Charles University in Prague

First Faculty of Medicine

Autoabstract of Ph.D. thesis

Molecular Basis of Hereditary Hyperuricaemic Nephropathies

Petr Vylet'al

Prague 2008

Doctoral Study Programmes in Biomedicine

Charles University in Prague
and the Academy of Sciences of the Czech Republic

Study Program: Molecular and Cellular Biology, Genetics and Virology

Program committee chairman: Petr Pikálek, Ph.D., Associate Professor

Supervising department: Institute of Inherited Metabolic Disorders and Center for Applied Genomics

Author: Petr Vyleťal, M.Sc.

Supervisor: Stanislav Kmoch, Ph.D., Research Assistant Professor

Opponents:
Doc. MUDr. Milan Jirsa, CSc.
Prof. MUDr. Ondřej Viklický, CSc.

Date of autoabstract distribution: .....

Date of defence: 23. 4. 2009 at 12:30 o'clock, Institute of Biology and Medical Genetics, University Hospital Motol

The thesis is available at the dean's office of the First Faculty of Medicine of Charles University in Prague.

Table of Contents

Souhrn

Familiární juvenilní hyperurikemická nefropatie (FJHN) a medulární cystické onemocnění ledvin typu 1 (MCKD1) a typu 2 (MCKD2) představují soubor autosomálně-dominantních tubulointersticiálních nefropatií charakterizovaných kombinací hyperurikémie, dnavé artritidy, progresivní renální insuficience a v některých rodinách také medulárních cyst. Fenotypové projevy těchto onemocnění nejsou konsistentní, vzájemně se překrývají a naznačují širší genetickou a alelickou heterogenitu. Jejich patofysiologie byla převážně neznámá. Předchozí studie lokalizovaly geny, podmiňující FJHN/MCKD, na chromosomy 16p11 a 1q21. Tato disertační práce byla primárně zaměřena na objasnění molekulární podstaty a mechanismů FJHN/MCKD. Za účelem naplnění tohoto cíle jsme se zaměřili na shromažďování a charakterizaci pacientů a rodin s FJHN/MCKD, identifikaci onemocnění podmiňujících genů, charakterizaci identifikovaných proteinů a jejich mutant a izolaci a charakterizaci interagujících partnerů nově identifikovaných proteinů. K tomu jsme použili a zavedli řadu molekulárně genetických, molekulárně biologických a biochemických metod. Shromáždili jsme jeden z největších souborů rodin postižených FJHN/MCKD na světě. Ve zhruba 26% těchto rodin jsme nalezli mutace v genu *UMOD* (kódující uromodulin) a různými přístupy charakterizovali 6 mutant uromodulinu. V jedné rodině jsme definovali nový genetický lokus na chromosomu 1q41, identifikovaly mutaci v genu *REN* (kódující renin) a různými přístupy charakterizovali mutantní renin. Ve zbylých rodinách jsme nebyli schopni prokázat genetickou vazbu k žádnému známému lokusu FJHN/MCKD. Na druhé straně jsme poskytli důkazy, že porušená biologie uromodulinu je společná pro geneticky heterogenní FJHN/MCKD a navrhli jsme několik patogenetických mechanismů, které by mohli vysvětlit vznik tohoto onemocnění. Nebyli jsme dále schopni identifikovat žádné interakční partnery uromodulinu za použití dvou různých přístupů. Praktickým výstupem této práce je rutinní selektivní prověřování stavů s hyperurikémií a unikátní, komplexní diagnostický servis pro tuto skupinu onemocnění prováděný na našem pracovišti.

associated kidney disease (UAKD) to chromosome 1q41. Kidney Int. 2005 Oct;68(4):1472-1482. **IF 4.927** (2005)

Vyleťal P., Kublová M., Kalbáčová M., Hodaňová K., Barešová V., Stibůrková B., Sikora J., Hůlková H., Živný J., Majewski J., Simmonds A., Fryns J. P., Venkat-Raman G., Elleder M. and Kmoch S.: Alterations of uromodulin biology: a common denominator of the genetically heterogeneous FJHN/MCKD syndrome. Kidney Int. 2006 Sep;70(6):1155-1169. **IF 4.773** (2006)

Vyleťal P., Hůlková H., Živná M., Berná L., Novák P., Elleder M. and Kmoch S.: Abnormal expression and processing of uromodulin in Fabry disease reflects tubular cell storage alteration and is reversible by enzyme replacement therapy. J Inherit Metab Dis. 2008 Aug;**31**(4):508-17. **IF 1.668** (2007)

Živná M., Hůlková H., Hodaňová K., **Vyleťal P.**, Kalbáčová M., Barešová V., Sikora J., Blažková H., Živný J., Ivánek R., Sovová J., Claes K., Matthijs G., Elleder M., Kapp K. and Kmoch S.: Preprorenin signal sequence mutation affecting renin biosynthesis and intra-renal RAS expression in a family with uromodulin associated kidney disease. Manuscript in preparation, 2008.

8.2 Publications unrelated to the thesis

Vyleťal P., Sokolová J., Cooper D. N., Kraus J. P., Krawczak M., Pepe G., Rickards O., Koch H. G., Linnebank M., Kluijtmans L. A., Blom H. J., Boers G. H., Gaustadnes M., Skovby F., Wilcken B., Wilcken D. E., Andria G., Sebastio G., Naughten E. R., Yap S., Ohura T., Pronicka E., Laszlo A., Kožich V.: Diversity of cystathionine beta-synthase haplotypes bearing the most common homocystinuria mutation c.833T>C: a possible role for gene conversion. Hum Mutat. 2007 Mar;28(3):255-64. **IF 6.473** (2006)

Abstract

Familial juvenile hyperuricemic nephropathy (FJHN) and medullary cystic kidney disease type 1 (MCKD1) and type 2 (MCKD2) are autosomal dominant tubulointerstitial nephropathies characterized by combinations of hyperuricaemia, gouty arthritis, progressive renal insufficiency, and in some but not all families, medullary cysts. The phenotypic expression of these diseases is inconsistent, overlaps and indicates broader genetic and allelic heterogeneity. Their pathophysiology was mainly unknown. Previous studies localized FJHN/MCKD genes to chromosomes 16p11 and 1q21. This thesis was primarily aimed at identification of molecular bases and mechanisms underlying FJHN/MCKD. To follow this aim, we focused on collection and characterization of FJHN/MCKD patients and families, identification of disease causing genes in affected families, characterisation of identified proteins and their mutated forms and the isolation and characterisation of interacting partners of newly identified proteins. We employed and established numerous molecular genetic, molecular biological and biochemical methods. We gathered one of the largest sets of families with FJHN/MCKD in the world. In about 26% of families we identified *UMOD* (uromodulin encoding) gene mutations and characterised by various approaches 6 uromodulin mutant proteins. In 1 family we defined new genetic locus on chromosome 1q41, identified the mutation in *REN* (renin encoding) gene and characterised by various approaches renin mutant protein. In the rest of families, we could not prove the genetic linkage to any of known or newly identified FJHN/MCKD loci. Nevertheless, we provided evidences that alteration of uromodulin biology is common to genetically heterogenous FJHN/MCKD and suggested several pathogenic mechanisms leading to the disease. We were unable to identify any uromodulin interacting proteins by two different protein-protein interaction approaches. As a practical consequence, we perform routinely the selective screening of hyperuricaemic conditions and provide unique, complex diagnostic service for this group of disorders.

