EUNEFRON Report Summary

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Executive Summary:

The EUNEFRON project is the result consortium of European researchers joining forces to study orphan nephropathies, in response to the Call HEALTH-2007-2.4.4-1: Natural course and pathophysiology of rare diseases principally affecting the genitourinary tract.

Diseases of the kidney are a large and increasing growing health burden in all societies. At least 60 rare inherited diseases affect the kidney. Because they are rare, on average, less than one person in every 2000 suffers from them; however, they have a large negative impact on the quality of life of both the patients and their families. Because of the rarity of these conditions and their phenotype variability, the care for these patients is less than ideal. The limited knowledge of the underlying mechanism(s) and natural course, lack of standardisation of diagnostic procedures, fragmentation of the clinical and biological data collections, small cohorts that restrict the power of clinical studies, without mentioning a lack of priority for the pharmaceutical industry and even public funding are all contributing factors preventing improvements in treatment and care.

In an attempt to address this problem, EUNEFRON has pursued specific objectives in rare inherited diseases affecting five critical structures of the kidney: Podocytes (Topic 1), Proximal tubule (Topic 2), Thick ascending limb (Topic 3), Distal convoluted tubule (Topic 4) and the Collecting duct (Topic 5). The data on the natural course and pathophysiology generated in these topics 5 were complemented by a sixth topic: the creation of a European Registry and a Network of Genetic Laboratories involved in rare kidney diseases.

After 4 years of research, the EUNEFRON principle investigators and management look back on many achievements in the field of orphan nephropathies research. New families with rare kidney diseases were identified and analysed. In many cases, new mutations in known genes were discovered (more than 200 new mutations found) and, in some cases, the exclusion of known disease genes and further analysis of patient material led to finding new disease genes. By combining the data of many patients and their particular genetic change, novel insights into the natural course (including genotype-phenotype correlations) and pathophysiology of rare nephropathies was gained.

Novel disease models and discovery tools were developed including mouse models as well as new cell culture models which have supported the elucidation of molecular disease mechanisms.

EUNEFRON research has also led to new diagnostic tools and several patent applications were submitted during the course of the project.

Through its many international members and connections, EUNEFRON has been able to set up a comprehensive European Registry of Rare Nephropathies to standardize phenotyping and foster studies of the natural course and genotype-phenotype correlations on a Europe-wide scale. In addition, through the EUNEFRON website, patients and clinicians have access to a European Network of Genetic laboratories for genotyping, using standardized and improved procedures.

Finally, through international collaborative efforts, for the collection of biomaterials and information on rare kidney disease detection, treatment and follow-up, repositories and databases could be set up in an intra-European way, allowing the analysis of much larger patient cohorts than was previously possible using databases from one country alone.
During the project period, EUNEFRON principle investigators published their work in more than 100 peer reviewed scientific publications and many spin-off projects are still developing. EUNEFRON research was also presented at nearly 200 conferences, workshops, meetings and other dissemination opportunities.

Project Context and Objectives:
The EUNEFRON project is the creation of a multidisciplinary European consortium for the study of orphan nephropathies, in response to the Call HEALTH-2007-2.4.4-1: Natural course and pathophysiology of rare diseases principally affecting the genitourinary tract.

Diseases of the kidney are a major and growing health burden in all societies. The elderly are disproportionately affected, but renal disease is also a condition that severely affects children. There is no cure to chronic renal disease. Progression may be slowed to delay the life-threatening end-stage renal disease (ESRD), but few strategies exist to prevent and treat kidney diseases. As leading renal physicians and scientists we know the urgent need to control the escalating costs of renal disease, and our responsibility to act through our research activities.

There are at least 60 rare inherited diseases affecting the kidney, which, although individually affecting less than one person in every 2000, have a large negative impact on the quality of life of the patients, often children, and their families. The care of patients with such rare nephropathies suffers from major problems. The rarity of these conditions, and their phenotype variability, implies limited knowledge of the underlying mechanism(s) and natural course, lack of standardisation of diagnostic procedures, fragmentation of the clinical and biological data collections, small cohorts that restrict the power of clinical studies, without mentioning a lack of priority for the pharmaceutical industry and even public funding. The establishment of multidisciplinary projects gathering a critical mass of expertise and patients, at the European level, is thus essential to maximise the impact of research on these rare diseases.

With the creation of the EUNEFRON consortium, we have mobilized a critical mass of expertise to investigate, on a Europe-wide scale, the natural history and pathophysiology of a series of rare inherited diseases affecting critical structures of the kidney. The project has used and developed multiple models with the aim to develop preventive, diagnostic and therapeutic interventions. A central part of the project has been the creation of a European registry and a network of genetic laboratories to foster a tight interaction between physicians and researchers, promote clinical and basic research, and ensure the efficient dissemination of knowledge.

EUNEFRON has pursued specific objectives in rare inherited diseases affecting five critical structures of the kidney:

- Podocytes (Topic 1)
- Proximal tubule (Topic 2)
- Thick ascending limb (Topic 3)
- Distal convoluted tubule (Topic 4)
- Collecting duct (Topic 5)

The informations on the natural course and pathophysiology generated from patients cohorts in Topics 1 to 5 are complemented by the creation of a European Registry and a Network of Genetic Laboratories involved in rare kidney diseases (Topic 6). Management and dissemination (Topic 7) supports all activities within the consortium (see figure 1).

In figure 2, an overview can be seen of the 16 rare inherited nephropathies that were investigated by the EUNEFRON consortium, grouped by segment/topic. These diseases are caused by mutations in 20 genes (indicated in italics, reflecting the knowledge status at the time of the start of the project) that encode proteins involved in a wide range of functions (enzyme, transport, structure, transcription, ).

Specific objectives: Disorders of the podocyte (Topic 1)
Feto-Maternal Allo-Immune Glomerulopathies (FMAIG)
Fabry Disease
Mechanisms of proteinuria and disease progression in genetic diseases of the podocyte

To investigate the natural course and pathophysiology of rare diseases affecting the podocyte, based on a complementarily expertise, and taking advantage of cell and animal models. These rare diseases provide a unique opportunity to analyze pure podocyte pathobiology, irrespective of confounding factors. We will also investigate the pathogenesis of proteinuria and interstitial fibrosis in congenital nephrotic syndrome and therapeutic modalities (FMAIG, Fabry).

Specific objectives: Disorders of the proximal tubule (Topic 2)
Cystinosis
Imerslund Gräsbeck disease
Maturity Onset Diabetes of the Young (MODY 3)
Hereditary Angiopathy with Nephropathy, Aneurism and Cramps (HANAC)

To investigate the natural course and pathogenesis of rare diseases affecting renal PT, based on a complementary expertise in clinical studies, epithelial physiology (ion transport), and cell biology (endocytosis and trafficking), and taking advantage of cell and mouse models. These studies will yield knowledge on PT protein handling, damage and adaptive mechanisms induced by oxidative stress and renal disease progression.

Specific objectives: Disorders of the thick ascending limb of Henle’s loop (Topic 3)
Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC)
Familial Juvenile Hyperuricemic Nephropathy (FJHN)

To develop novel mouse models and cell culture systems to provide insights into the pathophysiology of several human TAL disorders. We take advantage of the network to investigate the natural course of these disorders, their phenotype variability, and the response to interventions. Several results obtained in animal models, e.g. inhibition of nephrocalcinosis in CIC-5 and CLDN-16 KO mice, could be transferred directly to patient oriented research.

Specific objectives: Disorders of the distal convoluted tubule (Topic 4)
Gitelman syndrome (GS)
Pseudohypoaldosteronism type II (PHA2) or Gordon syndrome

To investigate the natural course and pathophysiology of GS and PHA2, based on complementary expertise in mouse models, cellular studies, and patient cohorts. We focus on the molecular bases for the phenotypic heterogeneity and investigate possible causes for the failure to detect the second mutation in GS. These investigations will yield new disease models and insights into regulation of blood pressure, response to diuretics, Ca2+ and Mg2+ metabolism, and transport mechanisms in the DCT and other nephron segments.

Specific objectives: Disorders of the collecting duct (Topic 5)
Genetic renal disorders of systemic pH homeostasis
Genetic renal disorders of systemic water homeostasis

To investigate the genotype-phenotype correlations and the molecular basis underlying dRTA with AE1 mutants, and study the (patho)physiological role and localization of newly-identified anion transporters, pendrin and AE1 interacting proteins. The role of pendrin as a potential target in hypertension and the role of CIC-5 in H+-ATPase-mediated urine acidification will be determined. NDI patients will be analyzed and we will test whether V2R (ant)agonists are able to rescue their encoded V2R mutants. A novel method for fast in vivo screening to evaluate the role of proteins in CD physiology and diseases will be...
Specific objectives: Registry and network (Topic 6)
Creation of a European Registry of Rare Nephropathies
Creation of a European Network of Genetic Laboratories

The creation of a European Registry of Rare Nephropathies is central to the overall project. It will be a critical tool to improve our knowledge on these rare diseases from clinical presentation (including natural course of the disease from childhood to adulthood) to pathophysiology; to facilitate the dissemination of information; to promote basic and clinical investigations; and to improve clinical care and follow up of the patients at the European level. As part of this program, we will extend the UK Cystinosis Registry to other European centres, with special attention to gender and ageing aspects, effect of cysteamine and additive therapies. We also aim to create a European Network of Genetic laboratories in order to standardize and improve the procedures for genetic diagnosis; facilitate access to genotyping; and search for new genes.

Specific objectives: Management and dissemination (Topic 7)
Management
Dissemination

The structure of the Eunefron project aims at a maximal integration between the topics. The studies in Topics 1 to 5 providing insights into the natural course and pathophysiology of the rare nephropathies. Topic 6 integrates the relevant informations into a European Registry, while establishing a network of Genetic Laboratories. The management and dissemination activities (Topic 7) have provided constant support for the research activities in Topics 1 to 6.

Project Results:

TOPIC 1: Disorders of the podocyte
WP 1-1 Feto-Maternal Allo-Immune Glomerulopathies (FMAIG)
Fetomaternal alloimmunization with antenatal glomerulopathies (FMAIG) is a recently described alloimmune disorder, which results from maternal antibodies that cross the placenta, bind to fetal glomerular podocytes, and mediate renal disease. The pathogenic antibodies are directed against neutral endopeptidase (NEP). The infants mother is NEP-deficient and thus she becomes immunized during pregnancy against NEP expressed by placental cells. We have found three families with the same mechanism of disease. They originate from the Netherlands, Portugal, and Morocco. The family study indicates that the NEP deficiency is a hereditary genetic defect. Anti-NEP-induced antenatal renal disease might account for idiopathic membranous nephropathy (MN) or chronic renal failure detected during adolescence or early adulthood.

1-1-1 Recruitment and phenotyping of new FMAIG families.
We have been looking for additional FMAIG families through the French Feto-Maternal Allo-Immunization Network and the Websites of European Pediatric Nephrology Societies and Patients Associations. We have also established direct contact with many children hospitals throughout Europe. We have tested more than 15 families but until now we have identified only two additional families living in Germany and Italy with the same mechanism of disease as previous ones. This low prevalence of FMAIG families is most likely due to the rarity of allo-immune mediated glomerulonephritis and perhaps even more importantly to misdiagnosis. We have not yet identified families with FMAIG due to a non-NEP antigen but in the wake of these studies, we found that some very young children with membranous nephropathy, but not their mothers, had both circulating cationic bovine serum albumin (BSA) and anti-BSA antibodies. In those children, BSA was present in immune deposits. These cases point to a role for environmental factors in the pathogenesis of membranous nephropathy. This important finding was made possible because we received sera of young children with membranous nephropathy from many sources in Europe. We have introduced these results in the Eunefron project.

1-1-2 Mapping of NEP epitopes and identification of additional antigens involved in FMAIG.
We have identified the peptides that mimic the epitope recognized by maternal alloantibodies against neutral endopeptidase (NEP). These peptides are good candidates for the specific removal of anti-NEP antibodies by extracorporeal
immunoadsorption from sera of NEP deficient pregnant mothers. We have not found yet a new alloantigen but we have identified a non-glomerular antigen involved in early-childhood MN. These new, unexpected data will have an impact on children's health care and food safety policy in early childhood. Looking for new FMAIG families, we found that some very young children with membranous nephropathy, but not their mothers, had circulating antibodies. By using a proteomic approach followed by mass spectrometry, the target antigen for the circulating antibodies was identified as bovine serum albumin (BSA). These patients had both high-level anti-BSA antibodies of IgG1 and IgG4 subclasses, and circulating cationic BSA. BSA was colocalized with IgG in subepithelial immune deposits. IgG1 and IgG4 eluted from kidney-biopsy specimen had anti-BSA reactivity. These data strongly suggest that in those patients, cationic BSA became planted into the anionic glomerular capillary wall, which led to the subsequent deposition of anti-BSA IgG. These cases appear to be the human counterpart of the experimental models using cationic BSA which led to the concept of antigen planting and subsequent in situ formation of immune complexes. These findings thus indicate that the repertoire of antigens involved in membranous nephropathy may include non-glomerular antigens. To our knowledge, they provide the first demonstration that food antigens can be responsible for immune mediated glomerulonephritis.

1-1-3 Establishment of an experimental model of FMAIG. The original program has been stopped because we were not able to establish the mouse model overexpressing NEP despite high-level expression of the transgene. Because, in the meantime, a major candidate podocyte antigen, PLA2R, was identified by David Salants group in Boston, we re-oriented part of the project towards the identification of predisposing genes in adult membranous nephropathy, which could be useful also for the understanding of risk to childhood membranous nephropathy. Because this groundbreaking finding reinforced our concept that podocyte antigens could serve as targets for pathogenic antibodies also in human pathology, we have extended our program to incorporate this antigen in our studies. In a genome-wide association study, we showed that single nucleotide polymorphisms (SNP) in the PLA2R gene were strongly associated with primary MN, which confirmed the involvement of this antigen using an unbiased genetic approach. The same study revealed a highly significant association of HLA-DQA1 alleles, with the risk of developing primary MN being multiplied by 80 in homozygous individuals. Genetic variations in both immune response and podocyte genes could lead to the production of autoantibodies. We have also analyzed the prevalence and significance of anti-PLA2R antibodies in recurrent and de novo MN and showed that PLA2R1 was involved in 5 of 10 patients with recurrent membranous nephropathy, but in none of the 9 patients with de novo membranous nephropathy. We provided evidence that some patients with PLA2R1-related idiopathic membranous nephropathy and anti-PLA2R1 antibodies at the time of transplantation will not develop recurrence, which suggests that donor kidneys lacking the relevant PLA2R SNPs might not express the appropriate epitopes and might thus be resistant to recurrence.

