increase fluid intake, dietary patterns, regular urine pH monitoring and concerns with medication compliance. Every newly developed calculus is reviewed in regular MDT meetings with the urology team, avoiding delayed interventions in the context of a large stone burden and aiming for complete stone clearance whenever possible.

Additionally, this regional service encourages early familial screening, and a considerable proportion of our cohort (n=12, 23%) was from relatives of primary cases who had started preventative measures before manifesting symptoms and were therefore at a lower risk of stone formation. In this later group only 4 individuals developed stones at some point and their yearly stone formation rates during follow up in our unit were: 0.0 (asymptomatic stone on diagnosis), 0.12, 0.18 and 0.94.

Patients who did not make any stones prior and during follow up where not including in the analysis, so our stone formation rate may in fact overestimate the rate for the entire cohort. Finally, considering that genetic testing was not performed in most of the patients, we cannot rule out the presence of a larger proportion of milder mutations

in this cohort to explain the less severe clinical phenotype compared to other published series.

4.4.5. Medical management:

Standard recommendations for fluid intake in children with cystinuria are usually >2 L/m^2/day (431, 494), whereas adults may target total volumes of 4-5 L/day. However, in practice it is particularly challenging for patients to keep up with such copious fluid intake (517). Indeed, from our cohort only 27% of the patients maintained an intake of >2 L/m^2/day while a slightly less stringent target of 1.5 L/m^2/day was reported in almost 2/3 of cases. In general, and as has been our experience in this clinic, children can maintain a sufficient high volume orally with no need for gastric tube insertion; however, we acknowledge that in some of the most challenging cases this could be considered (494).

In general, the higher the intake the better, with the key aim being to dilute urinary cystine. In routine practice, clinicians establish hydration targets according to cystine concentration in a 24-hour urine sample. The common goal set up according to cystine supersaturation levels in human urine (at a pH >7.0) is <250 mg/L (or <1000 µmol/L). In our cohort approximately 55% of the patients with an analysed 24-hour sample achieved concentrations below that recommended target. Unfortunately, available data on compliance regarding fluid intake is scarce; however, an adult report from a large cystinuria-dedicated clinic in the UK documented relatively similar rates with 66% of cystinuric patients achieving at least 3 L/day (no data was available on 24-hour urine cystine concentration, although 3 L/day is considered optimal for reduced stone formation) (517).

With respect to diet, our cohort only documented consumption of a low salt diet as reported by families and patients (55%). This paediatric cystinuria clinic does not receive routine dietician support, but patients and parents are encouraged to have a diet high in fruit and vegetables. Specific restrictions on protein intake are not recommended as is usual practice in paediatrics (as opposed to adult services) (494, 500). Specialised dietetic input in cystinuria clinics has been identified through patient questionnaires to have a positive impact on clinical care (550).

Urine alkali supplementation in this cohort was done in all cases by use of potassium citrate rather than the sodium-containing alkali salts that are common in adult practice. While increased sodium intake is a known risk factor for stone formation in cystinuria (520, 522) and avoiding a high-salt diet is part of the standard recommendations, evidence supporting any benefit of potassium over sodium containing alkali is lacking. Evidence is similarly lacking for the benefits of citrate over bicarbonate (122, 123). With ~75% of patients reporting regularly taking citrate supplements, compliance seems generally good, and this is backed up by a large proportion of individuals achieving a urine pH of 7.0 or higher.

Average adult doses are 60-80 mEq/day of alkali while in children they are usually reported between 1-2 mEq/kg/day (494, 510). In our cohort approximately 90% of individuals achieved therapeutic targets of urine pH >7.0 with lower doses (median 0.5 mEq/kg/day). Whether dietary patterns leading to a reduced acid load may be behind the lower alkali requirements in this specific cohort is unknown.

Specific analysis according to the phenotype stone formation vs. asymptomatic revealed that while individuals with symptomatic cystinuria are generally reporting to take regular alkali 38/43 (88%), this was less frequent in the non-stone formers 3/8 (37.5%) (p=0.004). This, of course, may imply a correlation with disease severity and patient awareness rather than a paradoxical relation between alkali intake and stone formation.

While CBTDs are usually recommended as a second line therapy in individuals whose response to dietary, hyperhydration and urine alkalinisation has proved insufficient, it can be offered as a first line therapy for those with very large cystine excretion and who are unable to maintain daily intakes above 3 L/m^2/day (or 4 L/day in adults) (494). In this specific cohort, the proportion of stone-formers who received CBTD at some point during their follow up was 56%, which is relatively similar to other reports from the literature (27-52%; including mainly adult patients) (492, 504, 517). Interestingly, some of the published paediatric studies have reported proportions as high as 90% of patients taking CBTD, predominantly tiopronin over penicillamine (presumably for its

safer profile in terms of adverse effects). High number of cases on CBTD may be explained by selection bias, such as decreased eGFR or prior history of stone formation (537, 551).

Use of CBTD showed a significant improvement in 24-h urine cystine excretion in our cohort, not only compared to measurements done around time of diagnosis but also to individuals who are only treated conservatively; an effect already reported in other studies before (432, 433, 494, 537). Indeed, up to 2/3 of the patients treated with CBTD had 24-hours urine cystine concentrations <1000 µmol/L.