1. Introduction

This work was focused on hereditary nephropathies associated with hyperuricaemia and gout, namely on familial juvenile hyperuricaemic nephropathy (FJHN) and nephronophthisis-medullary cystic kidney disease complex (NPH-MCKD). The introduction summarises the knowledge about these disorders until 2001 when I started to work on this subject.

FJHN (OMIM 162000)

This condition was first reported in 1960 by Duncan and Dixon [1]. Since then, tens of kindreds of various ethnic origin had been described in the literature by 2001.

The disorder is characterised by autosomal dominant inheritance with high penetrance, early onset hyperuricaemia due to urate underexcretion, gouty arthritis and renal insufficiency progressing to renal failure between 20^{th} and 40^{th} year of life. Hypertension may be present in later stages of renal disease and conversely accelerates the progression of chronic renal failure. Affected individuals require dialysis and later on renal transplantation. Proteinuria and abnormalities of urinary sediment are absent or minimal.

Renal manifestations of FJHN have features of non-specific interstitial nephropathy [2, 3]. Urate crystals were detected only in a fraction of examined biopsies [4, 5].

In management of clinical and biochemical symptoms, mainly the control of hyperuricaemia by allopurinol (xanthine oxidase inhibitor) alone or in combination with benzbromarone (tubular anion exchanger inhibitor) [6, 7] and the control of hypertension, if present, are followed.

The pathogenesis of FJHN, especially the basis of urate underexcretion and histological changes, was mainly unknown.

NPH-MCKD (OMIM 174000, 603860, 256100, 602088, 604387)

Medullary cystic kidney disease was first described by Thorn et al. in 1944 as "salt-loosing nephritis" [8]. By 2001, MCKD had been described in more than 50 families mainly from Europe and North America [9].

Reduced urine concentrating ability with salt wasting, preceding decreased GFR, can be the only early findings. Proteinuria is absent or minimal, urinary sediment is non-specific. Following substantial decrease in urine-concentrating ability, polyuria usually accompanied by polydipsia appears in early or later adulthood (average 28 years). Anemia, metabolic acidosis and uremia are related to progressive renal insufficiency. Hypertension is usual and often precocious, despite the presence of polyuria. The renal insufficiency usually slowly progresses to ESRD in the 3^{rd} to 5^{th} decade of life and affected individuals end up on dialysis and require renal transplantation [10].

Histological findings conform to diffuse tubulointerstitial nephritis and interstitial fibrosis. Tubular dilation is present and formation of cysts at the cortico-medullary border and/or in the medulla may be seen (in contrast to FJHN). Small and echogenic kidneys are observed.

42. Petermann R., Mossier B.M., Aryee D.N. and Kovar H.: *A recombination based method to rapidly assess specificity of two-hybrid clones in yeast.* Nucleic Acids Res **26**(9): p. 2252-2253, 1998.
43. Van Criekinge W. and Beyaert R.: *Yeast Two-Hybrid: State of the Art.* Biol Proced Online **2**: p. 1-38, 1999.
44. Serebriiskii I.G., Toby G.G., Finley R.L., Jr. and Golemis E.A.: *Genomic analysis utilizing the yeast two-hybrid system.* Methods Mol Biol **175**: p. 415-454, 2001.
45. Brown D.A. and Rose J.K.: *Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface.* Cell **68**(3): p. 533-544, 1992.
46. Rhodes D.C., Hinsman E.J. and Rhodes J.A.: *Tamm-Horsfall glycoprotein binds IgG with high affinity.* Kidney Int **44**(5): p. 1014-1021, 1993.
47. Rhodes D.C.: *Binding of Tamm-Horsfall protein to complement 1q measured by ELISA and resonant mirror biosensor techniques under various ionic-strength conditions.* Immunol Cell Biol **78**(5): p. 474-482, 2000.
48. Rhodes D.C.: *Binding of Tamm-Horsfall protein to complement 1q and complement 1, including influence of hydrogen-ion concentration.* Immunol Cell Biol **80**(6): p. 558-566, 2002.
49. Muchmore A.V. and Decker J.M.: *Evidence that recombinant IL 1 alpha exhibits lectin-like specificity and binds to homogeneous uromodulin via N-linked oligosaccharides.* J Immunol **138**(8): p. 2541-2546, 1987.
50. Sherblom A.P., Decker J.M. and Muchmore A.V.: *The lectin-like interaction between recombinant tumor necrosis factor and uromodulin.* J Biol Chem **263**(11): p. 5418-5424, 1988.
51. Welker P., Geist B., Fruhauf J.H., Salanova M., Groneberg D.A., Krause E. and Bachmann S.: *Role of lipid rafts in membrane delivery of renal epithelial Na+-K+-ATPase, thick ascending limb.* Am J Physiol Regul Integr Comp Physiol **292**(3): p. R1328-1337, 2007.

8. List of author's publications

8.1 Publications related to the thesis

Stibůrkova B., Majewski J., Hodaňonová K., Ondrová L., Jeřábková M., Zikánová M., **Vyleťal P.**, Šebesta I., Marinaki A., Simmonds A., Matthijs G., Fryns J. P., Torres R., Puig J. G., Ott J. and Kmoch S.: Familial juvenile hyperuricaemic nephropathy (FJHN): linkage analysis in 15 families, physical and transcriptional characterisation of the FJHN critical region on chromosome 16p11.2 and the analysis of seven candidate genes. Eur J Hum Genet. 2003 Feb;11(2):145-154. **IF 3.669** (2003)

Hodaňová K., Majewski J., Kublová M., **Vyleťal P.**, Kalbáčová M., Stibůrková B., Hůlková H., Chagnon Y. C., Lanouette Ch. M., Marinaki A., Fryns J. P., Venkat-Raman G., and Kmoch S.: Mapping of a new candidate locus for uromodulin-

