Nephrol Dial Transplant (2015) 30: 636–644
doi: 10.1093/ndt/gfu374
Advance Access publication 3 December 2014

Retrospective cohort study of familial hypomagnesaemia with hypercalciuria and nephrocalcinosis due to *CLDN16* mutations

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ORIGINAL ARTICLE

ABSTRACT

Background. Familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive tubular disorder exhibiting a high risk for progressive chronic kidney disease (CKD).

Methods. This is a retrospective multicentre study of 25 paediatric cases with FHHNC in Poland. Median age at diagnosis was 4 years and median follow-up time was 4.8 years.

Results. All cases of FHHNC carried recessive mutations in *CLDN16*. The founder mutation in *CLDN16*, Leu151Phe, was the most frequent cause of FHHNC in Polish patients, with 13 (52%) cases being homozygous and 5 (20%) carrying Leu151Phe allele in compound heterozygosity. All cases showed nephrocalcinosis, increased urinary fractional excretion of magnesium and hypercalciuria. Other disease features included hypomagnesaemia (76%), hyperparathyroidism (76%), hyperuricaemia (56%) and hypocitraturia (60%). Treatment with thiazides effectively reduced hypercalciuria in most cases. During follow-up, renal function declined in 60% of patients; 12% of patients reached CKD stage 3 or 4 and one patient developed end-stage renal failure.

Conclusions. We report one of the largest cohorts of FHHNC cases caused by *CLDN16* mutations. A missense variant of *CLDN16*, Leu151Phe, is the most common mutation responsible for FHHNC in Poland. Additionally, we found that normomagnesaemia does not exclude FHHNC and the calculation of fractional excretion of Mg can be diagnostic in the setting of normomagnesaemia. We also demonstrate the efficacy of a treatment with thiazides in terms of hypercalciuria in the majority of patients.

Keywords: *CLDN16*, FHHNC, hypercalciuria, hypomagnesaemia, nephrocalcinosis

INTRODUCTION

Familial hypomagnesaemia with hypercalciuria and nephrocalcinosis (FHHNC) is a rare autosomal recessive tubulopathy characterized by renal loss of calcium and magnesium, bilateral medullary nephrocalcinosis (NC) and progressive chronic kidney disease (CKD) [1]. The disorder is caused by mutations in either the *CLDN16* (OMIM # 248250) or *CLDN19* (OMIM # 248190) genes, encoding the tight junction proteins claudin-16 and -19. Both proteins play a crucial role in the paracellular reabsorption of magnesium and calcium in the thick ascending limb (TAL) of Henle's loop [2–4]. Patients usually present with polyuria, urolithiasis, hyperparathyroidism, hyperuricaemia, hypocitraturia and urinary tract infections [1, 3]. In addition, mutations in *CLDN19* are associated with severe ocular abnormalities [4].

The exact epidemiology of FHHNC is not known. Based on the MEDLINE database, since its first clinical description by Michelis *et al.* in 1972 [5], more than 120 cases were reported worldwide. Most were published as case reports and only a few

series of larger groups of patients were evaluated [1, 3, 6–9]. In this paper, we present all FHHNC cases reported to the Polish NEPHROCALCINOSIS registry.

MATERIALS AND METHODS

The Polish NEPHROCALCINOSIS registry was established in 2010 and approved by the Polish Society for Paediatric Nephrology to assess the epidemiology and aetiology of nephrocalcinosis in the Polish paediatric population. As an unexpected high number of FHHNC cases were reported in a short time period, we decided to investigate this rare disease phenotype more precisely. In June 2011, all 14 Polish centres of paediatric nephrology were invited to participate in this substudy. They were asked to identify all patients with the characteristic phenotype of FHHNC and to report their complete clinical characterization, including relevant laboratory data to the registry database. The collected data included family history, demographics, age at presentation, initial symptoms, concomitant medical conditions and complications, age at diagnosis, medication, results of genetic analysis and assessment of renal ultrasound (US). The following laboratory data were collected: serum concentrations of creatinine (sCr), magnesium (sMg), parathyroid hormone (sPTH), uric acid (sUA), venous pH and bicarbonate levels, 24-h urinary calcium (24h-uCa) and citrate excretion (24h-uCit), estimated glomerular filtration rate (eGFR) and fractional urinary Mg (uMg) excretion (FEMg%).