1-1-4 Analysis of podocyte and PT cell alterations induced by anti-NEP antibodies. Due to the absence of an experimental model of FMAIG, this program was modified. We have turned to in vitro experiments using differentiated cell lines to test the hypothesis that in addition to forming immune complexes, anti-NEP antibodies may directly alter podocyte biology. We are using a well-differentiated human podocyte cell line incubated with peptide affinity purified maternal antibodies. To have a broad view on the antibody-induced podocyte cell pathway alterations, we are using the cell signaling antibody array (Sigma) designed for multiplex analysis of the signal transduction pathway. This study is done in collaboration with the company MicroBiochip.

1-1-5 Development of assays for circulating nephritogenic antibodies. Antenatal MN due to NEP antibodies is a severe disease; therefore, it is of the utmost importance to identify quickly the mothers with alloimmune anti-NEP antibodies. For fast screening of the mothers at risk, we have developed an indirect immunofluorescence test on sections from human rabbit and rat kidneys. Although gene mutations were detected in all mothers and led to the production of anti-NEP antibodies, expression of the renal disease was variable, being determined by the mothers antibody response. Maternal production of anti-NEP IgG1 appears to be necessary for the disease to develop. Because pregnancies of NEP-deficient mothers should be carefully monitored, we have developed a quantitative ELISA test to determine antibody titer and distribution of NEP-specific IgG subclasses in the mothers sera.

WP 1-2 Fabry disease
Fabry-Anderson’s disease results from mutations of the GLA gene encoding the lysosomal enzyme α-galactosidase A (α-Gal). The lack of functional enzyme results in accumulation of globotriaosylceramide (Glo3) in a variety of cells of different organs including brain, heart, kidney and vascular, endothelial cells. The disease generally affects males earlier and more severely than females, resulting in brain manifestations, heart failure, renal failure and early death. Enzyme replacement therapy (ERT) results in significant clearance of the deposits.

One of the major issues is to determine which patients to treat and when to institute the ERT, which is not only very expensive but also places a significant burden on the patients and their families. The disease is very heterogenous and a number of patients do not develop symptoms until late in life. It is therefore important to identify mutations associated with early onset of the disease. We also found that mice lacking α-Gal A will accumulate it in their podocytes in the kidney after it is administered by infusion. The podocyte is one of the most severely damaged cells in Fabry disease marking it as a clear target for uptake studies in this disease both in cell cultures and in patients after ERT.

1-2-1 Analysis of renal biopsies for the expression of α-Gal A and reabsorbed proteins.
The aims of this task were to study the mutations in Fabry patients with deficiency in the lysosomal enzyme α-Galactosidase A and to compare these to the severity of disease as seen in renal biopsy materials and the severity of proteinuria. Over a period of 6 years, approximately 200 urine samples from 40 Danish Fabry patients were obtained. The samples have been analysed for albumin and a battery of low and medium molecular weight proteins to follow the development of proteinuria as a consequence of enzyme replacement treatment. In addition, these results are related to the mutation of the individual patient and compared to the biopsy material we have from about 10 of these patients. This study has resulted in the finding and publishing of a new Fabry disease causing mutation and a manuscript describing the results of the urine sample and biopsy material is in preparation.

1-2-2 Uptake of α-Gal A in glomerular podocytes.
Recombinant α-Gal A is used for the treatment of Fabry patients. In this task we have focused on the uptake mechanism by which this enzyme is taken up in renal glomerular podocytes and in endothelial cells. We have identified 2 new receptors for uptake of the enzyme α-Gal A in podocytes in renal glomeruli, megalin and sortilin and could also identify sortilin as a new receptor in renal endothelial cells.
The identification of megalin in human renal podocytes may be a very valuable finding for the explanation of certain glomerular diseases and this finding further prompted us to look for megalins endocytic partner in the renal glomeruli, cubilin. We were able to show that cubilin is expressed in rat and human glomerular podocytes illustrating that the two receptors also under physiological conditions in the glomerulus and mediate podocyte uptake of filtered proteins.

WP 1-3 Mechanisms of proteinuria and disease progression in genetic diseases of the podocyte
Inherited diseases of the glomerular filtration barrier represent a rare and heterogeneous group of disorders, characterized by proteinuria and frequently nephrotic syndrome with ensuing interstitial fibrosis and renal failure. A better understanding of the underlying pathological mechanisms is necessary to develop new therapeutic strategies aimed at delaying the onset and slowing disease progression. In this work package, we have investigated the mechanisms of proteinuria and interstitial fibrosis in two inherited glomerular diseases.
The X-linked Alport syndrome (XLAS) is caused by mutations in the COL4A5 gene resulting in the disruption of the 3α4α5(IV) collagen network in the glomerular basement membrane (GBM). The GBM of Alport patients has been shown to thicken unevenly, separate and eventually deteriorate. Previous studies have shown that urinary excretion of proteins in XLAS patients and XLAS models is increased. We have investigated the underlying cause of proteinuria in XLAS by studying the glomerular component, resulting from the altered GBM, as well as a tubular component.

A growing number of components of the podocyte have been involved in patients with inherited nephrotic syndromes. Mutations in the NPHS2 gene coding for podocin, an integral membrane protein involved in the slit diaphragm, lead to autosomal recessive steroid-resistant nephrotic syndrome (histologically focal segmental glomerulosclerosis, FSGS), one of the most intractable cause of ESRD in the first two decades of life. It is thought that the excessive endocytic uptake of the filtered proteins, leading to proximal tubule (PT) dysfunction and dedifferentiation, plays a major role in the interstitial changes. Accordingly, we have tested the potential role of PT-mediated endocytosis in the pathogenesis of inflammation and interstitial fibrosis associated with the loss of podocin.
1-3-1 Studies on XLAS dogs.
Our results show an increasing urinary excretion of proteins (?2-microglobulin, retinol binding protein (RBP), ?1-microglobulin, apolipoprotein A-1, DBP, albumin, transferrin and IgG) over time and a reduced content of the same proteins in proximal tubule cells. Besides the glomerular component of the proteinuria, a significant tubular component is seen, which is due to a progressive change in the uptake of low molecular weight ligands by megalin and/or cubilin. Furthermore the protein overload present in the lumen of the proximal tubule exceeds the reabsorption capacity of megalin and cubilin and results in a combined low and high molecular weight proteinuria. Also a shift in the distribution of lysosomes to the apical part of the proximal tubule cells is seen in the XLAS dogs suggesting changes in the lysosomal degradation pattern in response to the altered endocytosis. Overall the increased glomerular permeability and the subsequently altered megalin-mediated and megalin-dependent cubilin-mediated endocytosis lead to a partial LMW proteinuria and partial HMW proteinuria.

1-3-2 Studies on XLAS patients.
Concerning the human studies, we have obtained kidney biopsies and kidney necropsy tissue from patients from the group in Brussels and are seeking to obtain tissue and urine samples from the other partners in Europe and other colleagues. We are at present looking for a PhD-student or a post doc for this study.

1-3-3 Role of endocytosis in a mouse model of FSGS.
Glomerular proteinuria is a strong predictor of renal function decline in chronic kidney diseases, and the interaction between proximal tubule (PT) cells and proteins filtered in excess is involved in the genesis of fibro-interstitial damage. Exposure of PT cells to albumin induces the production of pro-inflammatory and pro-fibrotic cytokines as well as free radicals triggering oxidative stress which can lead to dedifferentiation and proliferation of PT cells. However, the potential involvement of endocytosis in albumin-induced toxicity, as well as the timing and signalling pathways involved in PT cells are poorly documented.

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We analyzed the phenotype of PT cells exposed to a strictly time-controlled glomerular proteinuria, using the Nphs2flox/-Cre+ mice, in which conditional inactivation of podocin induces a massive proteinuria followed by tubulo-interstitial lesions. Histological analysis revealed changes in PT morphology starting as soon as 5 days after inactivation of podocin as evidenced by the presence of dilated tubules with brush border disappearance progressing from the early to the straight proximal tubule and finally resulting in atrophic, dedifferentiated tubules 12 days post podocin inactivation. These changes in PT morphology coincided with the presence of a progressively increasing low-molecular weight (LMW) proteinuria, glucosuria and phosphaturia resulting in a renal Fanconi syndrome. Since the endocytic multi-ligand receptors, megalin and cubilin, are essential for the reabsorption of LMW-proteins, we analysed their expression. Immunostaining for megalin revealed a decreased expression starting at day 5 after podocin inactivation which progressively declined following the increase in LMW-proteinuria.

To further investigate the role of endocytosis and the signaling pathways that are activated after albumin-induced toxicity, we exposed primary cultured PT cells to high concentrations (10mg/ml) albumin and analysed the expression of markers for endocytosis, oxidative stress and fibrosis. Exposure of primary PT cells to high albumin decreased the expression of megalin and cubilin and induced oxidative stress and fibrosis; pointing to a dedifferentiation of PT cells. Therefore, we analysed the expression of transcription factors involved in the regulation of megalin and cubilin expression, cellular proliferation and (de)differentiation. We observed an increased expression of ZONAB, a transcription factor involved in epithelial cell dedifferentiation and proliferation and a direct repressor of megalin and cubilin while the expression of its counterpart, HNF1?, was decreased. Furthermore, we observed an increase in cell proliferation markers like PCNA and a loss of epithelial polarization as evidenced by the disappearance of tight junctions and apical megalin expression.

These results show that glomerular proteinuria induces pathways in PT cells that lead to oxidative stress, cellular
dedifferentiation and proliferation resulting in a generalized PT dysfunction as evidenced by the presence of renal Fanconi syndrome which occurs rapidly after podocin inactivation.

1-3-4 Studies in epithelial and fibroblasts co-cultures.

Exposure of PT cells to albumin induces the production of pro-inflammatory and pro-fibrotic cytokines which can lead to dedifferentiation and proliferation of PT cells as well as collagen synthesis by interstitial fibroblasts. However, the potential involvement of endocytosis in albumin-induced toxicity, as well as the timing and signalling pathways involved in PT cells are poorly documented.

To further investigate the signalling pathways that are activated after albumin-induced toxicity, we exposed primary cultured PT cells to various concentrations (control: 1 mg/ml vs. toxic: 10mg/ml) of bovine serum albumin (BSA) for 48h and analysed the expression of markers for endocytosis, oxidative stress and fibrosis. Exposure of primary PT cells to high albumin decreased the expression of megalin and cubilin and induced oxidative stress (DCF, CA3) and fibrosis (?SMA, fibronectin, Snail); pointing to a dedifferentiation of PT cells. Furthermore, we observed an increase in cell proliferation markers like PCNA and a loss of epithelial polarization as evidenced by the disappearance of tight junctions and apical megalin expression. These results show that glomerular proteinuria induces pathways in PT cells that lead to oxidative stress, cellular dedifferentiation and proliferation resulting in a generalized PT dysfunction as evidenced by the presence of renal Fanconi syndrome which occurs rapidly after podocin inactivation. Exposure to apical albumin also induce the expression of fibrosis markers, suggesting the transformation of PT cells. Further experiments are planned to investigate the consequences of the modified phenotype of PT cells on the activity of fibroblasts in co-culture.

TOPIC 2: Disorders of the proximal tubule

WP 2-1 Cystinosis

Cystinosis is the most frequent cause of inherited renal Fanconi syndrome in children, progressing towards end stage renal disease. The disease is caused by mutations of the CTNS gene encoding the lysosomal cystine transporter cystinosin. The existing treatment with cysteamine does not reverse Fanconi syndrome and only postpones the deterioration of the renal function. The pathogenesis of PT dysfunction in cystinosis is not yet understood. Patients with cystinosis present with low molecular weight proteinuria and albuminuria starting from early age, pointing to defective endocytosis via megalin and cubilin. Based on studies of the glutathione status, enhanced oxidative stress in cystinosis was suggested. However, it is not known how oxidative stress can be linked to lysosomal cystine accumulation. It can be hypothesised that cystinosin dysfunction might lead to the alterations of the integrity of the lysosomal membrane, making cystinotic cells prone for oxidative stress damage, accelerated aging and apoptosis. Based on the existing network between centres following cystinosis patients cohorts and animal models we have investigated the pathophysiology of nephropathic cystinosis.

2-1-1 Study of PT protein reabsorption in cystinosis.

Mice lacking the Ctns gene on a mixed genetic background were shown to accumulate cysteine but did not develop renal proximal tubulopathy. As the renal phenotype can be influenced by genetic background, during the course of the project, we generated congenic C57BL/6 and FVB/N Ctns-/- mice and showed that C57BL/6 mice presented with pronounced histological lesions of the proximal tubules as well as a tubulopathy, and progressively developed chronic renal failure. In contrast, renal dysfunction was not observed in the FVB/N strain. Thus the C57BL/6 strain represents the first Ctns-/- mouse model to show clear renal defects.

2-1-2 Study of the relation between cystine accumulation, apical PT transport, oxidative stress and overall lysosomal function.

In order to better understand the physiological role of cystinosin, we searched for proteins interacting with cystinosin using a proteomic approach. Thus, we showed that cystinosin consistently interacts with galectin-3 through a carbohydrate-dependant mechanism and that this interaction is maintained when cystinosin is deleted from its lysosomal targeting motifs. In addition, we further expanded the spectrum of galectin-3 subcellular localization to lysosomes and late endosomes. We also showed the interaction of cystinosin with various subunits of the V-ATPase, and other proteins such as lipid raft proteins (e.g. caveolin-2, flotillins) and proteins of the mTORC1 complex (the Ragulator complex protein PDRO and the RAS-related GTP-binding proteins A and B), which is reminiscent of the observation that mutant phenotypes resulting from the loss of the Ers1 gene encoding the yeast homolog of cystinosin, can be suppressed by overexpression of Meh1, a member of the EGO complex that functions upstream of TORC1 to mediate amino-acid signaling, and fits well with the recent identification of the V-ATPase as a
component of the mTOR pathway.