29. Tamm I. and Horsfall F.L., Jr.: *A mucoprotein derived from human urine which reacts with influenza, mumps, and Newcastle disease viruses.* J Exp Med **95**(1): p. 71-97, 1952.
30. James P., Halladay J. and Craig E.A.: *Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast.* Genetics **144**(4): p. 1425-1436, 1996.
31. Stiburkova B., Majewski J., Hodanova K., Ondrova L., Jerabkova M., Zikanova M., Vylet'al P., Sebesta I., Marinaki A., Simmonds A., Matthijs G., Fryns J.P., Torres R., Puig J.G., Ott J. and Kmoch S.: *Familial juvenile hyperuricaemic nephropathy (FJHN): linkage analysis in 15 families, physical and transcriptional characterisation of the FJHN critical region on chromosome 16p11.2 and the analysis of seven candidate genes.* Eur J Hum Genet **11**(2): p. 145-154, 2003.
32. Hart T.C., Gorry M.C., Hart P.S., Woodard A.S., Shihabi Z., Sandhu J., Shirts B., Xu L., Zhu H., Barmada M.M. and Bleyer A.J.: *Mutations of the UMOD gene are responsible for medullary cystic kidney disease 2 and familial juvenile hyperuricaemic nephropathy.* J Med Genet **39**(12): p. 882-892, 2002.
33. Serafini-Cessi F., Malagolini N. and Cavallone D.: *Tamm-Horsfall glycoprotein: biology and clinical relevance.* Am J Kidney Dis **42**(4): p. 658-676, 2003.
34. Vylet'al P., Kublova M., Kalbacova M., Hodanova K., Baresova V., Stiburkova B., Sikora J., Hulkova H., Zivny J., Majewski J., Simmonds A., Fryns J.P., Venkat-Raman G., Elleder M. and Kmoch S.: *Alterations of uromodulin biology: a common denominator of the genetically heterogeneous FJHN/MCKD syndrome.* Kidney Int **70**(6): p. 1155-1169, 2006.
35. Vylet'al P., Hulkova H., Zivna M., Berna L., Novak P., Elleder M. and Kmoch S.: *Abnormal expression and processing of uromodulin in Fabry disease reflects tubular cell storage alteration and is reversible by enzyme replacement therapy.* J Inherit Metab Dis **31**(4): p. 508-517, 2008.
36. Hodanova K., Majewski J., Kublova M., Vyletal P., Kalbacova M., Stiburkova B., Hulkova H., Chagnon Y.C., Lanouette C.M., Marinaki A., Fryns J.P., Venkat-Raman G. and Kmoch S.: *Mapping of a new candidate locus for uromodulin-associated kidney disease (UAKD) to chromosome 1q41.* Kidney Int **68**(4): p. 1472-1482, 2005.
37. Paul M., Poyan Mehr A. and Kreutz R.: *Physiology of local renin-angiotensin systems.* Physiol Rev **86**(3): p. 747-803, 2006.
38. Campbell D.J.: *Critical review of prorenin and (pro)renin receptor research.* Hypertension **51**(5): p. 1259-1264, 2008.
39. Walter P., Ibrahimi I. and Blobel G.: *Translocation of proteins across the endoplasmic reticulum. I. Signal recognition protein (SRP) binds to in-vitro-assembled polysomes synthesizing secretory protein.* J Cell Biol **91**(2 Pt 1): p. 545-550, 1981.
40. Morris B.J.: *Molecular biology of renin. I: Gene and protein structure, synthesis and processing.* J Hypertens **10**(3): p. 209-214, 1992.
41. Ma H., Kunes S., Schatz P.J. and Botstein D.: *Plasmid construction by homologous recombination in yeast.* Gene **58**(2-3): p. 201-216, 1987.

Before the development of ESRD, the control of water and electrolyte imbalance and later, when ESRD breaks out, dialysis followed by kidney transplantation are applied.

The pathogenesis of MCKD was mainly unknown.

In 1951, Fanconi et al. recognised very similar familial renal disease, designated nephronophthisis (NPH), affecting children 4-14 years old characterised by renal insufficiency, anemia, polyuria, hypostenuria and tubulo-interstitial involvement [11]. In the 1960s, inability to clearly distinguish both entities and confused diagnoses lead to proposal of NPH-MCKD complex and several studies tried to prove the identity of the two disorders [12-15]. However, differences, namely mode of inheritance, age of onset of clinical symptoms and progression to ESRD and extrarenal organ involvement were evidenced. Further evidences of distinctness were provided by molecular genetic studies [16-19].

On the other hand, there were recognised remarkable phenotype similarities between FJHN and MCKD associated with hyperuricaemia with or without gout. MCKD families with hyperuricaemia and/without gout had been reported [20-22] but also MCKD families in which portion of affected members showed hyperuricaemia without cysts, resembling thus FJHN phenotype [20, 23].

Molecular genetics of FJHN/ MCKD

Christodoulou et al. found the first genetic linkage to chromosome 1q21 in two large Cypriot MCKD families in 1998 [20]. One year later, Scolari et al. found linkage to another locus on chromosome 16p12 in large Italian family with MCKD associated with hyperuricaemia and gouty arthritis [23]. Thus were established MCKD1 (1q21) and MCKD2 (16p12). Further linkage studies in MCKD families revealed further genetic heterogeneity of the disease [24, 25] and no concordance of the phenotype with the identified locus [26].

Kamatani et al. found linkage to 16p12 region in a large Japanese FJHN family [27] and Stibůrková at al. in 2 of 3 Czech families [28] in 2000. Dahan et al. confirmed linkage to 16p12 locus in large Belgian FJHN family [22] and among others promoted hypothesis that FJHN and MCKD2 are in fact allelic disorders.

2. Aims of the study

The main goal of this thesis has been to contribute to understanding of FJHN and other inherited diseases presenting with familial occurrence of hyperuricaemia, gouty arthritis and progressive kidney failure at an early age. We have been especially interested in molecular bases and mechanisms underlying these disorders. The project was based on the following strategy:

1. Continuous collection and characterization of FJHN/MCKD patients and families.

2. Identification of disease causing genes in affected families by positional cloning approaches.

3. Characterisation of the identified proteins and their mutated forms by standard molecular biology methods and evaluation of mechanisms involved in the disease pathogenesis.

4. Isolation and characterisation of interacting partners of newly identified proteins as a parallel approach to the positional cloning in definition of potential candidate disease causing genes and disease related pathways.

3. Methods

3.1 Selected specific methods

Unless otherwise stated, water used in experiments was ultrapure water prepared from common distilled water in Milli-Q® RG Ultrapure Water Purification System (Millipore).

Bioinfomatics

Information on gene organisation and DNA sequences of exons, introns and gene promotors were obtained from The Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nuccore) and The UCSC Genome Browser database using BLASTN tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for pairwise alignment of nucleotide sequences. Results of sequencing were confronted with the UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) and the Entrez SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp).

3.1.1 Uromodulin and urine related specific methods

Isolation of uromodulin from urine

Urinary uromodulin was isolated from one litre of healthy male urine collected by the salt out method similar to original procedure reported by Tamm and Horsfall [29]. NaCl was added to urine to 0.58 M and uromodulin was precipitated at 4°C. The precipitate was collected in the centrifuge, washed once with 0.58 M NaCl solution, collected again and dissolved in distilled water. Insoluble material was removed in the centrifuge. Supernatant was dialysed against 4 litres of water at for 3 days and lyophilised. The lyophilisate was weighted and dissolved in water with NaN3. The purified uromodulin was evaluated by SDS-PAGE and WB.