eGFR was calculated using the original *Schwartz* method [10]. In patients >2 years eGFR < 90 mL/min/1.73 m^2 body surface area (BSA) was considered as decreased. In infants and younger children, physiological differences of eGFR were considered [11]. Grading of CKD was adopted from KDIGO (Kidney Disease: Improving Global Outcomes) guidelines: G1—normal eGFR; G2—eGFR between 60 and 89; G3a—GFR between 45 and 59; G3b—eGFR between 30 and 44; G4—eGFR between 15 and 29; G5—eGFR of <15 mL/min per 1.73 m^2 [12]. FEMg% was calculated from a random urine sample and serum concentrations of Mg and Cr using the following formula: (uMg*sCr/0.7*sMg*uCr)*100%. Values >4% were considered as excessive renal Mg loss [13]. Hypomagnesaemia was defined as sMg < 0.7 mmol/L (1.7 mg/dL) [14], hypercalciuria (HC) as 24h-uCa>4 mg (0.1 mmol)/kg of body weight [15], hyperparathyroidism as sPTH>66 pg/mL [16], hyperuricaemia as sUA >5.5 mg/dL (0.327 μmol/L) [17] and hypocitraturia as 24h-uCit <1.32 mmol (253 mg)/1.73 m^2 BSA and <0.92 mmol (177 mg)/1.73 m^2 BSA in boys and girls, respectively [18].

Imaging of medullary NC by ultrasound was classified according to *Hoyer*'s grading as follows: stage 1—slightly higher echogenicity of pyramids than that of cortex; stage 2a—white garlands at the border of pyramids; stage 2b—significantly increased echogenicity of pyramids without acoustic shadowing; stage 3—hyperechoic pyramids with acoustic shadowing [19].

Growth parameters were assessed using percentile charts for height/length and weight dedicated for Polish children [20]. Short stature was defined when height/length was <5th percentile for age and sex.

Mutational analysis was performed by Sanger sequencing of the entire coding sequence of the*CLDN16* gene and the adjacent intro/exon boundaries from both strands as previously described [21]. A written informed consent for genetic testing was obtained from legal representatives and patients if applicable.

The statistical analysis was performed by the software Statistica (StatSoft Inc., Tulusa, OK, USA) for Windows, version 7.1. As the majority of parameters were not normally distributed, the specific differences were tested by non-parametric Mann–Whitney U- test and correlations with the Spearman test. P-values <0.05 were considered statistically significant.

During the study period 25 patients (16 males and 9 females) with FHHNC from 18 families were reported by seven units of paediatric nephrology in different regions of Poland.

RESULTS

Patients and families

Twenty-five individuals (16 males and 9 females) from 18 families aged 1–22.4 years (median 10 years) were enrolled in the study (Table 1). All of them were of Caucasian origin and born to non-consanguineous healthy parents. Four patients had second-degree relatives with urolithiasis. In family 3 (F3), four male siblings were affected, 2 of them were dizygotic twins (F3.2 and F3.3). In families F5, F7 and F11 two male siblings were affected each, whereas in family F4 brother and sister suffered from FHHNC. Patients F1.1 and F2.2 were published previously [22, 23].

Initial clinical presentation and US imaging

The clinical data are shown in Table 1 and in Figures 1 and 2. The median age of initial symptoms was 0.9 (range 0.1–13) years, the median age of clinical diagnosis of FHHNC was 4 (range 0.4–16.3) years. Patients presented with non-specific symptoms as polyuria/polydipsia (76%), abdominal pain (36%), vomiting (32%), failure to thrive (28%). Urinalysis showed sterile leucocyturia in 60% of patients, microscopic haematuria in 24% of patients, whereas urinary tract infection was found in 44% of patients. Three (12%) patients developed tetany due to low sMg level, two (8%) patients showed signs of rickets.

With one exception, ultrasound examination revealed a higher degree NC. Stage 2b was the most frequent and was found in 16 (64%) patients, followed by stage 3 (8 patients: 32%) and 2a (1 patient: 4%). NC was accompanied by urolithiasis in six (24%). In patients F2.1 and F9.1, ESWL was performed but unfortunately the voided concrements were not available for chemical analysis. In the remaining cases, no intervention was necessary because only small non-obstructing stones were detected.