WP 2-2 Imerslund Gräsbeck disease

Imerslund-Gräsbeck syndrome (IGS), also named megaloblastic anaemia 1, is a rare vitamin B12 deficiency disease characterized by defective intestinal absorption of the vitamin B12-intrinsic factor complex. Generally IGS patients present at 2-4 years of age with signs of cobalamin deficiency and proteinuria. More than 200 human cases and familial clusters have been reported in Scandinavia, Middle East and Northern Africa. Null and missense mutations of the cubilin (CUBN) and amnionless (AMN) genes have been implicated in some IGS kindreds, but both loci have been excluded in others. This diversity in mutations may explain why only some patients with selective IF-B12 malabsorption have proteinuria. Mutations in CUBN affecting more binding sites or resulting in the absence of functional receptors are more likely to give proteinuria than mutations affecting only the IF-B12 binding site. Likewise, mutations in AMN are likely to give proteinuria due to its supposed function as chaperone for cubilin.

The objective of this workpackage was to compare the mutations of the two genes involved in the Imerslund Gräsbeck disease, AMN and CUBN, to the excretion of low molecular weight proteins in the urine of a cohort of patients and this objective has been fully obtained.

2-2-1 Identification of the patients with cobalamin deficiency.

We have contacted the Danish diagnosis registry, and hospitals and medical doctors in several European countries to find patients which presented in an early age with signs of cobalamin deficiency with the goal to identify patients with proteinuria. During the course of the project, we have together with Dr. Renata Kozyraki and Dr. Pierre Verroust in Paris recruited a total of 5 French families and together with Dr. Francesco Emma in Rome recruited 1 family in Italy. For this study we had access to 9 patients in total.

We have got permission from the Central Danish Region Committee on Biomedical Research Ethics and from the Danish Patient Registry to seek Danish patients but this seek was unsuccessful.

2-2-2 Analysis of urinary protein excretion pattern and its relevance for treatment including vitamin supplement treatment.

The urine has been analyzed for cubilin and non-cubilin ligands from the patients, their parents and 20 Danish control children. The excretion pattern has been compared to the mutations for the cubilin and AMN genes. The characterization of the proteinuria will be of further relevance for diagnosis and for the treatment including vitamin supplement treatment.

We have identified two new mutations for AMN and two new mutations for cubilin. Two AMN mutations were known with Turkish founders and one for the Finnish CUBN founder mutation.

2-2-3 Comparison of genotype and clinical phenotype.

For one of the Italian patients in which we identified a nonsense CUBN mutation a renal biopsy in addition to urine samples was available. For this interesting patient we have published a paper, in which we also present a new cubilin ligand, ?1-microglobulin. The study on the French/Italian cohort has now been submitted for publication which describes the genotype-phenotype correlation of proximal tubular function in Imerslund-Gräsbeck Syndrome.

In addition to this we have studied the disease in mouse models developed in our lab, in which the genes for cubilin or megalin or both were knocked out. The mouse model of proximal tubule endocytic dysfunction has resulted in the publication of a study on renal albumin handling.

WP 2-3 Maturity Onset Diabetes of the Young (MODY 3)

Our previous studies have demonstrated that a broad membrane trafficking defect involving the multi-ligand receptors (megalin and cubilin) and chloride channels/transporters (ClC-5, CFTR) results in defective endocytosis and generalized PT dysfunction in several inherited PT disorders. Hepatocyte nuclear factor 1 alpha (HNF1?) is a transcription factor expressed in liver, pancreas, kidney and intestine. Heterozygous mutations in TCF1, the HNF1?-coding gene, result in maturity onset diabetes of the young (MODY3), a rare, autosomal dominant form of diabetes associated with a variable renal phenotype that may include PT dysfunction. Mice lacking HNF1? show PT dysfunction leading to polyuria, aminoaciduria, and phosphaturia as well as LMW proteinuria due to defective expression of megalin and cubilin.

2-3-1 Transcriptional regulation of PT transporters by HNF1a.

The Cl-/H+ exchanger CIC-5 is essential for the endocytic activity of the proximal tubule cells and the tubular clearance of proteins filtered in the glomeruli. The mechanisms that regulate the expression of CIC-5 in general and its specific expression
in the proximal tubule are unknown. In this study, we investigated the hypothesis that the hepatocyte nuclear transcription factor HNF1α, which is predominantly expressed in proximal tubule segments, may directly regulate the expression of ClC-5. In situ hybridisation demonstrated that the expression of Clcn5 strikingly overlaps with that of Hnf1α in the developing kidney as well as in absorptive epithelia including the digestive tract and yolk sac. Multiple binding sites for HNF1α were mapped in the 5-regulatory sequences of the mouse and human Clcn5/CLCN5 genes. The transactivation of the Clcn5/CLCN5 promoter by HNF1α was verified in vitro, and the binding of HNF1α to the Clcn5 promoter in vivo was confirmed by chromatin immunoprecipitation in mouse kidney. The expression of Clcn5 was reduced in the proximal tubule segments of HNF1α-null kidneys and it was rescued upon transfection of HNF1α-null cells with wild-type but not with mutant HNF1α. These data demonstrate that HNF1α directly regulates the expression of CIC-5 in the renal proximal tubule and yield insights into the mechanisms governing epithelial differentiation and specialized transport activities in the kidney.

Loss of function mutations of HNF1α in mouse (Hnf1α-/-) and humans (MODY3) are associated with diabetes and dysfunction of the kidney PT cells, reflected by a defective water and solute reabsorption and low-molecular weight proteinuria. The PT cells reabsorb ultrafiltrated proteins by an effective endocytic process mediated by the multi-ligand receptors megalin and cubilin. To gain insights into the transcriptional mechanisms operating in PT cells, we performed in silico analysis and chromatin immunoprecipitation of megalin and cubilin promoters, which revealed several binding sites which interacted with HNF1α. These studies have shown that loss of function mutations in HNF1α induce a drastic decrease in megalin and cubilin expression and endocytic activity from Hnfα-/- mice. Therefore, HNF1α plays a key role in the constitutive expression of megalin and cubilin genes and in the regulation of endocytosis in PTC.

2-3-2 Adaptation to oxidative stress in rodent models of PT dysfunction.

Dysfunction of the renal proximal tubule is associated with impaired reabsorption and urinary loss of vital solutes. The metabolic consequences and adaptation mechanisms to such dysfunction remain poorly known, which prompted us to investigate mouse and human models of inherited proximal tubule disorders. We first demonstrated higher cell proliferation (cyclin E, PCNA, Ki67) and oxidative stress (Type 1 SOD, thioredoxin) in proximal tubule cells of CIC-5-deficient (Clcn5Y/-) mice, a well-established model of Dent’s disease taken as a paradigm of generalized proximal tubule dysfunction. Transcriptome and protein analyses showed a kidney-specific, 4-fold induction of type III carbonic anhydrase (CAIII) in Clcn5Y/- mice, with CAIII located in scattered PT cells. Kidney-specific CAIII upregulation was confirmed in mice lacking the multiligand receptor megalin and in a patient with Dent’s disease harbouring an inactivating mutation of CLCN5. CAIII was also specifically detected in the urine of mice lacking CIC-5 or megalin, and in patients with Dent’s disease. The specific induction of CAIII was substantiated in proximal tubule cell lines (HK2 and OK) exposed to H2O2. This study demonstrates that dysfunction of the proximal tubule due to the lack of CIC-5 in mouse and man is associated with increased cell proliferation, oxidative stress, and specific induction of CAIII. The induction of CAIII might reflect cell dedifferentiation and participate in oxidative scavenging in proximal tubule cells.

As part of this task, we have obtained a patent for CAIII as a diagnostic biomarker of PT dysfunction, and have started to prepare a detection kit that is currently validated in humans.

WP 2-4 Hereditary Angiopathy with Nephropathy, Aneurism and Cramps (HANAC)

HANAC (Hereditary Angiopathy with Nephropathy, Aneurysm and cramps) is a new autosomal-dominant syndrome due to mutations in exons 24 and 25 of COL4A1, which affect small vessels and large arteries (leukoencephalopathy, retinal arteriolar tortuositities, intracranial aneurysms), and includes hematuria or renal cyst, with substantial alterations of basement membranes (BM) of tubules, renal interstitial capillaries, and skin. The studies of the EUNEFRON consortium were aimed at providing a more extensive description of the disease, and understanding the pathomechanisms involved.

2-4-1 Recruitment, phenotyping and genotyping of new HANAC families.

During the study period, we have recruited 29 index patients presenting with either typical HANAC syndrome or at least of two or more organ involvement (kidney disease, brain or retinal vasculopathy, muscle cramps) (Table 1). In all these patients, detailed phenotypic characteristics were recorded and genetic analysis of COL4A1, COL4A2 and HSP47 genes were performed. Phenotypic study

Among the 29 index cases recruited, four patients presented with typical clinical HANAC features that associated retinal arteriolar tortuosity, muscle cramps with serum CPK elevation, bilateral renal cysts, brain vasculopathy inconstantly associated with unique and multiple cerebral aneurysms. The remaining 25 patients showed partial clinical HANAC phenotype,
with at least two major organ involvement, including central nervous system involvement, retinal arteriolar tortuosity, kidney defects, Raynaud phenomena and muscular symptoms. In one family, these symptoms were associated with bone symptoms, joint hyperlaxity, and delayed skin wound healing.

Functional vascular investigations performed in 4 HANAC patients revealed similar structural and functional defects with low intima/media thickness, and specific elevation of compliance and distensibility of the brachial artery, both suggesting alteration of the mechanical properties of the vessels. In addition, functional analysis suggested a primary endothelial dysfunction, with low dilation response to shear stress, that was corrected by exogenous nitrite oxide administration.

Genetic study
Four distinct COL4A1 missense mutations were characterized in the four patients with typical HANAC syndrome and in affected related family members. They were all located in exons 24 and 25, and substitutes highly conserved glycine residues in the CB3 domain of the COL4A1 protein. This domain contains several integrin binding sites. In two additional patients with partial HANAC phenotype that associated symptomatic cerebral small vessel disease, multicystic kidney disease and retinal arteriolar tortuosity, a frameshift mutation in exon 24 responsible for premature stop codon and a donor splice site mutation in intron 44 were characterized respectively.

Taken together with the three HANAC mutations identified in the first description, 8 of 9 COL4A1 HANAC mutations are localized in exon 24 and 25 and affected the CB3 domain of the protein is responsible for interaction with integrins.

Studies on trafficking and cell toxicity in fibroblasts of HANAC patients.
To get further insight into the pathogenesis of HANAC syndrome, we have established primary culture of skin fibroblasts from patients and analyzed potential defects in trafficking and secretion of the mutated COL4A1 chains and the induction of endoplasmic reticulum stress and unfolded protein response.

We have first analyzed the cellular localization of COL4A1 protein at the basal level by confocal microscopy using anti-collagen IV antibodies and anti-HSP47 antibodies. Intracellular distribution of COL4A1 was similar in normal human dermal fibroblasts (HDF) and COL4A1 mutated fibroblasts. After incubation with ascorbic acid that enhance the secretion of COL4A1, a decrease of intracellular content of type IV collagen was observed in control and G495V and G519R fibroblasts but not in G528E fibroblasts, suggesting an abnormal delayed secretion of the mutated G528E COL4A1 protein.

We then analyzed endoplasmic reticulum (ER) stress in COL4A1 mutant dermal fibroblasts at the basal level and after exposition to ER-stress chemical inducers. No significant difference in ER stress protein expression was observed at the basal level between HANAC patients skin fibroblasts and controls. Incubation of cells with ER-stress chemical inducers did not significantly upregulate ER stress protein markers, except in G498V cells which showed a two-fold higher CHOP mRNA. No spliced form of XBP1 mRNA expression was detected in control and mutated cells, whereas higher amounts of spliced XBP1 mRNA were detected after ER stress induction in G528E and G498V human dermal fibroblasts. We also analyzed apoptosis at the basal condition and after ER stress induction. No difference was observed between control HDF and mutated cells. By contrast, higher rates of apoptosis were recorded after ER stress induction in mutated cells.

Establishment and study of animal models harbouring COL4A1 and COL4A2 mutations.
For this task, two knock-in mouse strains bearing two distinct Col4a1 mutations responsible for HANAC syndrome in human (respectively p.Gly495V and p.Gly525Leu) were successfully generated by homologous recombination. The renal phenotype of heterozygous and homozygous mutants was analysed at birth and in adults.

Despite severe intracerebral bleeding and growth retardation at birth, homozygous mice lived through adulthood. At birth, albuminuria and glomerular hematuria were detected in homozygous and heterozygous p.Gly495V and p.Gly525Leu Col4a1 mutant animals. Homozygous neonate mutants showed morphological glomerular defects with ultrastructural abnormalities of podocytes and glomerular basement membrane. Other HANAC hallmarks were also present. Basement membrane expression of the COL4A1 protein persisted at a low level in homozygous mutants, demonstrating that the mutation did not abolish the trimer formation and secretion. Abnormal expression of nephrin was detected in both mutants. Albuminuria progressively disappeared during the first week and neonatal morphological changes resolved. However, at 3 months, renal defects developed in both heterozygous and homozygous animals, that associated bilateral glomerular cysts and large periglomerular and vascular inflammatory infiltrates. Papilla atrophy was also present in homozygous mutants. The renal phenotype of our Col4a1 HANAC mouse models highlights the crucial role of the COL4A1 protein during glomerular and collecting duct
embryogenesis and suggests that HANAC patients present a glomerulocystic kidney disease.

**TOPIC 3: Disorders of the thick ascending limb of Henle’s loop**

WP 3-1 Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC)

The TAL is critically important for the reabsorption of NaCl, K+, Ca2+ and Mg2+, both by cellular and paracellular pathways. Potentially life-threatening inborn errors of renal salt wasting are caused by mutations in the human genes coding for epithelial transport systems operating in the TAL. Mutations have been identified in genes coding for transcellular as well as paracellular systems. Ca2+ and Mg2+ are maintained within narrow limits and the kidney plays a major role in this process. To gain a deeper understanding into the pathophysiology of renal and overall calcium and magnesium homeostasis, we generated in the past a mouse model (CLDN-16 KO) which lacked the renal tight junction protein claudin 16. This protein is, when mutated, known to lead to renal loss of calcium and magnesium in humans. The resulting disease is termed familial hypomagnesemia with hypercalciuria and nephrocalcinosis (FHHNC). The disease starts at birth and in the vast majority of the cases, leads to end stage renal failure with the need for dialysis and renal transplantation. Within the EUNEFRON project, we choose several approaches to get detailed insights in this disorder.

3-1-1 Generation of CLDN-16 knock-down.

In this task we started to generate mice in which claudin-16 production should be almost completely suppressed by means of micro RNA. However, this task was abandoned at a relatively early stage, since this model was generated and published by competitors. The model itself shared major similarities with the cldn16 deficient mouse model we have established, i.e. the renal loss of calcium and magnesium. However due to the technique by which it has been generated, our model allows significantly more additional molecular genetic studies which have ultimately led in the EUNEFRON period to the identification of a novel human disease (see below, deviation).