One of the main concerns for clinicians when prescribing tiopronin or penicillamine is the potentially high risk of significant adverse effects such as: nephrotic syndrome, bone marrow toxicity and mucocutaneous reactions (432, 433, 494, 537). Although the proportion of patients who develop side effects in this scenario is non-negligible (6-15%), more than 20 years' experience in this clinic and various reports in the literature have indicated that generally both drugs are safe and effective, and providing patients undergo regular monitoring (3-6 months) including blood tests and urinalysis, its use is justified (494, 517, 552).

Tiopronin is generally the first choice of treatment as it has been found to be better tolerated than penicillamine, and indeed in our cohort the rate of adverse effects was up to 4 times higher with the latter (494). Another interesting detail taken from this cohort is that adequate clinical responses can also be achieved with doses lower than the usual recommendations (15 mg/kg/day of tiopronin and 20 mg/kg/day of penicillamine) with 50% of patients on tiopronin being stabilised on daily doses < 15 mg/kg/day and on occasions as low as 5.5 mg/kg/day. Similar experience has been reported by an Asian paediatric study with all patients on CBTD (n=7) receiving doses under 15 mg/kg/day (551). While for tiopronin total doses as high as 3 g/day have been reported, the routine practice in this unit is not to exceed a dose of 1 g/day; this is also in accordance with a previous report showing no added biochemical benefit from using higher doses such as 2 or 3 g/day (526).

Other therapeutic options such as selenium, glutamine and captopril were not offered

to these patients as they are not part of standard treatment in children (553-556).

4.4.6. Urological procedures:

Data from this study have shown not only a biochemical benefit of using CBTD with significant reduction of cystine excretion but also a significant reduction in the rates of new stone formation and subsequently in the number of procedures required per year. Individuals with cystinuria due to the chronicity and high recurrence rate of the condition are generally exposed to many procedures over their lifetime, with all the extra risks associated, including CKD. While medication compliance has been associated with a significant reduction in stone formation and procedure requirements, it is common for patients not to maintain sustained adherence and even those with adequate compliance may not remain stone free for prolonged period of times (557).

Paediatric patients tend to have a more severe phenotype than adults due to obvious earlier presentation but also faster stone formation rates. It is particularly important to achieve complete stone clearances, as residual fragments can quickly increase in size large enough to cause symptoms and prompt further surgical treatment (494).

Although cystine stones are generally known to be of harder consistence, shockwave lithotripsy (ESWL) continues to be the first option in most cases (providing stone size is ideally smaller than 10-15mm) due to the lower rate of complications compared to ureteroscopy and PCNL. In our cohort PCNL and ureteroscopy were more frequently performed than ESWL, which may reflect a more severe patient phenotype with faster stone growth making lithotripsy less likely to be effective as an initial therapy.

Patients on CBTD treatment did undergo a higher number of procedures; this paradoxical correlation can most likely be explained by the fact that only those with more severe disease and refractory to first line treatment were offered tiopronin or penicillamine. The reduction in stone formation rates was also significant in the group treated with CBTD and this is parallel to other previous observations from 1.6 to 0.5-0.7 procedures/year (466, 558).

4.5. CONCLUSIONS:

This large, exclusively paediatric cohort provides extensive information regarding multiple aspects of the clinical care of patients with a rare condition such as cystinuria. It has shown how children with cystinuria can start forming stones from early infancy hence the importance of awareness and rapid diagnosis.

Less common presentations of urolithiasis such as bilateral and/or bladder stones plus staghorn conformation are more frequent in this condition, hence there is a need to encourage urinary stone metabolic screening on first presentation, particularly in these cases. If positive screening, referral to tertiary units for multidisciplinary management should be done soon. Delaying urinary stone screening while patients have stones in-situ is a relatively common practice due to concerns with supersaturation of certain minerals and risk of misdiagnosis. However, delaying diagnosis and treatment in more severe conditions such as cystinuria and primary hyperoxaluria may put patients at increased risk. In this clinic early screening is performed in all paediatric patients on presentation.

Recurrent stone formers are at increased risk of developing impaired kidney function so regular monitoring of serum creatinine and blood pressure should be performed. Follow up in this referral unit has shown stable kidney function throughout paediatric age and better outcomes than previous reports hence the recommendation is for patients to be managed whenever possible by multidisciplinary teams as is the case in this unit (with nephrologists, urologists, radiologists and, if available, dietician support).

Suboptimal compliance with oral hydration is also frequent during paediatric age. Moreover, a large proportion of asymptomatic patients do not take alkali supplementation. While in this cohort no children required gastrostomy feeding tube insertion, we appreciate that this may offer optimal treatment support particularly in the most severe cases. CBTD treatment has proved effective in children to reduce not only cystine excretion but also stone formation and removal procedures rate as well. We have also shown that CBTD are often effective at lower doses than generally

recommended and the evidence extracted from this cohort may prove helpful for other units when significant concerns about adverse effects are raised. Our experience regarding CBTD and adverse effects has shown that tiopronin presents a safer profile with only 15% of individuals discontinuing because of toxicity.

Even though ESWL tends to be a preferable first treatment option from the paediatric urolithiasis' list of procedures, ureteroscopy and PCNL techniques are performed at a higher frequency in paediatric cystinuria presumably due to the more aggressive nature of the disease in children but also the typical harder consistence of the cystine stones making them less amenable to shockwave lithotripsy.

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