Selected laboratory data are shown in Table 2. Initial eGFR was decreased in 15 (60%) patients. The distribution of CKD grades was as follows: G1-10 (40%) patients, G2-14 (56%)

Table 1. Clinical characteristics of the FHHNC study group

Patient number	Sex	Age at manifestation (years)	Age at diagnosis (years)	Initial clinical presentation											*CLDN16* mutations
				NC (type)[a]	Polyuria/ polydipsia	Sterile leucocyturia	UTI	Abdominal pain	Vomiting	Failure to thrive	UL	Microscopic hematuria	Tetany	Rickets	
F1.1	f	8	14	3	+	–	–	–	–	–	–	–	+	–	Compound heterozygous Ser110Arg-Leu151Phe
F2.1	m	5.9	6.2	3	+	–	–	–	–	–	+	+	–	–	Homozygous Leu151Phe
F3.1	m	0.8	16.3	2b	+	–	–	–	–	–	–	–	–	–	Compound heterozygous Ser110Arg-Trp234Cys
F3.2	m	0.2	12.9	2b	+	–	+	–	–	–	–	–	–	–	Compound heterozygous Ser110Arg-Trp234Cys
F3.3	m	0.2	12.9	2b	+	–	+	–	–	–	–	–	–	–	Compound heterozygous Ser110Arg-Trp234Cys
F3.4	m	1.2	6.3	2b	+	+	–	–	–	–	–	–	–	–	Compound heterozygous Ser110Arg-Trp234Cys
F4.1	f	0.1	2	2b	+	+	–	–	–	–	–	–	–	–	Homozygous Leu151Phe
F4.2	m	7.8	7.8	2b	+	+	–	–	–	–	–	–	–	–	Homozygous Leu151Phe
F5.1	m	0.7	1.6	2b	–	–	–	+	–	–	–	–	–	–	Compound heterozygous Leu145Pro-Leu151Phe
F5.2	m	0.9	0.9	2b	–	–	–	–	–	–	–	–	–	–	Compound heterozygous Leu145Pro-Leu151Phe
F6.1	f	1	4	2b	+	+	+	+	+	+	+	+	+	+	Homozygous Leu151Phe
F7.1	m	1	1	2b	+	+	+	+	+	–	+	–	–	–	Homozygous Leu151Phe
F7.2	m	1	1	2b	+	+	+	+	+	+	+	+	–	–	Homozygous Leu151Phe
F8.1	f	1	1	3	+	+	+	+	+	–	–	–	+	+	Compound heterozygous Gly239Arg-Gly245Asp
F9.1	f	10	10	3	–	–	+	+	–	–	+	+	–	–	Homozygous Leu151Phe
F10.1	m	0.5	0.5	2b	–	+	–	–	–	+	–	–	–	–	Homozygous Leu151Phe
F11.1	M	0.3	0.4	2b	+	+	+	–	–	–	–	–	–	–	Compound heterozygous Ser120Arg-Tyr288Stop
F11.2	M	0.8	0.8	3	+	+	+	–	–	–	–	–	–	–	Compound heterozygous Ser120Arg-Tyr288Stop
F12.1	F	1.9	2	3	+	+	+	–	+	+	–	–	–	–	Homozygous Leu151Phe
F13.1	F	0.2	2	2a	+	–	–	–	–	+	–	–	–	–	Homozygous Leu151Phe
F14.1	F	3	6	3	+	+	–	+	+	+	–	+	–	–	Compound heterozygous Leu145Pro-Leu151Phe
F15.1	M	0.9	5.3	2b	+	+	–	–	–	–	–	–	–	–	Homozygous Leu151Phe
F16.1	M	0.2	8	3	–	+	–	–	+	–	–	–	–	–	Homozygous Leu151Phe
F17.1	F	0.5	0.8	2b	+	–	+	+	+	+	–	–	–	–	Homozygous Leu151Phe
F18.1	M	13	13	2b	–	+	–	+	–	–	+	+	–	–	Compound heterozygous Leu151Phe-Splice site mutation

[a]According to *Hoyer's* ultrasound staging of nephrocalcinosis [19]. NC, nephrocalcinosis; UTI, urinary tract infection; UL, urolithiasis.

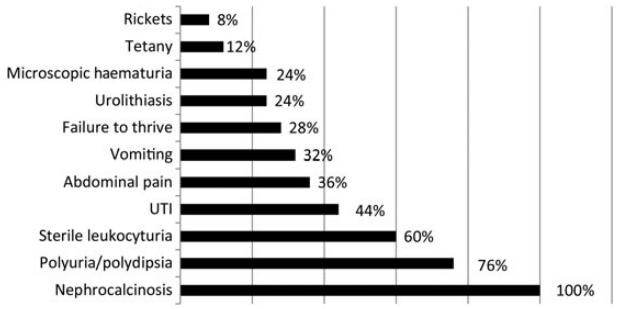

FIGURE 1: Frequency of initial clinical symptoms in the FHHNC study group. UTI, urinary tract infection.