3-1-2 Generation of inducible CLDN-16 KO mice.

In this second approach, our aim was to generate mice in which the gene for claudin-16 could be eliminated at various points of development, i.e. before birth, in newborns, younger and adult mice. Unfortunately, also this task had to be cancelled. After breeding the CLDN-16 KO mice strain, previously generated by our group, with a mouse strain that carried the necessary gene to eliminate cldn16, we discovered that these breeding cohorts were not producing any offspring, thus we stopped this project due to technical reasons (see below, deviation).

Deviation: Due to the early ending of the tasks 3-1-1 and 3-1-2, we investigated instead whether the CLDN-16 ko mouse exhibits genetic mechanisms that allows these mice to compensate via different other ways for their disrupted renal magnesium and calcium homeostasis. We identified several known transport systems for magnesium and calcium but also novel genes and molecules. We hypothesized them to be candidates of so far unexplained human magnesium and calcium associated disorders. Indeed, we could show that one of them, called CNNM2, causes, when mutated a form of human familial magnesium wasting through the kidney. Little was known about the function of CNNM2, therefore we started to characterize this protein and we could show that it exists in different variants which are present in many organs, also in the kidney. There it is located in an area where magnesium uptake takes place. Moreover we also could demonstrate that CNNM2 is located in the gut, where magnesium is taken up from the food. Thus, patients with defects in cnnm2 are not only deficient in the reuptake of large amounts of magnesium back from the pre-urine but are also unable to take necessary amounts of magnesium from the diet. Finally, we could also show how CNNM2 is located in the membrane that surrounds kidney cells and that it present at the side where cells are connected to the blood. This implicates that CNNM2 is involved in the transport of magnesium out of the cells after it has been taken up into the cell by other mechanisms.

3-1-3 Natural course of FHHNC patients.

A more clinical approach was the collection of more patients with FHHNC, in total we have identified during the EUNEFRON period 41 patients, 15 with mutations in CLDN-16, and 26 with mutations in CLDN-19. Survival analysis suggested that patients with CLDN19 mutations had a higher risk of progression to chronic kidney disease (CKD) and poorer renal survival than patients with CLDN16 mutations. In line with this, the first genotype-phenotype analysis on this set of patients has resulted in the conclusion that patients with CLDN-19 mutations may display more severe renal impairment than patients with CLDN-16 mutations. In addition, ocular abnormalities, a sub-phenotype of FHHNC, were observed in 91% of patients with CLDN19 mutations and none of the patients with CLDN16 providing a putative differentiating hallmark for future clinical detection and
adequate treatment. Currently, data of the middle- and longterm course are being collected. These results have been implemented into the FHHNC database (see topic 6). The results will help to achieve a more complete overview of frequency and natural course of patients with FHHNC.

WP 3-2 Familial Juvenile Hyperuricemic Nephropathy (FJHN)

Besides solute transport, the TAL is also the site of the production and secretion of the Tamm-Horsfall protein (THP, or uromodulin). THP is the most abundant protein in the normal urine, where it may inhibit urinary crystal formation and confer antibacterial properties, thus protecting from kidney stones and urinary tract infections. It was shown recently that mutations in the UMOD gene that encodes THP cause a set of rare human disorders, familial juvenile hyperuricemic nephropathy (FJHN, also named medullary cystic kidney disease type 2, MCKD2) and a variant form of glomerulocystic kidney disease (GCKD), that all lead to end-stage renal disease.

Familial juvenile hyperuricemic nephropathy (FJHN, or MCKD type 2) is an autosomal-dominant disorder characterised by abnormal tubular handling of urate and late development of chronic interstitial nephritis usually leading to progressive renal failure. Mutations in the UMOD gene account for only 40% of the affected families. The phenotype of individuals with UMOD mutations varies widely between and within affected families, and it is unknown whether early hyperuricemia plays a role in the development of chronic interstitial nephritis or, alternatively, is an independent manifestation of the disease. In families with FJHN, mutations in UMOD lead to an accumulation of uromodulin in TAL cells, together with a drop in the overall urinary excretion.

3-2-1 Natural course and phenotype in patients with FJHN.

In a collaborative effort of several EUNEFRON partners, clinical and genetic data were gathered for 109 patients from 45 families. Overall, 37 different UMOD mutations were identified, 19 of which are novel. As for previously reported studies, about 50% of the identified mutations affect one of the 48 conserved cysteine residues in the uromodulin sequence. Detailed clinical data were available for 70 patients. The vast majority of patients (about 90%) had a family history of gout or renal disease compatible with autosomal dominant way of inheritance. Hyperuricemia appears a key hallmark of the disease. Serum uric values higher than the 75th and 90th percentile values corresponding to GFR were observed, respectively, in 71% and 50% of patients carrying UMOD mutation and not undergoing allopurinol treatment. On the contrary, renal cysts were found in only a fraction (34%) of FJHN patients with UMOD mutations and had unspecific localisation in most of the cases. Renal survival ranged between 25 and more than 70 years, with a median value of 54 years. Large intrafamilial variability was recorded together with an apparent absence of genotype-phenotype correlation in terms of age at ESRD. A slight, though not statistically significant, trend for higher renal survival was observed in patients carrying polar substitution compared with those with cysteine-affecting mutations.

An UMOD mutation was identified in 17% of the 136 FJHN probands. Many of the probands with no UMOD mutation were clinically undistinguishable from those carrying uromodulin mutations, although their uricemia was less frequently out of proportion with GFR.

A small proportion (5 probands) of UMOD-negative cases resulted to be mutated in the HNF1B gene, while the majority could be linked to REN mutations, to MCKD1 or FJHN3 loci, or to yet to be identified FJHN genes/loci.

As urinary concentration of uromodulin is markedly decreased in patients carrying an UMOD mutation, we developed an ELISA-based assay to measure uromodulin urinary levels as a diagnostic tool besides genetic screening. This method was successfully validated in a large collection of individuals with normal renal function and in chronic kidney disease patients.

3-2-2 Generation of a knock-in mouse model of mutant THP.

This project was aimed at the generation of a mouse model of FJHN to gain insight into the molecular bases of the disease. We originally envisaged a strategy to obtain a knock-in mouse model, ie a mouse with targeted insertion of a mutation in the Umod gene, but eventually followed an alternative strategy and generated mouse lines carrying either wild type or mutant Umod transgenes. The two lines that were chosen for further analysis were matched for transgene expression and showed specific expression in the TAL segment of the kidney nephrons, where endogenous uromodulin is normally expressed. Remarkably, transgenic mice expressing mutant uromodulin develop a renal disease that is very similar to the human condition, showing urinary concentrating defect, tubulo-interstitial damage, tubular cysts and renal failure. Further analysis of the mouse model allowed gaining insight into the mechanisms of disease pathogenesis and set the bases for testing potential
therapeutic strategies in the in vivo model. Our data clearly demonstrate a gain-of-toxic function mechanism of uromodulin mutations that progressively leads to damage of TAL segments and renal failure. Retention of mutant protein in the ER is a primary event in the disease pathogenesis, already present in young pre-symptomatic animals. A detailed analysis of the its possible consequences on cell physiology revealed that intracellular accumulation of mutant protein is not leading to cell death nor to the activation of well-known ER stress pathways, as the Unfolded Protein Response, aimed at restoring the ER function. Rather, we observed very early induction of the inflammatory process, well before any histological sign of renal damage.

3-2-3 Cellular responses to mutant THP expression.

We generated cell lines expressing either wild type or mutant uromodulin isoforms. These models were used to characterise the response signals that are elicited upon accumulation of mutant uromodulin in the ER, the cellular compartment where most proteins are synthesised and matured to their final conformation. In our cell models mutant uromodulin shows ER retention to an extent that was different for each of the several mutations that were tested. Despite this difference, no clear genotype-phenotype correlation could be drawn.

In these models, ER retention of mutant uromodulin did not result in the formation of intracellular aggregates, as seen in patient biopsies and in transgenic mice kidney sections. Also, mutant protein retention did not have any dominant negative effect on the trafficking of the wild-type one when they were co-expressed.

Extensive analysis was carried out to identify the cellular response pathways that are activated upon ER retention of mutant uromodulin. Rather surprisingly, we could not detect the activation of the UPR nor any toxic effect on cell viability. The same observations were made in standard culturing conditions or after induction of different cellular stresses (e.g. oxidative stress or hyperosmotic conditions).

Interestingly, we demonstrated that protein aggregation could be induced in the same cellular systems when expressing soluble mutant uromodulin isoforms lacking GPI-anchoring to the membrane. Consistently with what observed in patient kidney biopsies, soluble mutant uromodulin forms aggregates within the ER, supporting the hypothesis that loss of GPI-anchoring could play a role in vivo in mutant uromodulin aggregation. This mechanism could represent an interesting therapeutic target and will be further explored in the mouse model of FJHN.

Notably, in all cell systems analysed we consistently observed that mutant uromodulin was partly escaping the ER quality control and reached the plasma membrane. We demonstrated that this mechanism has also relevance in vivo, as we collected evidence of mutant uromodulin secretion in the urine of both patients and transgenic mice. This could be of significance for the disease pathogenesis, since mutant protein reaching the plasma membrane tends to form extracellular aggregates that trap wild type uromodulin likely interfering with its function.

3-2-4 Role of CIC-5 in the trafficking of THP.

CIC-5 is a chloride-proton exchanger primarily localized to endosomal membranes. Mutations in its gene (CLCN5) have been found in Dent disease and renal tubular disorders complicated by nephrolithiasis. The role played by the chloride-proton exchanger CIC-5 in regulating intracellular vesicle trafficking in the proximal tubule, and its co-expression with uromodulin in the epithelial cells lining the thick ascending limb (TAL) raised the possibility that CIC-5 may regulate the trafficking of uromodulin in that segment. This hypothesis was particularly relevant when considering that patients with defective CIC-5 (as mice knock-out for CIC-5) may show kidney stones and/or nephrocalcinosis, and that the abundant uromodulin excretion in the normal urine has been suggested to protect against urinary stones formation.

To test a potential role of CIC-5 in regulating uromodulin trafficking in vivo, we measured the urinary excretion of uromodulin in 1 year-old mice lacking CIC-5 and in patients with Dent's disease with inactivating mutations of CLCN5. These studies clearly showed that the lack of CIC-5 is reflected by an increased urinary excretion of uromodulin that cannot explain the propensity for kidney stone formation in Dent disease patients.

The potential interaction between CIC-5 and uromodulin was tested in HEK293 and MDCK cells, stably expressing wild-type or mutant uromodulin isoforms through transduction with lentiviral vectors. These cell lines were considered the ideal model where to study uromodulin trafficking due to their homogenous expression level. Endogenous CIC-5 resulted to be expressed at low but detectable level by RT-PCR in both cell systems. Uromodulin-expressing cells were transfected in order to overexpress either wild type or mutant CIC-5 and secretion of uromodulin was measured. Uromodulin levels in the culturing medium resulted to be similar in all the experimental conditions, suggesting that uromodulin secretion is not regulated by CIC-5.
**TOPIC 4: Disorders of the distal convoluted tubule**

**WP 4-1 Gitelman syndrome (GS)**

Gitelman syndrome (GS) is an autosomal recessive tubulopathy caused by inactivating mutations in SLC12A3, the gene that codes for the thiazide-sensitive NCC. These mutations disrupt the function and impair the folding of NCC when expressed in Xenopus oocyte, thereby activating the quality control mechanism and targeting them for degradation. A Slc12a3 KO mouse model recapitulates some of the human phenotype of GS. Despite these advances, mutations in SLC12A3 are only detected in a subset of GS patients and, often, at the heterozygous state. GS is characterized by a significant inter- and intra-familial phenotype variability, and males appear to be more severely affected than females. In this task, we performed collaborative studies to investigate the molecular bases for the phenotypic heterogeneity in GS, the regulation of NCC in DCT cells, and the mechanisms that explain the failure to detect mutations in up to 40% of patients.

**4-1-1 Natural course and genotype-phenotype correlations in patients with GS.**

Gitelman syndrome is characterized by significant inter- and intrafamilial phenotype variability, with early onset and/or severe clinical manifestations in some patients. No correlations between the disease variability and the position/nature of SLC12A3 mutations have been investigated thus far. In this study, extensive mutational analyses of SLC12A3 were performed in 27 patients with GS, including genomic DNA sequencing, multiplex ligation-dependent probe amplification, cDNA analysis, and quantification of allele-specific transcripts, in parallel with functional analyses in Xenopus laevis oocytes and detailed phenotyping. Twenty-six SLC12A3 mutations were identified in 25 patients with GS, including eight novel (detection rate 80%). Transcript analysis demonstrated that splicing mutations of SLC12A3 lead to frameshifted mRNA subject to degradation by nonsense-mediated decay. Heterologous expression documented a novel class of NCC mutants with defective intrinsic transport activity. A subgroup of patients presented with early onset, growth retardation, and/or detrimental manifestations, confirming the potential severity of GS. The mutations that were associated with a severe presentation were the combination at least for one allele of a missplicing resulting in a truncated transcript that was downregulated by nonsense-mediated decay or a nonfunctional, cell surface-absent mutant. The most recurrent mutation on the second allele was a newly described NCC mutant that affected the functional properties of the co-transporter. These data suggest that the nature/position of SLC12A3 mutation, combined with male gender, is a determinant factor in the severity of GS and provide new insights in the underlying pathogenic mechanisms of the disease. These results have been integrated with the Gitelman database created as part of work package 6.

**4-1-2 Role of parvalbumin and caveolin-1 in the DCT.**

The thiazide-sensitive Na+-Cl- cotransporter (NCC) plays an essential role in NaCl reabsorption in the distal convoluted tubule (DCT). We have previously shown that the EF-hand, Ca2+-binding protein, parvalbumin (PV), which is selectively expressed in the DCT, regulates the transcription of NCC in mouse kidney and mDCT cells. This effect could be mediated by purinergic signaling, as 6h exposure to 10mM ATP significantly reduced NCC mRNA, whereas pre-incubation with the Ca2+ chelator EGTA-AM induced a 2-fold overexpression of NCC mRNA in mDCT cells.