Urine biochemical analysis

Urine total protein, creatinine, uric acid, magnesium, calcium, and phosphate were determined in random spot urine samples stored at -80°C by the Protein (urine) (BioSystems), CREA (Roche), UA Plus (Roche), Mg (Roche), Ca (Roche), and PHOS (Roche) kits, respectively, on a Hitachi Modular analyser (Roche). Sodium, potassium, and chloride were determined by ion-selective electrodes. Osmolality was determined by freezing point method using FISKE 2400 osmometer (Advanced Instruments-Fiske Associates).

18. Hildebrandt F., Singh-Sawhney I., Schnieders B., Centofante L., Omran H., Pohlmann A., Schmaltz C., Wedekind H., Schubotz C., Antignac C. and et al.: *Mapping of a gene for familial juvenile nephronophthisis: refining the map and defining flanking markers on chromosome 2. APN Study Group.* Am J Hum Genet **53**(6): p. 1256-1261, 1993.
19. Haider N.B., Carmi R., Shalev H., Sheffield V.C. and Landau D.: *A Bedouin kindred with infantile nephronophthisis demonstrates linkage to chromosome 9 by homozygosity mapping.* Am J Hum Genet **63**(5): p. 1404-1410, 1998.
20. Christodoulou K., Tsingis M., Stavrou C., Eleftheriou A., Papapavlou P., Patsalis P.C., Ioannou P., Pierides A. and Constantinou Deltas C.: *Chromosome 1 localization of a gene for autosomal dominant medullary cystic kidney disease.* Hum Mol Genet **7**(5): p. 905-911, 1998.
21. Scolari F., Ghiggeri G.M., Casari G., Amoroso A., Puzzer D., Caridi G.L., Valzorio B., Tardanico R., Vizzardi V., Savoldi S., Viola B.F., Bossini N., Prati E., Gusmano R. and Maiorca R.: *Autosomal dominant medullary cystic disease: a disorder with variable clinical pictures and exclusion of linkage with the NPH1 locus.* Nephrol Dial Transplant **13**(10): p. 2536-2546, 1998.
22. Dahan K., Fuchshuber A., Adamis S., Smaers M., Kroiss S., Loute G., Cosyns J.P., Hildebrandt F., Verellen-Dumoulin C. and Pirson Y.: *Familial juvenile hyperuricemic nephropathy and autosomal dominant medullary cystic kidney disease type 2: two facets of the same disease?* J Am Soc Nephrol **12**(11): p. 2348-2357, 2001.
23. Scolari F., Puzzer D., Amoroso A., Caridi G., Ghiggeri G.M., Maiorca R., Aridon P., De Fusco M., Ballabio A. and Casari G.: *Identification of a new locus for medullary cystic disease, on chromosome 16p12.* Am J Hum Genet **64**(6): p. 1655-1660, 1999.
24. Auranen M., Ala-Mello S., Turunen J.A. and Jarvela I.: *Further evidence for linkage of autosomal-dominant medullary cystic kidney disease on chromosome 1q21.* Kidney Int **60**(4): p. 1225-1232, 2001.
25. Kroiss S., Huck K., Berthold S., Ruschendorf F., Scolari F., Caridi G., Ghiggeri G.M., Hildebrandt F. and Fuchshuber A.: *Evidence of further genetic heterogeneity in autosomal dominant medullary cystic kidney disease.* Nephrol Dial Transplant **15**(6): p. 818-821, 2000.
26. Hateboer N., Gumbs C., Teare M.D., Coles G.A., Griffiths D., Ravine D., Futreal P.A. and Rahman N.: *Confirmation of a gene locus for medullary cystic kidney disease (MCKD2) on chromosome 16p12.* Kidney Int **60**(4): p. 1233-1239, 2001.
27. Kamatani N., Moritani M., Yamanaka H., Takeuchi F., Hosoya T. and Itakura M.: *Localization of a gene for familial juvenile hyperuricemic nephropathy causing underexcretion-type gout to 16p12 by genome-wide linkage analysis of a large family.* Arthritis Rheum **43**(4): p. 925-929, 2000.
28. Stiburkova B., Majewski J., Sebesta I., Zhang W., Ott J. and Kmoch S.: *Familial juvenile hyperuricemic nephropathy: localization of the gene on chromosome 16p11.2-and evidence for genetic heterogeneity.* Am J Hum Genet **66**(6): p. 1989-1994, 2000.

Presymptomatic detection of familial juvenile hyperuricaemic nephropathy in children. Pediatr Nephrol **12**(5): p. 357-364, 1998.
3. Simmonds H.A., Warren D.J., Cameron J.S., Potter C.F. and Farebrother D.A.: *Familial gout and renal failure in young women.* Clin Nephrol **14**(4): p. 176-182, 1980.
4. Richmond J.M., Kincaid-Smith P., Whitworth J.A. and Becker G.J.: *Familial urate nephropathy.* Clin Nephrol **16**(4): p. 163-168, 1981.
5. Farebrother D.A., Pincott J.R., Simmons H.A., Warren D.J., Dillon M.J. and Cameron J.S.: *Uric acid crystal-induced nephropathy: evidence for a specific renal lesion in a gouty family.* J Pathol **135**(2): p. 159-168, 1981.
6. Lhotta K., Gruber J., Sgonc R., Fend F. and Konig P.: *Apoptosis of tubular epithelial cells in familial juvenile gouty nephropathy.* Nephron **79**(3): p. 340-344, 1998.
7. Hisatome I., Kosaka H., Ohtahara K., Tsuboi M., Manabe I., Ohtahara A., Sawaguchi M., Igawa O., Tanaka Y., Fujimoto Y., Yoshida A., Takeda A. and Shigemasa C.: *Renal handling of urate in a patient with familial juvenile gouty nephropathy.* Intern Med **35**(7): p. 564-568, 1996.
8. Thorn G.W., Koepf G.F. and Cliniton M.: *Renal failure simulating adrenocortical insufficiency.* New Eng J Med **231**: p. 76-85, 1944.
9. Scolari F., Viola B.F., Ghiggeri G.M., Caridi G., Amoroso A., Rampoldi L. and Casari G.: *Towards the identification of (a) gene(s) for autosomal dominant medullary cystic kidney disease.* J Nephrol **16**(3): p. 321-328, 2003.
10. Hildebrandt F. and Omram H.: *New insights: nephronophthisis-medullary cystic kidney disease.* Pediatr Nephrol **16**(2): p. 168-176, 2001.
11. Fanconi G., Hanhart E., von A.A., Uhlinger E., Dolivo G. and Prader A.: *[Familial, juvenile nephronophthisis (idiopathic parenchymal contracted kidney).].* Helv Paediatr Acta **6**(1): p. 1-49, 1951.
12. Strauss M.B. and Sommers S.C.: *Medullary cystic disease and familial juvenile nephronophthisis.* N Engl J Med **277**(16): p. 863-864, 1967.
13. Mongeau J.G. and Worthen H.G.: *Nephronophthisis and medullary cystic disease.* Am J Med **43**(3): p. 345-355, 1967.
14. Gardner K.D., Jr.: *Evolution of clinical signs in adult-onset cystic disease of the renal medulla.* Ann Intern Med **74**(1): p. 47-54, 1971.
15. Habib R.: *Nephronophthisis and medullary cystic disease.* In: *Pediatric nephrology. Current concepts and management*, Strauss J., Editor. Symposia Specialist, Miami: p. 393-401, 1962.
16. Omran H., Fernandez C., Jung M., Haffner K., Fargier B., Villaquiran A., Waldherr R., Gretz N., Brandis M., Ruschendorf F., Reis A. and Hildebrandt F.: *Identification of a new gene locus for adolescent nephronophthisis, on chromosome 3q22 in a large Venezuelan pedigree.* Am J Hum Genet **66**(1): p. 118-127, 2000.
17. Antignac C., Arduy C.H., Beckmann J.S., Benessy F., Gros F., Medhioub M., Hildebrandt F., Dufier J.L., Kleinknecht C., Broyer M. and et al.: *A gene for familial juvenile nephronophthisis (recessive medullary cystic kidney disease) maps to chromosome 2p.* Nat Genet **3**(4): p. 342-345, 1993.