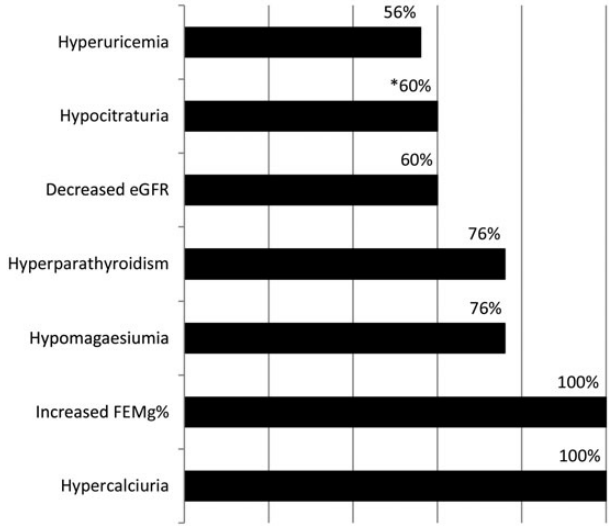

FIGURE 2: Frequency of biochemical disturbances at diagnosis of FHHNC in the study group (*urinary citrate excretion was measured only in 15 patients). FEMg%, fractional magnesium excretion; eGFR, estimated glomerular filtration rate.

patients, G3a-1 (4%) patients. Median eGFR for children >2 years was 86 (range 59–128) mL/min/1.73 m^2.

At the time of clinical diagnosis median sMg level was 0.59 (range 0.29–0.96) mmol/L and hypomagnesaemia was found in 19 (76%) patients. All patients showed increased FEMg%-median 10.6% (range 6.2–26.7). HC was found in all patients and median 24h-uCa was 8.9 (range 4.5–17.8) mg/kg. Median sPTH level was 93.6 (range 43.7–840) pg/mL and hyperparathyroidism was diagnosed in 19 (76%) patients. Median sUA level was 6.0 (range 4.1–9.2) mg/dL and hyperuricemia was found in 14 (56%) patients. 24h-uCit was measured only in 15 patients with median of 0.82 mmol/1.73 m^2 (range 0.1–4.14). There were nine (60%) patients with hypocitraturia in this group. Venous blood pH and bicarbonate level were normal in all patients.

Mutation analysis

The results of the mutation analysis are summarized in Table 1. Either homozygous or compound heterozygous mutations in *CLDN16* were identified in all cases, 52% of the patients carried Leu151Phe in a homozygous state. This mutation was found in 72% (26/36) of the expected mutant alleles. The remaining alleles were private missense ($n = 6$), nonsense ($n = 1$) or splice-site mutations ($n = 1$). Co-segregation analysis was performed whenever possible. All mutations were absent in 100 ethnically matched controls. The median age of genetic confirmation of FHHNC was 9 (range 0.7–17.4) years.

Treatment

Due to the retrospective design of the study a precise evaluation of treatment and its impact on assessed parameters was not possible in most patients. However, treatment was focused on Mg supplementation and reduction of urinary Ca excretion (Table 2). Magnesium citrate or carbonate was prescribed in all patients, usually at a dose of 10–20 mmol Mg^{2+} (0.4–0.8 mmol) per kg of body weight three times a day. To reduce hypercalciuria, all but six patients were treated with hydrochlorothiazide (HCT) at a standard dose of 0.5–1.5 mg per kg body weight/24 h either in monotherapy or in commercially available formulation with amiloride (AMI) at a dose of 0.05–0.15 per kg of body weight/24 h (Tialorid®). Citrate substitution was administered in 18 patients as potassium citrate at a daily dose of 50–100 mg (0.5–1 mmol) per kg of body weight. In patients with severe hyperparathyroidism and renal failure, 1alpha-hydroxycholecalciferol was administered. Most patients who received HCT required potassium supplementation. Additionally, a high fluid intake and dietary salt restriction was generally advised. Patient 2 discontinued treatment at 13 years of age due to family problems as previously described [23].

Clinical and laboratory follow-up

The median time of the follow-up was 4.8 (range 0.5–9.9) years.