In the present study, we investigated the mechanisms of Ca2+-dependent regulation of NCC expression in mDCT cells, as well as the potential role played by the purinergic receptor P2Y2 in these cells. qPCR studies and cytosolic calcium (Fura-2) measurements after stimulation with 10µM ATP/UTP in absence of extracellular Ca2+ showed that P2Y2 is responsible for inducing intracellular Ca2+ signaling in mDCT cells. Transient transfection of mDCT cells with P2Y2 siRNA selectively inhibited (+/- 83%) the P2Y2 mRNA and resulted in an 85% inhibition of intracellular Ca2+ release. UTP, as well as ATP stimulation induced a 40% decrease in NCC mRNA level, an effect that was blocked after P2Y2 silencing. We next used cytoplasmic or nuclear PV mutants that chelate Ca2+ in specific cell compartments, in order to test whether variations in the intracellular Ca2+ levels may control NCC transcription. The decrease in NCC mRNA upon nucleotide stimulation was preserved in mDCT cells overexpressing cytosolic PV mutants, whereas it was totally abolished in cells overexpressing PV restricted to the nucleus. We conclude that (1) the P2Y2 receptor is essential for the intracellular Ca2+ signaling induced by ATP/UTP in mDCT cells; (2) the P2Y2-mediated signaling downregulates the expression of NCC and (3) regulation of NCC expression is influenced (or depends from) by high nuclear Ca2+ status. These data give new insights into the regulation of NaCl reabsorption in the distal nephron and the influence of purinergic signaling in the kidney.

Most patients with Gitelman syndrome (GS) are compound heterozygous. However, up to 30% of GS patients carry only a
single mutant allele, and a normal SLC12A3 screening is also observed in a small subset of patients. Locus heterogeneity could explain the lack of detection of mutant SLC12A3 alleles in GS patients. The renal phenotype of the parvalbumin knockout mice pointed to PVALB as a candidate gene for GS in SLC12A3 negative cases. PCR and direct sequencing of PVALB was performed in 132 GS patients in whom only one (N=53) or none (N=79) mutant SLC12A3 allele was found. The possible interference of biallelic SNPs on normal transcription or normal splicing was investigated. Genotyping of 110 anonymous blood donors was performed to determine the allelic frequency in normal population. No sequence variants resulting in amino acid substitution or truncated protein within the PVALB gene were found in the 264 chromosomes tested. Ten biallelic SNPs, including six novel, were identified: five in the 5' UTR, none of them affecting predicted regulatory elements; three in the coding region, without alteration of the consensus splice sites; and two in the 3'UTR. The observed allelic frequencies did not differ significantly between GS patients and controls. Thus, our results strongly suggest that mutations in PVALB gene are not involved in GS patients who harbour a single or no mutant SLC12A3 allele.

4-1-3 Search for large deletions in SLC12A3.

Because 18 to 40% of suspected GS patients carry only one SLC12A3 mutant allele, large genomic rearrangements may account for unidentified mutations. Here, we directly sequenced genomic DNA from a large cohort of 448 unrelated patients suspected of having GS. We found 172 distinct mutations, of which 100 were unreported previously. In 315 patients (70%), we identified two mutations; in 81 patients (18%), we identified one; and in 52 patients (12%), we did not detect a mutation. In 88 patients, we performed a search for large rearrangements by multiplex ligation-dependent probe amplification (MLPA) and found nine deletions and two duplications in 24 of the 51 heterozygous patients. A second technique confirmed each rearrangement. Based on the breakpoints of seven deletions, nonallelic homologous recombination by Alu sequences and nonhomologous end-joining probably favor these intragenic deletions. In summary, missense mutations account for approximately 59% of the mutations in Gitelman's syndrome, and there is a predisposition to large rearrangements (6% of our cases) caused by the presence of repeated sequences within the SLC12A3 gene.

WP 4-2 Pseudohypoaldosteronism type II (PHA2) or Gordon syndrome

Pseudohypoaldosteronism type II (PHA2) is due to mutations in two WNK kinases, WNK1 and WNK4. Studies in Xenopus oocytes revealed that PHA2 could thus result either from loss-of-function mutations of WNK4, responsible for the loss of NCC inhibition, or gain-of-function WNK1 mutations, which inhibit WNK4 activity and activate NCC. Furthermore, WNK1 and WNK4 stimulate clathrin-dependent endocytosis of the K+ channel ROMK1, and PHA2-causing mutations of WNK4 increase the endocytosis, thereby inhibiting K+ secretion. Some of the effects of the WNKs are independent of their kinase function, suggesting that they are dependent on specific protein-protein interactions. In particular, a short (KS-WNK1) kinase-deficient WNK1 isoform, which is predominant in kidney, inhibits the full-length (L-WNK1) which itself is responsible for the WNK4 inhibition. Our aim in this task was to get more insight into the respective role of each WNK1 isoforms in the regulation of ion handling in the distal tubule and the pathophysiology of PHA2. In addition, several mice models were created aiming at the specific inactivation of the L or KS-WNK1 in the kidney.

4-2-1 Elucidation of the molecular mechanisms of PHA2-causing WNK1 mutations.

Large deletions in intron 1 of the WNK1 gene lead to the overexpression of WNK1 in the leucocytes of patients with PHA2. Phylogenetic and functional analysis of the minimal intron 1 deletion region identified one repressor and one insulator, potentially preventing interactions between the regulatory elements of L- and KS-WNK1. We have pursued the characterization of the insulator and found that it could bind CTCF, the main insulating element known in vertebrates. In follow-up studies, we will also be testing the implication of methylation in the regulation of the differential expression of the two WNK1 isoforms; preliminary experiments suggest that the regulatory elements of KS-WNK1 expression are methylated in cells that do not originate from the distal convoluted tubule.

4-2-2 Dissection of tissue-specific role of the WNK1 isoforms.

In vitro studies showed that the ubiquitous isoform (L-WNK1) and the renal kinase-defective isoform (KS-WNK1) of WNK1 regulate a number of Na+ and K+ transport mechanisms. In order to define the roles of WNK1 isoforms in vivo in the kidney, we have generated a knock-out mouse model allowing the tissue-specific inactivation of all WNK1 isoforms or the complete inactivation of KS-WNK1 only. The characterization of the inactivation of KS-WNK1 resulted, as expected, in an increased expression and phosphorylation of the thiazide-sensitive Na-Cl cotransporter NCC in WNK1+/?i1 mice while that of the epithelial sodium transporter ENaC was decreased. Na+ and K+ balance was affected as evidenced by increased diastolic
blood pressure, decreased urinary aldosterone level and modified K+ channels expression. Our data therefore showed that KS-WNK1 is an important regulator of Na+ and K+ transport in the distal convoluted tubule. In addition, our study suggested that NCC activation is not sufficient to induce the development of PHA2. We are currently comparing the two models in order to further characterize their differences which could help unravel the pathophysiological mechanisms of PHA2.

In addition to the two tasks above, a new line of research has become more and more interesting during the course of the EUNEFRON project: the regulation of WNK1 by micro-RNAs (miRNA).

An increase in WNK1 expression leads to the development of hypertension in humans. Conversely, reducing the expression of the same gene by 50% leads to a decrease in blood pressure in mice. The characterisation of the mechanisms regulating WNK1 expression could therefore lead to the identification of new regulators of blood pressure. This is why we have developed an additional project which was not included in the initial proposal. Micro-RNAs are recently discovered small (21-24 nucleotides) non-coding RNAs which negatively regulate gene expression by enhancing degradation or blocking translation of their target transcripts. A computational analysis identified a potential target sequence for miR-192 and miR-215, two kidney-specific microRNAs (miRs), in the 3’ untranslated region (3’UTR) of the human WNK1 gene. It could be shown that miR-192, but not miR-215, is able to regulate WNK1 expression at the post-transcriptional level only. We also found that miR-192 expression is strongly inhibited by Na+ depletion, K+ loading and aldosterone infusion, three situations of elevated plasma aldosterone. In addition, L-WNK1 expression, while unaffected by any of the conditions at the RNA level, was increased at the protein level by aldosterone. Taken together, these results suggest that down-regulation of miR-192 could be involved in the stimulation of WNK1 expression by aldosterone in the kidney. More generally, this study has led to a new working hypothesis under which microRNAs could play a role in the regulation of ion transport in the kidney.

TOPIC 5: Disorders of the collecting duct
WP 5-1 Genetic renal disorders of systemic pH homeostasis
Collecting ducts (CD) consist of principal cells and intercalated cells (ICs), which play essential roles in fine-tuning the sodium/water and acid-base homeostasis, respectively. Identification of gene mutations in rare diseases has revealed several key proteins in these processes. AE1 mutations (SLC4A1) in patients cause different forms of red blood cell deformities or dRTA. Recessive or dominant dRTA depends on the residues mutated, but the underlying mechanism is poorly understood. Pendrin (SLC26A4) is mutated in Pendred syndrome, which is characterized by goiter, hypothyroidism and sensorineural deafness. Pendrin is prominently expressed in the kidney, but the renal function has not been investigated in Pendred patients. Pendrin mediates excretion of HCO3- during metabolic alkalosis in mouse, but it may also play a role in Cl-reabsorption, hence in blood pressure regulation. If so, Pendred patients should be protected against hypertension.

Mutations in SLC4A11 were found in Harboyan syndrome and non-syndromic corneal endothelial dystrophy, whereas SLC26A3 mutations cause congenital chloride diarrhoea and can lead to end stage renal disease. Both anion transporters are expressed in the CD, but their exact localization and renal function is unclear. The co-distribution in endosomes and the changed polarity and expression of H+-ATPase observed in IC from Dents disease patients suggest that CIC-5 and H+-ATPase interact to regulate urine acidification. Impaired urinary acidification occurs in 50% of Dents disease patients, but the role of CIC-5 in ?-type IC-mediated urine acidification has not been substantiated.

Our objective in this work package was to investigate the genotype-phenotype correlations and the molecular basis underlying dRTA with AE1 mutants, and study the (patho)physiological role and localization of newly-identified anion transporters, pendrin and AE1 interacting proteins. In addition, the role of pendrin as a potential target in hypertension and the role of CIC-5 in H+-ATPase-mediated urine acidification would be determined. Finally, NDI patients would be analyzed and tested to see whether V2R (anti)agonists are able to rescue their encoded V2R mutants.

5-1-1 Investigation of genotype-phenotype correlations for different AE1 mutant classes in dRTA.
Mouse models for two different human AE1 mutants that cause distal renal tubular acidosis have been generated by our collaborator Christian Hübner, Department of Genetics, University of Jena. Preliminary analysis of the mice revealed under normal conditions only very mild changes in renal function whereas acid-loading uncovered more severe inability to maintain acid-base homeostasis. The mutations are predicted from cell culture work to lead either the retention of the mutant protein in the endoplasmatic reticulum and Golgi or to traffic to the apical instead of the basolateral membrane. However, histological
analysis of the kidneys from heterozygous and homozygous mice failed to detect any such abnormalities but observed nearly normal localization of mutant proteins. However, the intercalated cells carrying the mutant proteins appeared hypertrophic suggesting that the mutant proteins affected overall cell function and cause compensatory changes.

Further experiments are now needed to examine these discrepancies between cell culture work and mouse in vivo data. These experiments include parallel expression of the mutant mouse and human AE1 proteins in cell lines, Electron microscopy of kidney with fine localization of wildtype and mutant AE1 proteins, and functional assays such as in vitro microperfusion of collecting ducts from the different mice.

It was not possible to obtain enough biopsy material from various AE1 mutant patients since most patients lacked a clinical indication that would have justified a biopsy. In total we collected biopsies from three different human mutations. However, one biopsy proved useless since it contained only scarred tissue (e.g. connective tissue). One biopsy showed major abnormalities in the localization of various marker proteins and the third biopsy is currently being processed for further analysis.

5-1-2 Role of interacting proteins in AE1 regulation.
We demonstrated that AE1 is also expressed at very low levels in the podocytes of the renal glomerulum. This localization was further corroborated by the fact that an interaction of AE1 with the podocyte specific protein nephrin could be detected by several methods including yeast-two-hybrid and co-immunoprecipitations. Moreover, we showed that mice lacking AE1 had subtle changes in their glomeruli supporting a role of AE1 in glomerular function. In addition, we found that these mice also had higher urinary albumin levels, an indication of a reduced selectivity of the glomerular filter. Similarly, mice with loss of nephrin had altered glomerular AE1 expression and localization, suggesting that the interaction between AE1 and nephrin is important for the subcellular positioning of the two proteins and in total for glomerular function.

5-1-3 Role of pendrin in the normal and diseased kidney.
In this task we wanted to explore the function of an additional pathway in blood pressure regulation by the kidney, the chloride-bicarbonate exchanger pendrin. Pendrin has been implicated into salt retention by the kidney, one major pathway that can cause hypertension.

We contacted several centers seeing patients with pendrin mutations (Pendred patients) in the Czech Republic, Italy, and Belgium) and will be able to conduct a limited series of experiments and collect various parameters related to kidney function and blood pressure regulation. The start of experiments has been very much delayed due to the fact that only few centers with Pendred patients exist and were difficult to identify, that many patients are still in their teens and therefore ethical restrictions apply, and that we first needed to conduct experiments in animals to identify conditions that would allow us to uncover consequences of reduced pendrin function in human patients. It is planned that the ethical protocols will be approved by early 2013 and we will be able to complete this task even after the end of the EUNEFRON project.

We also performed experiments in animals (mice) and on human kidney biopsies. In human kidney we further characterized the abundance, exact localization and subtype of pendrin expressing cells. In mice we examined the regulation of pendrin by acid-base status versus electrolyte intake as well as the regulation of pendrin and its functional counterpart AE1 during changes in dietary electrolyte balance.

5-1-4 Localization, regulation and function of SLC4A11 and SLC26A3 in health and disease.
This task was abandoned due to major technical limitations and because we found out that some of the data from the literature on which we had based the hypothesis were not reproducible.

In the case of SLC4A11 we failed to obtain antibodies suitable for localization and regulation studies. Without these studies, further experiments with the very rare patients with SLC4A11 were deemed inappropriate.

The literature had reported expression of SLC26A3 in human and rodent kidney based on immunohistochemical data. We approached this question using several highly specific antibodies that demonstrated strong reactivity in colonic tissue (the site of major expression) but failed to show any staining in human or rodent kidney. Second, we searched for SLC26A3 transcripts using several pairs of primers whose specificity and reactivity had been tested again on colonic tissue. Again, all these primers failed to detect SLC26A3 expression in human or rodent kidney. We concluded that this gene/protein is not expressed in rodent and human kidney.