Uromodulin enzyme-linked immunosorbent assay (ELISA)

We established sandwich ELISA for quantitative analysis of urinary uromodulin, with mouse monoclonal anti-THP antibody (Cedarlane) as capture antibody, rabbit polyclonal anti-THP antibody (Biogenesis) as detecting antibody and HRP-linked goat anti-rabbit IgG (Pierce) as secondary antibody. F96 Maxisorp immuno-plate (Nunc) was coated by capture antibody overnight at 4°C. Wells were then washed by washing buffer (0.137 M NaCl, 0.027 M KCl, 0.078 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.3, 0.05% Tween 20) blocked by the blocking buffer (0.137 M NaCl, 0.027 M KCl, 0.078 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.3, 2% BSA). Following washing, urine samples diluted in TEA buffer (0.5% Triton X-100, 0.02 M EDTA, pH 7.5), calibration curve solutions and blanks were incubated in wells. Following washing, detecting antibody was added to wells and incubated. After washing, secondary antibody was added to wells and incubated. After final washing, detection solution (0.09% (w/v) o-phenylenediamine and 0.03% H_2O_2) was added and after colour development stop solution (2 M H_2SO_4). Absorbances were acquired by ELISA reader (SLT) at 492 nm with 620 nm filter and data were processed by KimW software (Schoeller Pharma).

Urine concentration

If concentrated for SDS-PAGE and/or WB analysis, total urine was loaded on Microcon YM-30 filter (Millipore) and 1.5 mL tube assembly and centrifuged at 12000g until volume on the filter reached less then about 50 µL. Ultrafiltrate was discarded and retentate was recovered to a new tube at 10000g.

Total urine deglycosylation

For analysis of uromodulin glycosylation patterns in urines, we used GlycoPro™ Enzymatic Deglycosylation Kit (ProZyme) according to manufacturer's instructions. Spot whole urine aliquots were used directly for deglycosylation reactions. Reactions were then analysed by SDS-PAGE and WB.

Uromodulin protein-protein interactions specific methods

Yeast vector and strain

Plasmid pGBD-B for construction of bait fusion proteins contains single *Bam*HI restriction site flanked by the *attB1* and *attB2* sequences that direct homologous recombination with the tagged insert in yeasts. It is a multi-copy shuttle vector containing TRP1 gene for selection in yeasts and ampicillin resistance gene (Amp^r) for the selection in *E. coli*.

S. cerevisiae host strain PJ69-4A was used for expression of baits [30]. Its genotype is *MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3GAL2-ADE2 met2::GAL7-lacZ.*

Primer design for bait construction

Oligonucleotides for PCR amplification of bait fragments were designed using Oligo 6.44 software (Molecular Biology Insights) and to contain two distinct parts - 3'

regions were UMOD specific and 5' regions were tags including sequences identical to *attB1* and *attB2* regions in pGBD-B vector.

PCR for bait fragments

Bait fragments were amplified by "touch-down" approach using Taq DNA polymerase (Promega, #M1861-discontinued) together with Deep VentR™ DNA Polymerase (New England Biolabs) from pCR3.1-UMOD construct provided by my colleagues.

Lithium acetate yeast transformation and bait cloning

For small-scale transformations, PJ69-4A yeast strain was propagated from fresh colony overnight at 30° in appropriate medium. The culture was then diluted and brought to mid-log phase. Yeast cells were harvested in the centrifuge, resuspended in LiAc solution (0.1 M lithium acetate, pH 7.5) and washed twice in the same solution. The pellet was resuspended in transformation buffer (0.1 M LiAc, 40% (w/v) polyethylene glycol 3,350, 0.01 M Tris-Cl, 0.001 M EDTA, pH 7.5) with the addition of heat-denatured salmon sperm DNA (Sigma, #D-9156) and linearised pGBD-B vector solution. The mixture of he aliquot of this cell suspension the aliquot of a bait fragment PCR reaction was incubated at 30°C/30 minutes, 42°C/1 minute and 30°C/1 minute. After transformation, the mixture was diluted by sterile water and spread over appropriate selective dropout plate and let grow for three days at 30°C.

Identification of yeast clones containing baits

For identification of colonies containing inserts, we used the same PCR reactions as for amplification of bait fragments from UMOD cDNA (chapter *PCR for bait fragments*).

Isolation of DNA from yeasts

Cultured yeast cells were harvested in the centrifuge and the pellet was resuspended in prespheroplasting buffer (0.05 M EDTA, 1% BME, pH 8). The suspension was incubated at RT/20 minutes, cells were harvested, resuspended in lyticase buffer (0.8 M KCl, 0.01 M EDTA, 0.1% BME) and incubated with lyticase (5 mg/mL) at 28°C/1 hour. Lysis solution (10% SDS) and Proteinase K solution (20 mg/mL) were added and after the incubation at 56°C/30 minutes, equal DNA was extracted by phenol/chloroform and precipitated by NaAc (3 M NaAc, pH 5.5) and 96% ethanol at -20°C/1 hour. Dry pellet was dissolved in water.