At last observation, a decreased eGFR was found in 17 (68%) patients (Table 2). In comparison to the initial values of eGFR, we observed a decline in renal function in 17 patients (68%), while the remaining 8 patients had stable eGFR at median 4.8 years of follow-up. The distribution of CKD grades was: G1-8 (32%) patients, G2-13 (52%) patients, G3a-1 (4%) patients, G3b-1 (4%) patients, G4-1 (4%) patient and G5-1 (4%) patient. The latter patient had started haemodialysis at the age of 15 years and 1 year later received a renal allograft [23]. Last median eGFR in children who started follow-up at the age >2 years was significantly lower than that at initial presentation: 69 (range 12–100) mL/min/1.73 m^2 and 86 (59–128) mL/min/1.73 m^2 ($P < 0.05$), respectively.

In comparison to the initial values, at last observation an increase in sMg levels was observed in 13 (52%) patients, however, hypomagnesaemia was still found in 20 (80%) patients (Table 2). In the study group, the last median sMg level did not differ significantly from that at initial presentation: 0.6 (range 0.29–0.8) and 0.59 (range 0.29–0.96) mmol/L ($P = 0.64$), respectively. In the follow-up period, no significant correlation between serum magnesium concentration and eGFR was observed ($r = -0.052$).

In comparison to the initial values, at last observation a decrease in 24h-uCa excretion was observed in 22 (88%) patients,

Table 2. Selected laboratory data in the FHHNC study group

Patient number	Sex	Follow-up period/age (years)	eGFR (mL/min/1.73 m^2)		Mg (mmol/L)		UCa (mg/kg/24 h)		sPTH (pg/mL)		FEMg% at diagnosis	sUA (mg/dL) at diagnosis	Ucitrate (mmol/1.73 m^2/24 h) at diagnosis	Treatment
			first	last	first	last	first	last	first	last				
F1.1	F	8.4/14–22.4	**88**	**53**	**0.62**	**0.58**	**12**	1.5	**1036**	**398**	**26.7**	**7.6**	**0.983**	HCT, Mg, potassium citrate
F2.1	M	9.7/6.2–15.9	**59**	**12**	**0.58**	0.75	**8.6**	2.8	**117**	**3977**	**12**	**6.8**	**0.104**	HCT, Mg, potassium citrate
F3.1	M	7.7/11.3–19	**81**	**80**	0.85	**0.66**	**6.6**	**7.6**	**88**	**74**	**10.8**	**9.1**	**0.637**	HCT, AMI/HCT, Mg, potassium citrate
F3.2	M	7.9/8.1–16	**86**	**73**	0.74	**0.6**	**10.2**	3.6	**140**	**118**	**9.6**	**6.9**	**0.818**	HCT, AMI/HCT, Mg, potassium citrate
F3.3	M	7.7/8.1–16	**86**	**65**	0.75	0.8	**7.8**	2.8	**109**	**78**	**9.1**	**6.3**	0.975	HCT, AMI/HCT, Mg, potassium citrate
F3.4	M	8.6/1.4–10	**78**	110	0.71	0.7	**8.7**	**5.4**	**92**	**155**	**8.3**	**5.7**	**0.506**	HCT, AMI/HCT, Mg, potassium citrate
F4.1	F	1.9/2–4.1	116	**82**	**0.59**	0.74	**12.8**	**6.5**	**78**	**175**	**14.6**	**6.4**	1.380	HCT, AMI/HCT, Mg, potassium citrate
F4.2	M	2.2/7.8–10	100	**64**	**0.59**	**0.56**	**9**	3.5	**225**	**435**	**9.6**	**7**	**0.148**	HCT, AMI/HCT, Mg, potassium citrate
F5.1	M	4.8/4.2–9	**77**	**77**	**0.37**	**0.54**	**6.7**	**4.8**	40	**105**	**9.2**	4.8	No data	AMI/HCT, Mg, potassium citrate
F5.2	M	3.7/2.3–6	**61**	**64**	**0.57**	**0.6**	**10**	3.9	**134**	**141**	**8.7**	5.1	No data	AMI/HCT, Mg, potassium citrate
F6.1	F	9.9/4–13.9	**82**	**81**	0.82	**0.45**	**11**	2.4	32	**68**	**20**	4.1	No data	HCT, Mg
F7.1	M	5.5/1.5–7	113	94	**0.57**	**0.53**	**8.6**	**6.5**	**68**	**120**	**8.1**	4.2	2.860	AMI/HCT, Mg, potassium citrate
F7.2	M	2.8/0.2–3	65	**82**	**0.56**	**0.49**	**6**	**5.5**	51	**176**	**8**	5.5	No data	HCT, Mg
F8.1	F	3.5/1.5–5	93	**85**	**0.64**	0.74	**17.8**	**6.7**	**112**	**247**	**6.2**	5.3	4.136	HCT, Mg
F9.1	F	8/10–18	**73**	**43**	0.96	**0.67**	**8.8**	3.8	63	**68**	**21.4**	**8.4**	No data	HCT, Mg
F10.1	M	1/0.5–1.5	**42**	93	**0.54**	**0.6**	**5.1**	**6**	44	**138**	**9.9**	5.5	No data	Mg
F11.1	M	3.7/0.3–4	**39**	**73**	**0.29**	**0.55**	**4.3**	3.5	**283**	54	**19.1**	**6**	No data	Mg, potassium citrate
F11.2	M	2/14–16	**89**	93	**0.37**	**0.42**	**4.9**	2	**177**	**81**	**12**	**6.2**	**0.293**	Mg, potassium citrate
F12.1	F	7.5/2–9.5	128	97	**0.45**	**0.64**	**5.8**	**6.8**	**72**	**71**	**21.4**	4.8	3.966	Mg, potassium citrate
F13.1	F	1/2–3	**68**	100	**0.56**	**0.67**	**5**	**6.5**	**104**	**92**	**11.2**	**6.8**	No data	AMI/HCT, Mg
F14.1	F	8/6–14	124	**64**	**0.64**	**0.57**	**11**	2.6	**119**	**210**	**9.8**	**7.6**	1.740	Mg, potassium citrate
F15.1	M	4.5/1–5.5	66	126	**0.6**	**0.29**	**8**	**5.4**	56	64	**7.5**	4.7	**0.373**	HCT, Mg, potassium citrate
F16.1	M	9/8–17	98	**73**	**0.54**	**0.41**	**13.5**	**6.7**	**71**	**98**	**13.6**	5.1	No data	HCT, Mg, potassium citrate
F17.1	F	0.5/0.5–1	73	93	**0.62**	**0.63**	**11.7**	**8.5**	**94**	**87**	**15.3**	5.3	No data	Mg
F18.1	M	3.5/13–16.5	**86**	**25**	**0.48**	**0.5**	**4.5**	1.2	**99**	**191**	**10.6**	**9.2**	**0.389**	Mg, potassium citrate