5-1-5 Identification of the role of ClC-5 in urine acidification.
The ClC-5 protein mediates chloride-proton exchange, is localized to endosomes in the proximal tubule, the brush border...
membrane lining the proximal tubule as well as to cells in the collecting duct, mostly type A intercalated cells. The latter play an important role in urinary acidification. Patients with Dents disease suffer from low molecular weight proteinuria often combined with rickets and in a subset of patients also a distinct urinary acidification defect is observed. We used a mouse model lacking Clc-5 to examine its role in urinary acidification and maintenance of acid-base homeostasis. Clc-5 KO mice were examined under baseline conditions as well as under various protocols that increase urinary acidification in wildtype animals. We also employed detailed analysis of the subcellular localization of proton pumps and the distribution of the various subtypes of intercalated cells along the collecting duct together with microperfusion experiments measuring directly proton pump activity in intercalated cells. The results demonstrate that the absence of Clc-5 impairs the renal capacity to respond to an acid-load and to acidify urine. The distribution of proton pumps suggests that the defect is localized mostly in the early collecting duct whereas the late collecting duct (e.g. medullary collecting duct) tries to compensate for defective urinary acidification upstream. Microperfusion experiments support this interpretation showing rather enhanced rates of proton pumping in the medullary collecting duct.

Ongoing experiments aim to examine the consequences for bone health and to understand the impact on phosphate homeostasis.

WP 5-2 Genetic renal disorders of systemic water homeostasis

Our previous studies had revealed that many V2R mutants in NDI can generate cAMP after AVP binding. As all these mutants were misfolded and retained in the ER, their inability to reach the cell surface was the main reason for causing NDI. One hypothesis was that cell-permeable V2R antagonists (CPAns) may form a pharmaceutical therapy, because most V2R mutants are rescued in their cell surface expression by CPAns at clinically-relevant concentrations, after which CPAns can be displaced and the V2R mutant activated by AVP. Indeed, treatment with V1R CPAns relieved NDI in patients to some extent. Prior to the start of EUNEFRON we discovered that FDA-approved cell-permeable V2R agonists (CPAs) directly activate ER-retained V2R mutants, which precludes restored transport and displacement by vasopressin. It was unclear, however, whether the V2R CPA(n)s show V2R mutant selectivity and whether they can relieve NDI in patients. We also planned to develop the use of lentivirus infection as an alternative tool to establish the physiological relevance of selected genes in CD cells in vivo, as an effort to overcome some limitations of the transgenic mouse approach.

5-2-1 Recruitment, phenotyping and genotyping of NDI patients.

One of the main advantages of a European network on rare renal disorders is the recruitment of patients with novel mutations in a disease. Through our contacts, 20 new NDI patients were recruited, registered, and phenotyped for V2R or AQP2 mutations.

Remarkably, all 20 newly recruited patients had mutations in the V2R gene. Further studies on these mutations may reveal their cell biological cause and whether their diseases may be treatable with any of the treatments available now or in the future.

5-2-2 Rescue of V2R mutant protein in vitro and in vivo.

Four cell-permeable agonists (CPAs) from three different companies were obtained. Dose-response analyses revealed that all four CPAs were 10-fold less potent to activate the human V2R than dDAVP, but had a similar maximal activity. All four CPAs were tested on ca. 30 missense V2R mutants in NDI stably-expressed in MDCK cells. It appeared that nearly 80% of these V2R mutants were intrinsically-functional (could generate cAMP in response to vasopressin) and that all these V2R mutants were activated by the CPAs to generate cAMP, without changing their intracellular location, which was corroborated by the absence of any change in maturation. In contrast, treatment with V2R antagonists rescued the cell surface expression of the same V2R mutants, which was underscored by a switch of these mutants from immature to mature glycosylated forms. Treatment with dDAVP or the three CPAs VA88, VA89 and OPC51 revealed a clear mutation-specific cAMP response (similar for 5 mutants; VA88>VA89=OPC51 for 6 mutants; VA88>VA89>OPC51 for 5 mutants), which was independent of the location of the mutation in the protein or the extent of retention. Two mutants (L44F, V88M) showed a significantly-reduced response to VA88 compared to dDAVP, which could be explained by mutation-specific effects on the ligand binding. Despite mutant-specific differences in the response to the three CPAs, VA88 yielded the highest activation for most V2R mutants. Compound Y has a similar profile as VA88. These data indicated that the VA88-relative VA483 and Y, which are in clinical phase II for nocturnia, are the most-promising CPAs to develop into a medicine for NDI due to V2R misfolding.

The compounds, however, have not been released for clinical studies, but are available for studies in mice. Therefore, to allow
testing the CPAs in vivo, milestone M5-2.4 was changed for M-2.5 to determine the relief of NDI by CPAs in a conditional V2R mutant knock-in mouse model. The mouse model has been designed and generated by ICS in France. Hemizygous (male) V2R knock-in mice show signs of a disturbed urine concentrating defect, indicating that the homologous recombination worked out. At present, the V2R mutant KI mice are breed with Ubiquitin-CRE to allow tamoxifen-induced deletion of the wild-type V2R gene and expression of the V2R mutant protein.

5-2-3 Development of a technique for fast generation of in vivo collecting duct disorder models.

The objective of this task was the development of a technique for fast generation of in vivo collecting duct disorder models. First, we optimized the expression of AQP2-containing lentiviral constructs. Based on other publications and information from a lentivirus expert lab, sufficient titers of CMV-green-fluorescent protein (GFP) viruses were produced. Following urethral or parenchymal injection of mice with these viruses, however, GFP expression was not observed at 7 or 28 days following injection. Troubleshooting revealed that expert-induced quantification did not work out and that the titers were 100-1000 too low. Based on these data, the titers were optimized for AQP2-AQP2 construct containing viruses. With the AQP2-GFP construct viruses, clear dDAVP-induced expression of GFP was obtained following infection of collecting duct cells in vitro, indicating that proper expression of GFP (and indirectly of AQP2 from the AQP2-AQP2 construct) was obtained. Following generation of high titers of AQP2-AQP2 and CMV-AQP2 constructs, AQP2-F204V NDI mice were injected via the urether and parenchymally. Within 5 days, however, all virus-injected mice died. Considering the difficulty and laborious effort to generate high-enough titers of the desired viruses, the difficulty to determine their titers, the costs to generate these viruses and the disappointing results obtained in mice, this task was ended.

5-2-4 Determination of the underlying mechanism why AQP2 mutations lead to Nephrogenic diabetes insipidus.

In general, mutations in the AQP2 gene leading to recessive NDI are misfolded and retained in the ER, usually resulting in severe NDI whereas AQP2 gene mutations leading to dominant NDI result in proteins that are properly folded and interact with wt-AQP2. Dominant NDI may be a bit less severe than recessive NDI. Until now, nearly all mutations causing recessive NDI are found in between the 1st and last transmembrane domain of AQP2, whereas all mutations causing dominant NDI are found in its C-terminal tail. AQP2 gene mutations causing dominant or recessive NDI AND of which the mutation locates differently from the above or which result in a different (sub)clinical phenotype were investigated to determine the underlying mechanism for their involvement in NDI.

Four AQP2 mutants in NDI were investigated. Patient 1 (case 1) was heterozygous for a novel AQP2-V24A mutation, who responded to minrin. Patient 2 (case 2) had a dominant inheritance of NDI and was heterozygous for a R254Q mutation. Patient 3 (case 3) with dominant NDI encoded AQP2-E258K, which interacts with wild-type AQP2 and is sorted to the multivesicular bodies. Patient 4 and 5 (case 4) encode AQP2-P262L in conjunction with AQP2-R187C (patient 4) or A190T (patient 5). AQP2-R187C and AQP2-A190T are classical misfolded mutants and do not interact with AQP2-P262L. Detailed molecular biology studies of each of these cases could resolve the molecular mechanism for three of them. Identification of the underlying mechanism for the involvement of mutants in a disorder is the first step for the rational design of a personalized treatment.

TOPIC 6: Registry and network

WP 6-1 Creation of a European Registry of Rare Nephropathies

Already before the start of EUNEFRON, since 2002, partner 2 had established a French network including information on 750 families affected by more than 10 different inherited tubulopathies, in collaboration with the national reference center for renal rare diseases (MARHEA, Necker Hospital, Paris). In addition, other networks existed in several countries involved in this project. Our aim in this task was to create a European Registry of Rare Nephropathies based on the harmonization between these centers, which would also be the basis for additional collaboration in human studies. Several sub-tasks were identified and addressed:

6-1-1 Standardization of the phenotyping.

For each subtype of tubulopathy, we have defined a minimal set of requirements. Phenotypic questionnaires with minimal requirements have been developed by the different centres involved. Questionnaires have been shared and analysed in order to define and unify the diagnostic criteria and phenotypic forms for each disease. At present, questionnaires containing minimal diagnostic criteria are available via the website. http://www.eunefron.org/pages/Catalogue_websummary.htm
Decisional algorithms have been developed for disorders that are genetically heterogeneous in order to orientate the diagnosis and gene tests to perform. Decision trees have been sent around through the EUNEFRON network. For each participant a link to the Orphanet website was created, referenced as diagnostic or research laboratories and we have created a link for the Network as a whole, using the disease catalogue available on the EUNEFRON website to share information about genes tested, techniques used for genotyping and referent physicians with a direct link to OMIM for each pathology.

6-1-2 Creation of specific databases.
For 8 different tubulopathies, specific databases have been built in Access Format. Each database contains more than 100 clinical and biological items as well as a repertoire of mutations detected.

One of the hurdles to be taken for being able to create databases with patient information was to obtain ethics approval. The technical document for the declaration to the French ethical committees has been presented on to ethical committees involved and we have obtained the agreement of the French person protection committee (deliberative function), the Consultative Committee for the Treatment of Research Information in the health field (advisory function) and from the French data protection authority (advisory and consultation function about the data treatment). An information note and a specific informed consent, validated by French ethics committees, were created and are available upon request.

After obtaining ethics approval, we started with the Gitelman Syndrome database because 3 of the 8 partners in the EUNEFRON consortium are actively involved in diagnosis and clinical research on this disease, and are responsible for the follow-up of a large and increasing cohort of genetically confirmed patients (France: 550, Belgium: 150, Netherlands, 130 patients). Each countries dataset was created independently and the patient is identified by a single number delivered automatically by the software program. Then the datasets from the 3 centers was merged. For Gitelman and Bartter syndromes, data from more than 350 patients have been entered. Similarly, for Dent syndrome data on 60 patients have been entered.

For the disease Familial Hypomagnesaemia with hypercalciuria and nephrocalcinosis, data concerning 23 French families have also been processed and 18 German families will be added. This database has been constructed in a slightly different way, using the OB3 language to allow access and maintenance of patient data from a distance. For each pathology, patient files have been studied and data selected according to the database items (biological and clinical data at diagnosis and for each visit) to facilitate data entry.

6-1-3 Analysis of the characteristics and natural course of rare nephropathies.
From the questionnaires (6-1-1) and based on our experience with patients, we have created a list of possible complications for each tubulopathy. For each visit by the patient, the follow-up form will be filled using selected items. A statistical analysis plan based on the study protocol and depending on the number of patients and data available will help us to analyse the large data set collected. The crossed data will help us to get new insights into the molecular effects of each subtype of mutation regarding to age and sex, to reveal phenotype-genotype correlations and also get new insights about the natural course of each disease (appearance of end stage renal failure and other complications).

Recruitment of large cohorts at the national and European level, collecting phenotypic characteristics at the time of diagnosis and during the follow-up will help us get insights into the molecular effects of each subtype of mutation.

6-1-4 Development of the UK cystinosis registry.
Cystinosis is an extremely rare genetic disorder (1 in 150,000 births) causing kidney tubular damage in the first year of life with severe fluid, electrolyte and mineral losses leading to progressive kidney failure, poor growth and early death.

Cysteamine is a medicine used to treat cystinosis but hasn’t been subjected to a randomised controlled trial to assess its efficacy.

We have established a secure, web-based database for cystinosis, embedded within the nationally accredited and widely used CEMARA program. The CEMARA program contains an information system, called "Decision Support Information System for Rare Disease reference centres" (CEMARA) and the Service of Biostatistics and Medical Informatics is based at the Necker Institute in Paris (Necker SBIM). This system allows the production and recording of personal information relating to patients with rare diseases and the use of consolidated predefined data in the form of indicators, figures and dashboards on the one hand and specific studies on the other.

We have collated data from CEMARA incorporating patient information from patients in Paris and Louvain, with data from Rome...
and from the UK, a total of 312 patients. This is, to date, the largest data set for this condition and consists of patients born in the 1970s through to 2010. Establishing this data set allowed us to analyse the progress and effect of treatment in cystinosis. We showed that the kidney survival (i.e. time before the patient needs kidney dialysis or transplantation) is significantly improved for patients born in 1980s and 1990s compared to those born in the 1970s. The evidence from the EUNEFRON project is strongly supportive of the efficacy of cysteamine in improving the kidney survival in cystinosis.

6-1-5 Dissemination of knowledge.

We have collected a repertoire of SLC12A3 gene mutations: Excel file recording 320 literature mutations to be shared on the website and throughout the scientific community. This work is also in progress for CLCNKB, SLC12A1, KCNJ1 and BSND, genes involved in the Bartter syndromes and will continue also after the EU funding for Eunefron has run out. As can be seen in the list of dissemination activities, Eunefron members have been very active in disseminating the results of the consortium to the scientific community. Some specific examples for the dissemination of the databases mentioned above are:

On 2008, December 6th, the French reference centre for rare renal diseases, MARHEA and the French association of patients with Insipidus Diabetes (AFDI) organized a conference with the participation of local and international specialists.

On 2009, January 19th, Rosa Vargas-Poussou, Anne Blanchard, Olivier Devuyst and Xavier Jeunemaitre, took part of the annual symposium of the French National Tubulopathies Network, presenting the EUNEFRON program and recent advances.

On 2009, June 13th, Rosa Vargas-Poussou presented the Euenefron network and its objectives during a public presentation organized by one of the three French centres of reference for rare nephropathies in Toulouse.

The annual meeting of the French Tubulopathies Network took place in Paris on February, 8th 2010 and the topic was the Bartter syndrome. See annex.