Preparation of yeast protein extracts

Overnight cultures were grown to mid-log phase. Cultures were chilled on ice, cells were harvested and washed in ice cold water. Pellets were frozen to -70°C, thawed by the addition of prewarmed cracking buffer (8 M urea, 5% SDS, 0.04 M Tris-Cl, 0.1 mM EDTA, 1% BME, 0.04% BPB) with 1% PIC, resuspended, transferred to tubes with glass beads (425-600 µm, Sigma, #G-87723), together incubated at 70°C/10 minutes and then vortexed vigorously. Cell debris, unbroken cells and glass beads were pelleted and supernatants were collected. Pellets were further incubated at

6. List of abbreviations

BME	β- mercaptoethanol
BPB	bromphenol blue
BSA	bovine serum albumin
cDNA	complementary or coding DNA
CDS	coding sequence
ConA	Concanavalin A
DCT	distal convoluted tubule(s)
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESRD	end-stage renal disease
FACS	fluorescent automated cell sorting
FJHN	familiar juvenile hyperuricaemic nephropathy
GFR	glomerular filtration rate
GPI	glycosyl phosphatidylinositol
HEK	human embryonic kidney
HRP	horseradish peroxidase
LiAc	lithium acetate
MCKD	medullary cystic kidney disease
NaAc	sodium acetate
NPH	nephronophthisis
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIC	protease inhibitor cocktail
RAS	renin-angiotensin system
RT	room temperature
SDS	sodium dodecylsulphate
TALH	thick ascending loop of Henle
TBS	tris-buffered saline
THP	Tamm-Horsfall protein
UAKD	uromodulin associated kidney disease
WB	Western blot(ting)
YTHS	yeast two-hybrid system
ZP	zona pellucida

7. References

1. Duncan H. and Dixon A.S.: *Gout, familial hypericaemia, and renal disease.* Q J Med **29**: p. 127-135, 1960.
2. McBride M.B., Rigden S., Haycock G.B., Dalton N., Van't Hoff W., Rees L., Raman G.V., Moro F., Ogg C.S., Cameron J.S. and Simmonds H.A.:

5. Conclusions

1) We were able to gather one of the largest sets of families with UAKD in the world. Through international collaboration and urinary uromodulin screening, we were able to gain biological materials from members of altogether 31 families (including unpublished results).

2) Using genotyping, linkage analysis and *UMOD* sequencing we proved genetic heterogeneity of the disease. We identified *UMOD* mutation in 8 families (including unpublished results) and characterised molecular properties and aberrant cellular trafficking of 6 mutant recombinant UMOD proteins.

3) By genome wide linkage analysis we identified new UAKD locus on chromosome 1q41. In large Belgian family (BE1) with atypical FJHN, we identified single locus and single haplotype segregating with the disease and performed sequence analysis of 9 candidate genes in which we found no classical deleterious mutation. Finally, we found heterozygous deletion mutation in *REN* gene and characterised molecular properties and numerous cellular effects of mutant recombinant renin and suggested probable pathogenic mechanisms.

4) We provided evidences that alteration of uromodulin biology is common to genetically heterogenous UAKD. We showed decreased UMOD urinary excretion in almost all investigated families. Accordingly, immunohistochemical analyses of kidney sections revealed abnormal uromodulin expression in FJHN/MCKD patients with different genetic backgrounds.

5) We were not able to identify any uromodulin interacting proteins by means of yeast two-hybrid system and concanavalin A pull-down assays.

Practical outputs

1) We introduced numerous molecular genetic, molecular biological and biochemical methods for the analysis of recombinant as well as native UMOD and renin proteins.

2) Western blot of urinary uromodulin is used routinely in our institute for selective screening of hyperuricaemic conditions. By this approach, we identified 5 Czech UAKD families and subsequently UMOD mutation in 3 of them (including unpublished results).
Through international collaboration, we similarly identified 1 Danish family with UMOD mutation and 5 families from the United Kingdom, 3 of them with UMOD mutation (unpublished results).

3) We provide unique, complex diagnostic service for this group of disorders.

100°C/3-5 minutes and vortexed vigorously again. Debris, unbroken cells and beads were pelleted and supernatants were combined with first supernatants. If no supernatants were obtained in this second round, cracking buffer was added to pellets and previous four steps were repeated. Extracts were denatured at 100°C/5 minutes and stored frozen at -70°C.

Construction of UMOD bait by restriction/ligation

As an alternative approach to $UMOD^{25-640}$ bait construction by homologous recombination cloning, we cut out the central part of a pGBD-B-$UMOD^{25-640}$ construct and replaced it with the same part from pCR3.1-UMOD construct (chapter *PCR for bait fragments*). This approach was designed to avoid PCR in bait construction.

Extraction of total proteins from cells by Triton X-114

HEK293 cells or Tris-Cl homogenate of random kidney tissue were resuspended in ice-cold TBS (0.01 M Tris-Cl, 0.15 M NaCl, pH 7.5), 1/5 volume of precondensed Triton X-114 (Sigma) and 1/100 volume of PIC (Sigma). Cells were extracted for 15 minutes on ice, lysate was centrifuged and supernatant was transferred to a new tube. Pellet was resuspended in ice-cold TBS and homogenised by sonication. Supernatant was warmed to 37°C until it became cloudy. Emulsion was then centrifuged and upper and lower phases were collected in separate tubes. Fractions of the kidney tissue were then tested for uromodulin by SDS-PAGE and WB.

Immobilisation of urinary uromodulin on Concanavalin A-sepharose 4B

ConA-sepharose (Sigma #C-9017) suspension was centrifuged to remove storage buffer, beads were washed three times with immobilisation buffer (0.01 M Tris-Cl, 0.15 M NaCl, 0.001 M $MnCl_2$, 0.001 M $CaCl_2$, pH 7.5) and finally resuspended in the same buffer with the addition of uromodulin. The suspension was incubated at 37°C/1 hour with gentle mixing, beads were collected by centrifugation and washed three times with immobilisation buffer and finally resuspended in TBS to the original volume. For blank assay, ConA beads were processed in the same way but without the addition of uromodulin.

ConA pull-down assay

To uromodulin coated beads suspension or blank beads suspension, HEK293 or kidney tissue fraction was added. The mixture was incubated for 90 minutes on ice with occasional gentle mixing. Beads were then collected in the and washed tree times by ice-cold TBS. Supernatant was aspirated and beads were resuspended in 1X SDS-PAGE sample buffer (0.002% BPB, 5% glycerol, 57.8 mM SDS, 1% BME) and denatured at 95°C/10 minutes. Beads were separated in the centrifuge and an aliquot of the supernatant was analysed by SDS-PAGE followed by Sypro Ruby (Molecular Probes) staining.

3.1.2 Renin related specific methods

Preparation of cell culture medium for secreted renin analysis

For secreted renin analysis, the foetal calf serum free medium was centrifuged twice to remove residual cells and cellular debris. After addition of PIC (Sigma), the supernatant was concentrated to a small volume and brought to PBS (0.137 M NaCl, 0.027 M KCl, 0.078 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.3) by two successive rounds of concentration-refill on Microcon YM-10 filter and 1.5 mL tube assembly (Millipore) in the centrifuge. Retentate was recovered and analysed by SDS-PAGE and WB as is or after the deglycosylation.

Cultured cells lysis

Cells were harvested to PBS buffer (0.137 M NaCl, 0.027 M KCl, 0.078 M Na_2HPO_4, 0.015 M KH_2PO_4, pH 7.3) collected by centrifugation at 800g/5 minutes and washed in PBS. The pellet was then resuspended in PBS with protease inhibitor cocktail (PIC) (Sigma) and sonicated twice for 30 seconds at output of 50% in the cuphorn filled with mixture of ice and water and connected to sonicator. Cell debris was removed in the centrifuge. The supernatant was analysed or stored at -20°C.

3.1.3 Renin and uromodulin related specific methods

Uromodulin and renin deglycosylation

For analysis of glycosylation patterns of wildtype and mutant uromodulins and renins expressed in cell cultures, we used GlycoPro™ Enzymatic Deglycosylation Kit (ProZyme) according to manufacturer's instructions. Reactions were then analysed by SDS-PAGE and WB.

Antibodies fluorescein labelling

For FACS analysis, we conjugated polyclonal anti-THP antibody (Biogenesis) or anti-renin antibodies (Yanaihara Institute) with fluorescein by Fluorescein Protein Labelling Kit (Roche) according to manufacturer's instructions.

3.2 General methods

Besides specific methods we used following standard molecular biology methods:

SDS-PAGE
Western blotting
DNA cloning in *E. coli*
Isolation of DNA from *E. coli*
DNA electrophoresis in agarose gel
Isolation of DNA fragments from the agarose gel
Determination of DNA concentration
DNA cycle sequencing.

yeasts were suggestive of bad tolerance of UMOD cDNA by yeast cells, resulting in higher mutation rate and absence of bait fusion expression. However, correct sequence of the $UMOD^{25-640}$ construct prepared by restriction/ligation spoke against this possibility. In these clones, absence of bait fusion expression could be rather caused by preserving the UMOD C-terminal segment (containing GPI attachment site) in bait constructs that could interfere with proper protein processing. This segment should have been probably omitted in bait design. Finally, PCR amplified bait fragments should had been sequenced in advance of gap repair cloning, which could probably a priori identify potential sequence errors introduced by PCR and suggest the employment of different amplification system

4.6.2 ConA pull-down assay

We used uromodulin isolated from healthy male urine as bait to coat ConA-sepharose beads. Coated beads were incubated with two different cell lysates prepared by means of detergent Triton X-114.

WB of Triton X-114 fractions of kidney homogenate showed uromodulin to be most abundant in the pellet (nonionic detergent insoluble material). Detergent insolubility of some GPI proteins develops shortly after synthesis [45], and is thought to be due to aggregation of GPI modified proteins with glycosphingolipids during secretion.

Uromodulin coated beads were incubated with all Triton X-114 phases (pellet, detergent-rich and detergent depleted phase) of both cell sources. As blanks, we used ConA beads with no uromodulin in ConA binding reaction and otherwise treated in the same way as coated beads. Material bound on beads during incubation with lysates fractions was released under denaturing condition and analysed by SDS-PAGE.

By this approach, however, we have never observed a protein(s) specifically present in uromodulin-containing incubations, i.e. not present also in blank reactions.

However, uromodulin isolated from urine might not be suitable for this assay. It is known that peptide as well as glycosyl structure of kidney-synthesised uromodulin and its counterpart found in urine differ. Moreover, binding of uromodulin to solid phase through its glycosyl chains might block its potential binding sites. Low amounts of potential interactors in examined samples or transient character of interaction could also explain our results. Another possibility is that uromodulin has no interacting proteins in situ.

In the literature, there were described interactions between uromodulin and immunoglobulin G (IgG) [46], complement components 1q and 1 [47, 48], recombinant interleukin-1 (rIL-1) [49] and recombinant tumour necrosis factor (rTNF) [50] in vitro. Uromodulin was also co-immunoprecipitated with Na+-K+-ATPase (NKA) from rat kidney protein extract and cultured TALH cells, which probably reflects their common localization in lipid rafts [51].

renal RAS system which is still not properly understood in humans (manuscript in preparation).

4.6 UMOD protein-protein interaction studies

4.6.1 Yeast two-hybrid system (YTHS)

After invention of uromodulin causative role in FJHN/MCKD2 we prepared three types of uromodulin-based bait clones comprising i) the whole CDS except for N-terminal signal sequence ii) ZP domain and iii) EGF domains cluster.

Bait fragments were PCR amplified from UMOD cDNA and transformed together with the vector employing homologous recombination cloning [41, 42]. Resulting clones were tested for the presence of the construct. Positive clones were tested for autoactivation of reporter genes. Significant autoactivation was observed only in case of some UMOD$^{25\text{-}640}$ bait clones. Only clones showing no autoactivation were selected for further analyses.

Several clones of each bait were tested for expression of bait fusion protein by WB with both anti-GAL4 BD monoclonal antibody and anti-THP polyclonal antibody. Only two clones with ZP domain bait showed specific signal on WB of correct MW (45 kDa) with anti-GAL4 BD monoclonal antibody. However, this bait fusion protein was not detected by WB with anti-THP polyclonal antibody. One clone with EGF bait showed specific signal on WB with anti-GAL4 BD monoclonal antibody, of which MW (about 19 kDa), however, corresponded to GAL4 BD only (theoretical MW of bait fusion protein was about 30 kDa). In bait clones with the whole UMOD CDS, no specific signal could be detected on WB with either antibody.

Selected constructs of each bait were also sequenced on slab gel platform to verify correctness of insert sequences. We found that all analysed constructs repeatedly showed multiple single nucleotide changes.

To exclude the possibility that these errors arose during PCR amplification of bait fragments, we prepared another UMOD$^{25\text{-}640}$ construct by classical restriction/ligation approach. Resulting construct was then transformed to E. coli to be amplified for sequencing before the transformation to yeasts. Sequence of the insert was correct. Construct was then transformed to yeasts and selected clones were screened and tested as described above. No apparent autoactivation was observed. The sequence of the construct isolated from single yeast clone was proved to be correct. However, expression of bait fusion protein could not be detected by WB with either antibody mentioned above.

In conclusion, we failed to proceed to further steps of two-hybrid screening due to inability to avoid obstacles described above. We also got insecure that YTHS was appropriate tool for studying uromodulin interactions. One of the main drawbacks of classical YTHS is unavailability of proper posttranslational modifications and folding of baits due to the omission of the original signal sequence and due to the fusion to BD directing the resulting fusion protein to the nucleus [43, 44]. In case of uromodulin, which is heavily glycosylated and extensively stabilised by disulfide bridges, this could be critical. Sequence errors found in constructs prepared by gap repair cloning in

4. Results and discussion

A couple of years ago, MCKD and FJHN were partially genetically characterised and their close relationship came out [22, 23, 28]. The following section describes and discusses results obtained in our four partially overlapping projects addressing strategic concepts outlined in chapter 2. Results are presented with respect to four published manuscripts, one manuscript prepared for the publication and one unpublished work.