On 2011, May 14th, MARHEA (centre de reference des maladies Rénales Héréditaires de l’adulte et de l’Enfant) and AIRG (Association pour l’Information et la Recherche sur les maladies Rénales Génétiques) France and Belgium networks organized a meeting for patients and families with Gitelman and Bartter syndromes. Participation of Olivier Devuyst, Rosa Vargas-Poussou and Anne Blanchard for clinical and genetics aspects, and Diana Kahila for databases

WP 6-2 Creation of a European Network of Genetic Laboratories

The genotyping of rare nephropathies is available only in a few number of genetic laboratories, and thus inaccessible to most of physician and patients. The main reasons are the rarity of these diseases, the relative difficulty to analyze the gene(s) involved and the cost of the genotyping procedure. In this task we have aimed to increase the accessibility of genotyping platforms among EUNEFRON partners by providing information and contact details of the researchers involved. The organization of genotyping at a European level will provide several advantages: access to the laboratories specialized for each type of rare nephropathy; exchange and improvement of molecular diagnostic procedures; decreased costs and delays for reliable results; sharing of expertise in interpreting the results; and identification of negative cases that may provide opportunities for the identification of new genes.

6-2-1 Organization of genotyping across European laboratories.

On the EUNEFRON website we have installed an overview of all the rare genetic kidney diseases for which genetic testing is available with one or more EUNEFRON partners. Links to information on the disease and contact details of the researchers involved are being provided. Patient data collected in this manner is also included in the databases created by EUNEFRON (if consent was given).

In addition to organizing genotyping access, we have also developed a clinical utility gene card (CUTG) for the specificity/sensitivity of genetic analysis in Gitelman syndrome. CUGCs are disease-specific guidelines regarding the clinical utility of genetic testing. Clinical utility refers to the ability of a genetic test to significantly affect the clinical setting and patient outcome. CUGCs cover all elements relevant for assessing risks and benefits of genetic test application. Due to their clear and concise format, they enable quick guidance to all stakeholders, including clinicians, geneticists, referrers, service providers and payers.

6-2-2 Data collection and capture and biobanking.

For the creation of databases for specific diseases, please see section 6-1 above. During the EUNEFRON project, data and samples of patients was continuously collected on rare nephropathies in all partner centers according to the proper ethical procedures. Of note is the large collection of data collected at the French Institute of Health and Medical Research, Paris,
Hôpital Européen Georges Pompidou (HEGP) for a subset of hereditary kidney diseases, which contains about 2300 DNA samples and urines and plasma according to the national clinical trial procedure GITAB. According to this procedure, before performing a genetic test, written consent is obtained from the patient. The patient can choose to give his consent for diagnosis and/or further research studies. DNA samples are stored depending on the consent of the patient willing and only after signing of the informed consent form. Plasma and urines have been collected through the French national clinical trial GITAB (cross over open label study), which main objective is to test the efficacy of indometacin compared to potassium sparing diuretics (amiloride or eplerone) or magnesium and potassium supplementation alone in patients with genetically proven Gitelman syndrome.

This was done for 10 of the rare nephropathies studied in EUNEFRON and is of huge significance in phenotype-genotype correlation studies.

6-2-3 Analyses of genotype-phenotype relationships.
Throughout the consortium, more than 20 rare nephropathies have been investigated. In many cases, these studies included the analyses of phenotype-genotype correlations (for example, see 2-2-3, 2-4-1, 3-1-3, 3-2-1, 4-2-1, 5-1-1 and 5-2-1). For several of these studies, this has resulted in an improved understanding of clinical sub-phenotypes that depend on the (type of) mutation involved and to scientific publications, most recently for Gitelman syndrome.

Near to completion is also the analysis of the international cystinosis data collection, which combines data from 4 EUNEFRON partners as well as from one external collaborator.

6-2-4 Identification of families that need further genetic investigation.
A substantial proportion of patients with rare tubular disorders have negative genetics results that may correspond to either a limited sensitivity of the genetic test, or the presence of unidentified genes. Identifying and analyzing such patients is often the route to finding thus far unknown disease genes. The identification by EUNEFRON researchers of CNNM2 as the gene responsible (when mutated) for hereditary magnesium wasting is an excellent example of this procedure.

We have selected patients with Gitelman’s syndrome, with NDI, and with isolated hypomagnesemia with clear disease phenotypes but without mutations/deletions in the known disease genes. For Gitelman syndrome, this means no or only a single heterozygous mutation or deletion in SLC12A3 or CLCNKB. For NDI, this means no mutations in either the AVPR2 gene or the AQP2 gene. For isolated renal hypomagnesemia, this means no mutation in any of the known disease genes (SLC12A3, Claudin genes, TRPM6, FXYD2, EGF, and the recently identified CNNM2).

Although their analysis will be carried out after the EUNEFRON contractual time has run out, these patients will be subjected to exome sequencing in order to find new disease genes.

Potential Impact:
TOPIC 1: Disorders of the podocyte
WP 1-1 Feto-Maternal Allo-Immune Glomerulopathies (FMAIG)
1-1-1 Recruitment and phenotyping of new FMAIG families.
Alloimmune antenatal MN due to NEP antibodies is a severe disease, with threatening renal complications for the neonates. Our results point to a role for environmental factors in the pathogenesis of membranous nephropathy. Although renal function improves in postnatal life, the immunologically mediated antenatal nephron loss may lead to chronic renal failure detected during adolescence. Some of these patients will need renal replacement therapy with substantial economic healthcare burden.

It is therefore essential to identify families at risk of alloimmune MN to avoid these complications.

1-1-2 Mapping of NEP epitopes and identification of additional antigens involved in FMAIG.
The delineation of pathogenic epitopes on the NEP protein will have a major impact on diagnosis and therapy of alloimmune mediated neonatal MN. Elimination or neutralization of circulating maternal anti-NEP antibodies early in pregnancy is needed to prevent the development of neonatal MN.

Secondly, our findings also indicate that the repertoire of antigens involved in MN may include nonglomerular antigens and point to environmental factors such as food antigens as potential cause of MN. A patent for diagnostic tests of BSA-related MN has been submitted (Early childhood membranous nephropathy due to cationic bovine serum albumin). From a clinical point of view, a diagnosis of BSA-related MN should be considered in young children with MN, which should prompt a search for BSA in immune deposits. If BSA is detected, eliminating this protein from the diet could be beneficial.
1-1-3 Establishment of an experimental model of FMAIG.
A genetically determined predisposition to adult idiopathic MN is now well established. Based on our GWAS data and our studies on recurrent MN, we hypothesize that sequence variations in PLA2R1 and HLA-DQA1 could play a critical role. The sequencing studies that we are planning will help further delineate the molecular mechanisms and triggering events of the disease, both in native and grafted kidneys, which will hopefully result in excluding donor kidneys at high risk of recurrence in patients with PLA2R1-related MN on native kidneys.

1-1-4 Analysis of podocyte and PT cell alterations induced by anti-NEP antibodies.
Due to the early stages of this project we cannot yet estimate its potential impact but we hope to provide a more extensive description of the cellular processes causing the disease, which may lead to a new therapeutic approach based on specific tolerization induced by epitope-driven therapy.

1-1-5 Development of assays for circulating nephritogenic antibodies.
Because future pregnancies in NEP-immunized mothers are at high risk for the children, the two new assays of circulating pathogenic antibodies during pregnancy which we have developed, will have a major impact on the monitoring and treatment modalities.

WP 1-2 Fabry disease
1-2-1 Analysis of renal biopsies for the expression of α-Gal A and reabsorbed proteins.
The results of these studies will allow us to evaluate the progression of disease related to enzyme treatment and related to the mutation of the individual patients. Our main goal for these studies was to be able to characterize mutations in such a way as to be able to determine for which patients to institute ERT at an early age and for which patients treatment should be delayed.

1-2-2 Uptake of α-Gal A in glomerular podocytes.
Our main goal for these studies was to be able to characterize the uptake mechanism for the recombinant enzyme in order to improve enzyme replacement treatment of Fabry patients. Indeed we found two new receptors involved in uptake of the enzyme which certainly will enhance potential development of new modifications of recombinant enzyme.

WP 1-3 Mechanisms of proteinuria and disease progression in genetic diseases of the podocyte
1-3-1 Studies on XLAS dogs.
We have characterized the effects of the initial glomerular defect on the molecular components of the renal tubular endocytic machinery, their role in protein and vitamin metabolism, and their contribution to protein overload induced nephrotoxicity in XLAS dogs. The new insights into the development of tubular and interstitial defects will also be relevant to a number of other renal diseases.

1-3-2 Studies on XLAS patients.
The insights gained from the studies on XLAS dogs have enabled us to study the role of the renal tubular endocytic machinery with regard to vitamin metabolism. Further analysis of the cohort of patients to study potential vitamin deficiencies originating from the renal disease will be essential in order to translate these finding into practical clinical applications.

1-3-3 Role of endocytosis in a mouse model of FSGS.
These studies substantiated the effects of an initial glomerular lesion and show that glomerular proteinuria induces pathways in proximal tubule (PT) cells that lead to oxidative stress, cellular dedifferentiation and proliferation resulting in a generalized PT dysfunction as evidenced by the presence of renal Fanconi syndrome which occurs rapidly after podocin inactivation. The better understanding of the disease pathways in mouse will facilitate better understanding and options for treatment in humans.

1-3-4 Studies in epithelial and fibroblasts co-cultures.
Like other projects under Task 1-3, these studies substantiated the effects of the apical exposure of PT cells to an excess of albumin, leading to novel insights into the contribution of PT cells to protein overload induced nephrotoxicity. These results reveal new insights into the development of tubular and interstitial defects relevant to a number of renal diseases in general.

TOPIC 2: Disorders of the proximal tubule
WP 2-1 Cystinosis
2-1-1 Study of PT protein reabsorption in cystinosis.
Based on the studies performed on CTNS-KO mice and additional functional studies, we have been able to propose a model of proximal tubular damage in cystinosis. This animal model is the only animal model available for cystinosis and represents an invaluable model to study cystinosin function and test emerging therapies. It has been (or is in the process of being) provided to numerous research groups all around the world (6 in Europe and 8 outside Europe) and to two SMEs.

2-1-2 Study of the relation between cystine accumulation, apical PT transport, oxidative stress and overall lysosomal function. This study has involved a proteomics approach to find interactors of cystinosin and the analysis of both cellular and animal models including the application of the cystine depleting drug cysteamine. Cysteamine was proved to be a potent anti-oxidant increasing intracellular glutathione levels and restoring intracellular redox state. Cysteamine, however, is unable to restore defective apical reabsorption. Searching for novel therapies for improving sodium-dependent proximal tubular reabsorption and receptor-mediated endocytosis appears a promising research strategy for the near future.

WP 2-2 Imerslund Gräsbeck disease

2-2-1 Identification of the patients with cobalamin deficiency. Both the search for and the identification of additional patients has increased the international visibility of this disease. The insights in the disease processes involved will increase our general understanding of endocytic processes in the proximal tubule of the kidney, as well as compensatory mechanism in more distal nephron segments.

2-2-2 Analysis of urinary protein excretion pattern and its relevance for treatment including vitamin supplement treatment. The characterization of the proteinuria will be of further relevance for diagnosis and for the treatment including vitamin supplement treatment. Especially when combined with the results of genotype-phenotype correlations, personalized treatment may be possible in the future depending on the needs of the individual patient.

2-2-3 Comparison of genotype and clinical phenotype. The studies mentioned under 2-2-2, especially when combined with the genotype-phenotype correlations which resulted from this task, will be of great importance for family advice and treatment of patients. The mouse KO studies have added significant information concerning the IGS disease and furthermore towards the general understanding of proteinuria mechanisms. The development of the mouse model for proximal tubule endocytic dysfunction will be of great value in further studying this process.

WP 2-3 Maturity Onset Diabetes of the Young (MODY 3)

2-3-1 Transcriptional regulation of PT transporters by HNF1a. These studies have documented the transcriptional regulatory network mediating apical transport in proximal tubule cells and provided new insights into adaptive mechanisms regulating PT function in the kidney. They also showed that low-molecular-weight proteinuria can be a marker of PT dysfunction in a monogenic form of inherited diabetes (MODY3).

2-3-2 Adaptation to oxidative stress in rodent models of PT dysfunction. The observations we have made in this task suggest that generalized PT dysfunction due to the loss of CIC-5 is associated with increased cell proliferation, dedifferentiation and oxidative stress. Our in vitro studies in PT cells support the hypothesis that CAIII production may play a role in the response to oxidative damage. The selective detection of CAIII in simple urine samples of mice and patients lacking CIC-5 suggests that it may represent a useful biomarker of renal Fanconi syndrome and we have obtained a patent for CAIII as a diagnostic biomarker of PT dysfunction.

WP 2-4 Hereditary Angiopathy with Nephropathy, Aneurism and Cramps (HANAC)

2-4-1 Recruitment, phenotyping and genotyping of new HANAC families. Because COL4A1 mutations localized in the C-terminal half of the protein outside the CB3 domain are responsible for a distinct clinical phenotype with severe small vessel brain disease sometimes associated with eye defects, but without muscle symptoms or renal involvement, we speculate for a phenotype-genotype correlation in COL4A1-related diseases, even if the number of patients with COL4A1 mutations remains limited to date. Moreover, this observation suggests that the HANAC phenotype may be due to defective interactions between COL4A1 and integrins expressed at the cell surface. In addition to the further improvement of our understanding of this disease entity, our results may lead to a more efficient determination of HANAC sub-phenotypes and facilitate more targeted treatment.

2-4-2 Studies on trafficking and cell toxicity in fibroblasts of HANAC patients. Our results in this task indicate that HANAC COL4A1 mutations may be associated with defects in extracellular secretion of the mutated protein and/or be responsible for a higher susceptibility to ER stress and cell apoptosis. Thus, drugs interfering with
ER stress should be tested to prevent these kinds of cellular damage.

2-4-3 Establishment and study of animal models harbouring COL4A1 and COL4A2 mutations.

For this task, two knock-in mouse strains bearing two distinct Col4a1 mutations responsible for HANAC syndrome in human were successfully generated. The renal phenotype of our Col4a1 HANAC mouse models highlights the crucial role of the COL4A1 protein during glomerular and collecting duct embryogenesis and suggests that HANAC patients present a glomerulocystic kidney disease. These models provide new insights in the disease and provide a means for developing future disease treatment strategies.

TOPIC 3: Disorders of the thick ascending limb of Henle’s loop

WP 3-1 Familial Hypomagnesemia with Hypercalciuria and Nephrocalcinosis (FHHNC)

3-1-1 Generation of CLDN-16 knock-down.

The original knock-down project was abandoned because it was published by competitors. Our alternative approach to analyse the CLDN-16 knock-out model has led to the identification of a previously unknown disease gene, CNNM2. Already, the first genetic tests for the screening of mutations of this gene in families with hereditary magnesium wasting are being offered by diagnostic labs, highlighting the significance of our finding.