4.1 Collection and characterisation of additional FJHN/MCKD patients and families

In this study we performed linkage analysis in additional 15 FJHN families. Linkage to 16p11.2 locus was confirmed in six families, which suggested that, in a large proportion of FJHN kindreds, the disease is likely to be caused by a gene or genes located outside of 16p11.2. Haplotype analysis of the new and previously analysed families provided two non-overlapping critical regions on 16p11.2 – FJHN1, and FJHN2. Considering MCKD2 to be a distinct molecular entity, the analysis suggested that as many as three kidney disease genes might have been located in close proximity on 16p11.2. From genomic databases we compiled integrated physical and transcription maps of whole critical genomic region in which 45 known genes and 129 predicted loci was localised. We selected, analysed and found no pathogenic mutations in seven candidate genes [31].

At the time of acceptance of our manuscript (end of the year 2002), Hart et al. came out with work in which they clearly described mutations in the *UMOD* gene, encoding for uromodulin, in two FJHN families as well as in one MCKD2 family, proving thus allelism of both disorders [32]. They suggested using the term “uromodulin-associated kidney disease” (UAKD) for both FJHN and MCKD2.

Uromodulin (UMOD; OMIM 191845) or Tamm–Horsfall protein (THP) is a protein selectively expressed in the thick ascending limb of Henle’s loop (TALH), the macula densa segment and the distal convoluted tubule (DCT). Normally the synthesized protein is glycosylated, glypiated, secreted and GPI-anchored in the apical membrane of the polarised renal tubular epithelial cells, from where it is continuously released by a specific but not yet identified protease. UMOD is excreted in the urine at the rate of 50–100 mg/day, which makes it one of the most abundant urinary proteins. UMOD precipitates in the urine and is the main constituent of urinary casts [33].

4.2 UMOD mutations in our cohort and their characterisation

In 19 FJHN/MCKD families, we investigated relevant biochemical parameters, performed linkage analysis to known disease loci, sequenced uromodulin gene, expressed and characterised mutant uromodulin proteins, and performed immunohistochemical and electronoptical investigation in kidney tissues.

We proved genetic heterogeneity of the disease. *UMOD* mutations were identified in six families. Expressed mutant proteins showed distinct glycosylation patterns, impaired intracellular trafficking, and decreased ability to be exposed on the plasma membrane, which corresponded with the observations in the patient's kidney tissue. We found a reduction in urinary uromodulin excretion as a common feature shared by almost all of the families. This was associated with case-specific differences in the uromodulin immunohistochemical staining patterns in kidney.

Our results suggest that various genetic defects interfere with uromodulin biology, which could lead to the development of the common disease phenotype. We thus gave support for UAKD concept [34].

4.3 UMOD in other diseases with renal involvement

To gain knowledge of the spectrum of UMOD changes in various genetic diseases with renal involvement, we examined urinary UMOD excretion and found significant quantitative and qualitative changes in 15 male patients at various clinical stages of Fabry disease (OMIM 301500). In untreated patients, the changes ranged from normal to a marked decrease, or even absence of urinary UMOD. This was accompanied frequently by the presence of aberrantly processed UMOD lacking the C-terminal part following the K432 residue. The abnormal patterns normalized in all patients on enzyme replacement therapy and in some patients on substrate reduction therapy. Immunohistochemical analysis of the affected kidney revealed abnormal UMOD localization in the TALH and the DCT, with UMOD expression inversely proportional to the degree of storage.

Our observations warrant evaluation of tubular functions in Fabry disease and suggest UMOD as a potential biochemical marker of therapeutic response of the kidney to therapy. Extended comparative studies of UMOD expression in kidney specimens obtained during individual types of therapies would be therefore of great interest [35].

4.4 Identification of a new UAKD locus

To identify additional UAKD loci, we analysed one of our families (BE1) with features suggestive of FJHN and without linkage to any of known UAKD loci.

Clinical, biochemical and immunohistochemical investigations were used for proper phenotype characterisation. Genotyping, linkage and haplotype analyses were employed to identify the candidate disease region. Bioinformatics and sequencing were used for candidate gene selection and analyses.

We identified a new candidate UAKD locus on chromosome 1q41. We analysed and found no linkage to this region in eight additional families, who did not map to the previously established loci. In the new locus, we selected and analysed sequences of nine candidate genes, but found no "classic" deleterious mutation within the analysed promoter regions, exon/intron junctions, and coding sequences.

We noted that affected individuals showed, in addition to the characteristic urate hypoexcretion, significant reductions in urinary excretion of calcium and UMOD.

Immunohistochemical analysis showed that low UMOD excretion resulted from its reduced expression, which is a different mechanism to intracellular UMOD accumulation observed in cases with UMOD mutations [36].

Besides those candidate genes mentioned in the article, we also analysed the sequence of renin precursor (preprorenin) encoding gene (*REN*). In affected member of BE1 family, we found heterozygous mutation in exon 1 of *REN* gene, causing the deletion of one leucine from pentaleucine hydrophobic core the preprorenin signal peptide. The mutation was present neither in healthy family member nor in 200 healthy controls. However, we postponed the publication of this finding to further characterise and to functionally study wildtype and mutant preprorenin and the impact of the mutation on cell biology and its potential role in the disease pathogenesis.

Renin and prorenin are secretory proteins active in the renin-angiotensin system (RAS) [37] and the (pro)renin receptor signal transduction pathway which is distinct from RAS receptor signalling [38]. Both pathways play an important role in kidney physiology and pathology. *REN* is expressed most profoundly in the ovary, the uterus and the kidney (http://symatlas.gnf.org/SymAtlas). In the kidney, the expression is restricted to (granular) cells of the juxtaglomerular apparatus. The hydrophobic core structure of the signal peptide binds to signal recognition particle, which ensures insertion of the signal peptide into translation-translocation channel in the membrane of the ER [39]. Following proteosynthesis, glycosylation, signal sequence removal and proteolytic processing, both the prorenin and renin are sorted to secretory granules from which they are released in highly regulated manner into the circulation [40].

4.5 Evaluating *REN* as a candidate gene in 1q41 region

Using positional cloning, we confirmed linkage to chromosome 1 region in the family from the previous study (chapter 4.4)

We found that the mutation decreases signal peptide hydrophobicity required for efficient ER translocation, changes property of the off-cleaved signal peptide and limits biosynthesis of secretion-competent and catalytically active prorenin and renin proteins. Cells stably expressing mutant protein retained prorenin and renin intracellularly and showed reduced growth rate and signs of activated ER stress, unfolded protein response and pronounced autophagy. The immunohistochemical analysis of kidney tissues showed in patients reduced expression of prorenin and renin in site of juxtaglomerular apparatus, absence of renin expressing cells along the tubules and enhanced ectopic expression of prorenin and renin in arterioles and arteries.

Our results indicate that the identified mutation leads to aberrant prorenin and renin biosynthesis, ER-stress mediated cytotoxicity and slowly progressing damage of renin expressing cells. This probably affects the structure and function of juxtaglomerular apparatus, changes sensitivity of the tubuloglomerular feedback mechanism and alters renal blood flow autoregulation. This leads to ischaemia, loss of glomeruli, tubulointerstitial injury and end-stage renal disease.

We believe that the here presented genetic defect and phenotype define and document the range and functional importance of juxtaglomerular cells and the intra-