3-1-2 Generation of inducible CLDN-16 KO mice.

Although we were able to generate the inducible CLDN-16 KO mice, they turned out not to produce any offspring, forcing us to abandon this project. We have focused instead on the important results obtained in the research on CNNM2 (see 3-1-1). The realization that the localization of the protein is on the blood side of the cells has forced us to review the previous conceptions of Mg handling in the kidney and will continue to improve our understanding of this system. Currently we seek to clarify if cnnm2 itself transports magnesium or if it is indirectly involved in this mechanism. Once the question of the exact function and structure of cnnm2 are solved, approaches for a pharmacological manipulation, thus treatment of patients can follow. Moreover, we hope to identify more families with mutations in cnnm2 in order to get better insights into the natural course of the disease and overall magnesium handling in humans.

3-1-3 Natural course of FHHNC patients.

The cohort of patients we have collected for this disease forms a unique resource for improving our understanding of the natural course of FHHNC and they have been implemented into an FHHNC database. The results of studying this dataset will help to achieve a more complete overview of the frequency and natural course of patients with FHHNC and may further improve their treatment.

WP 3-2 Familial Juvenile Hyperuricemic Nephropathy (FJHN)

3-2-1 Natural course and phenotype in patients with FJHN.

Our studies provided novel important findings on the natural course of FJHN with relevance for disease diagnosis and prognosis. Considering our data, we propose that screening for UMOD mutation should be advised for all patients with tubulo-interstitial nephritis associated with uricemia greater than the 75th percentile value corresponding to GFR and/or with familiar history of gout or renal disease. The ELISA method that was developed could significantly help the disease diagnosis.

3-2-2 Generation of a knock-in mouse model of mutant THP.

The TgUmodC147W mice produced in this project represent a unique model that recapitulates most of the features associated with FJHN. Our data clearly demonstrate a toxic function of uromodulin mutations and provide insights into the pathogenetic mechanism of the disease. Recent results highlight the importance of inflammation at the bases of renal damage in FJHN and provide a promising clue to design therapeutic approaches aimed at blocking disease onset and progression. We believe that results on TgUmodC147W mice could have broader relevance for other renal tubule-interstitial disorders and for the growing group of conformational diseases.

3-2-3 Cellular responses to mutant THP expression.

Our studies significantly contributed to gain insight into the molecular bases of FJHN. The cellular lines developed during this project have proven to be a good model, ideally complementing the in vivo one. Indeed, similar results on the induction of UPR and apoptosis upon uromodulin ER retention were collected in vitro and in vivo. Also, extracellular aggregation of mutant uromodulin was originally observed in MDCK cells and later confirmed in the mouse model.

These cell lines represent a unique tool that could help designing a therapeutic strategy through screening of small-molecule
libraries aimed at restoring mutant protein folding in the ER and its trafficking to the plasma membrane.

3-2-4 Role of CIC-5 in the trafficking of THP.

Our studies reveal that urinary levels of uromodulin are increased in the absence of CIC-5 both in mice and Dent disease patients. These results seem to rule out the possibility that uromodulin could play a role in the propensity of renal stone formation in Dent disease. Despite the fact that our cellular studies failed to demonstrate a direct role of CIC-5 in uromodulin trafficking, the association of increased urinary uromodulin with lack of CIC-5 function leaves the possibility open for a role of CIC-5 affecting uromodulin secretion that should be further explored in more differentiated cell systems, eg TAL primary cultures, on in animal models.

TOPIC 4: Disorders of the distal convoluted tubule
WP 4-1 Gitelman syndrome (GS)
4-1-1 Natural course and genotype-phenotype correlations in patients with GS.

Although limited by the relatively small number of severe cases, these data suggest that the combination of male gender with the presence of one allele of a splice defect that results in a truncated transcript or, less frequently, a nonfunctional intracellularly retained mutation could explain the clinical severity of Gitelman syndrome. These results gave new insight into the pathophysiology and molecular genetics of GS, and help to better understand the phenotype variation and gender-effect associated with the disease.

4-1-2 Role of parvalbumin and caveolin-1 in the DCT.

These studies investigated the molecular bases for the phenotypic heterogeneity in Gitelman syndrome, the regulation of NCC in the distal nephron (DCT segment), and the mechanisms that explain the failure to detect mutations in up to 40% of patients. Gitelman syndrome is probably the most frequent inherited tubulopathy. Based on a mouse model, we tested whether allelic variants in PVALB could be involved in GS patients who are simple heterozygous or negative for SLC12A3 mutations. Our negative results illustrate the limits of reverse genetics, in relation with inter-species differences in tubular functions and/or regulatory events. Further investigations will be necessary to evaluate the potential contribution of PVALB to the pathophysiology of human tubulopathies. Our results are relevant for the handling of NaCl and divalent cations, the action of diuretics, and the pathogenesis of bone mineralization.

4-1-3 Search for large deletions in SLC12A3.

The molecular analysis of a large cohort of 448 GS patients, including the first search for large-scale mutations, showed that, despite the high efficiency of direct genomic sequencing in detecting the vast majority of the SLC12A3 mutations found in this disorder, a complementary technique is necessary to achieve a high mutation detection rate, especially for those patients in whom only one mutation had been detected. We confirmed that MLPA is an efficient technique for analyzing large genomic rearrangements, which account for 76% of mutations detected in our patients with GS. Moreover, we showed that nonallelic homologous recombination by Alu sequences and nonhomologous end-joining are most likely to be responsible for intragenic deletions. Finally, we detected CLCNKB mutations in 3% and excluded mutations and large rearrangements of the SLC12A3 gene in 8% (n = 36) of our GS patients, which questions the clinical diagnosis of GS and raises the possibility of genetic heterogeneity in this inherited tubulopathy.

WP 4-2 Pseudohypoaldosteronism type II (PHA2) or Gordon syndrome
4-2-1 Elucidation of the molecular mechanisms of PHA2-causing WNK1 mutations.

We have been able to show that the minimal area of deletion of the first intronic region of the WNK1 gene which causes PHA2, contains a genetic repressor and an insulator. Our results will not only be important for better understanding how these mutations cause PHA2 in these patients but also more generally, provide another study-case for understanding genomic regulation of gene expression.

4-2-2 Dissection of tissue-specific role of the WNK1 isoforms.

Our data have shown that KS-WNK1 is an important regulator of Na+ and K+ transport in the distal convoluted tubule (DCT). In addition, our study suggests that NCC activation is not sufficient to induce the development of PHA2, as suggested by the previous characterization of a WNK4 mouse model.

We are currently comparing the two models in order to further characterize their differences which could help unravel the pathophysiological mechanisms of PHA2.
TOPIC 5: Disorders of the collecting duct
WP 5-1 Genetic renal disorders of systemic pH homeostasis

5-1-1 Investigation of genotype-phenotype correlations for different AE1 mutant classes in dRTA.
Two mouse lines were generated, R609H and L919X corresponding to relatively common AE1 mutations. We are exploring the mouse lines for features and clinical symptoms observed in patients carrying these AE1 mutations. The mouse models may thus become useful models to test small molecules that could act as chemical chaperones rescuing defective trafficking and function of mutant proteins thereby establishing novel and causative therapies for these patients. Several such molecules have been described in cell culture and will be tested by us along with related molecules.

5-1-2 Role of interacting proteins in AE1 regulation.
The identification of AE1 as a protein potentially impacting on glomerular function adds another player to the increasing number of genes and factors important for normal glomerular function. Normal glomerular function has been associated in many studies with overall kidney function as well as with general morbidity and mortality. The relevance of our findings for human patients remains to be investigated but may provide new clues to the pathogenesis of glomerular diseases.

5-1-3 Role of pendrin in the normal and diseased kidney.
Pendrin may be a major determinant of either acid-base status and/or salt retention and hence blood pressure. The latter has major clinical implications since pendrin may functionally couple if not even regulate sodium absorption by the epithelial sodium channel ENaC. ENaC has been found in many studies to be a strong regulator of blood pressure and is targeted by strong drugs including amiloride. Pendrin itself has not been pharmacologically targeted to date but may provide an attractive target for potent blood pressure lowering drugs. On the other hand, data from animal studies must be taken with caution since our preliminary data suggest that pendrin abundance may be much lower in human kidney than in rodent kidney. Clearly, our future experiments with Pendred patients may provide more insights.

5-1-4 Localization, regulation and function of SLC4A11 and SLC26A3 in health and disease.
This task was abandoned due to major technical limitations and because we found out that some of the data from the literature on which we had based the hypothesis were not reproducible.

5-1-5 Identification of the role of ClC-5 in urine acidification.
Our results in this task explain the occurrence of urinary acidification defects in some patients with Dents disease. Defects in urinary excretion are often associated with adverse consequences for bone health predisposing to osteopenia or osteoporosis. In the setting of Dents disease causing renal phosphate wasting and often low vitamin D3 levels due to defective renal vitamin D3 synthesis, urinary acidification defects add to the risk factors for low bone density. In fact, Dents patients often suffer from rickets that is classically treated with Vitamin D3 supplementations. In view of the urinary acidification defect, future clinical trials may test the addition of alkali supplements such as potassium citrate or potassium bicarbonate to alleviate the metabolic acid load. If successful, such a simple treatment could reduce the disease burden for Dents patients.

WP 5-2 Genetic renal disorders of systemic water homeostasis

5-2-1 Recruitment, phenotyping and genotyping of NDI patients.
All 20 newly recruited patients had mutations in the V2R gene. The patients are secured that an explanation for their disorder has been identified. Further studies on these mutations may reveal their cell biological cause and whether their diseases may be treatable with any of the treatments available now or in the future.

5-2-2 Rescue of V2R mutant protein in vitro and in vivo.
Cell-permeable agonists of the V2R may form the ideal therapeutic for patients of which their NDI is caused by misfolded, but intrinsically-functional V2R mutants. Based on our in vitro data, VA88 [483] and compound y are the best compounds to develop towards therapeutic for NDI patients. Our to be obtained studies in our conditional V2R mutant knock-in mice will reveal which compound relieves NDI in vivo the best.

5-2-3 Development of a technique for fast generation of in vivo collecting duct disorder models.
Development of a technique for fast generation of in vivo collecting duct disorder models would be great. Unfortunately, using injection of virus from the ureter and into the parenchyma, and subsequent infection, as tried here, the results do not support our idea that this methodology would be useful for this purpose.
5-2-4 Determination of the underlying mechanism why AQP2 mutations lead to Nephrogenic diabetes insipidus. Identification of the underlying mechanism for the involvement of mutants in a disorder is the first step for the rational design of a personalized treatment. Of three of the 4 cases investigated, the molecular cause of their NDI could be resolved.

TOPIC 6: Registry and network
WP 6-1 Creation of a European Registry of Rare Nephropathies
6-1-1 Standardization of the phenotyping.
The development of standardized phenotyping is a slow process but we have been able to set up a defined minimal set of requirements and have proposed questionnaires containing minimal diagnostic criteria for each subtype of tubulopathy. This is a first step to unifying standardization and phenotyping which will facilitate the easy merging as well as comparison of datasets in the future, resulting in stronger statistical analysis of disease and disease progression parameters.

6-1-2 Creation of specific databases.
Databases for 8 different tubulopathies have been built in Access format, each containing more than 100 clinical and biological items as well as a repertoire of mutations detected. Several of these databases have already been filled with actual patient information, for example the Gitelman database and the FHHNC database. These type of databases allow genotype-phenotype correlation studies as exemplified by a recent publication on FHHNC and will become more and more important to assess disease processes that develop over time, as well as comparisons between and determination of side-effects of treatments. Several databases have now been constructed on the OBD language which is better suited for long distance (digital) access and which will facilitate addition of new patients from colleagues all over the world.

6-1-3 Analysis of the characteristics and natural course of rare nephropathies.
In the future, we hope that the collection of described complications and genotype-phenotype correlations will become large enough to allow insights in the relation of certain genotypes and the development of those complications. Ultimately, this will lead to a better understanding of the disease processes and, equally important, to more personalized treatment.

6-1-4 Development of the UK cystinosis registry.
This collaboration has established the power and utility of sharing data in rare diseases. No single centre or even country could produce a dataset of this significance. In this respect, integrating European data across member states enables accurate assessment of the course and treatment effects in rare diseases.

The improvement in kidney survival in cystinosis over the last 3 decades demonstrated in this project, reinforces the efficacy of cysteamine and the need to start this early and at correct dosage. An approximate 50% prolongation of kidney survival has been achieved in this timeframe with consequent huge improvements in patient well-being as well as economic savings in later resort to costly renal replacement therapies.

The data on the effect of cysteamine on the commonly used biomarker in cystinosis (leucocyte cystine level) reflect wide variability in practice, casting some doubt on this marker and reinforcing that patients should be treated with full recommended doses of cysteamine.

6-1-5 Dissemination of knowledge.
The dissemination of the databases created during the Eunefron project is and will remain essential to its ultimate success. Only this way, can the collections of patient information continue to grow and produce the results they were designed for. Already during the project time dissemination of knowledge was actively pursued and will continue even after funding for Eunefron by the EU has run out.

WP 6-2 Creation of a European Network of Genetic Laboratories
6-2-1 Organization of genotyping across European laboratories.
Easier access to genotyping platforms on a European scope will benefit many patients and provide clinicians with additional possibilities to find the gene responsible for the disease in their patients. The on-going creation of clinical utility gene cards serves the same goal.

6-2-2 Data collection and capture and biobanking.
The continued data collection together with biological materials of patients is becoming more and more important for the correct determination of sub-phenotypes of diseases and for selecting the appropriate treatment. For this reason the development of standardized data capture, collection and biomaterial storage methods is of the utmost importance.
6-2-3 Analyses of genotype-phenotype relationships.
With the onset of omics technologies, personalized medicine is expected to become a feasible treatment option. For this, knowledge of genotype-phenotype relationships will be essential and the EUNEFRON consortium has made many contributions to this.

6-2-4 Identification of families that need further genetic investigation.
A substantial proportion of patients with rare tubular disorders have negative genetics results that may correspond to either a limited sensitivity of the genetic test, or the presence of unidentified genes. The identification of a new disease gene for hereditary magnesium wasting through this method, the EUNEFRON consortium has confirmed both the validity and the importance of this approach. The onset of exome sequencing now facilitates sequencing the whole exome for abnormalities in patients with an unknown cause of hereditary disease and many more disease genes are expected to be identified in the near future.

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Related information

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