Values outside the reference range are given in bold. The references are given in the text. HCT, hydrochlorothiazide, AMI, amiloride.

however, HC was still found in 13 (52%) patients. In the study group, the last median 24h-uCa was significantly lower than that obtained initially 4.8 (range 1.2–8.5) and 8.9 (range 4.5–17.8) mg/kg ($P < 0.001$), respectively. 24h-uCa excretion in 18 patients who received HCT is shown in Figure 3 (the results from patient 14.1 were excluded due to early discontinuation of treatment with HCT). In the follow-up period, no significant correlation between 24h-uCa excretion and eGFR was observed ($r = 0.143$).

The 24h-uCa follow-up is shown in Figure 3.

At last follow-up, the median sPTH did not differ significantly from that at initial presentation: 118 (range 54–3977) and 93.6 (range 43.7–840) pg/mL ($P = 0.1$), respectively.

Growth

Median height-for-age percentiles of patients did not significantly differ ($P = 0.63$) between first and last presentation with 30 (2–80) and 40 (2–90), respectively. The number of short-statured patients at first and last observation were 3 (12%) and 4 (16%), respectively.

Median weight-for-age percentiles of patients at first presentation did not significantly differ ($P = 0.28$) from those at last observation with 25 (2–80) and 32 (2–90), respectively.

Complications, co-morbidity

Patient F1.1 developed acute lymphoblastic leukaemia at the age of 9 years, which was successfully treated [22]. She was also diagnosed with a primary hypothyroidism in her late puberty, and developed low grade myopia (−2.5 diopters).

In patient F2.1, extreme hyperparathyroidism and high-grade bilateral slipped capital femoral epiphysis was observed after self-discontinuation of medication [23]. Similar to patient F1.1 he had slight myopia (−1 diopters).

Patient F13.1 suffered from PFAPA syndrome (periodic fever, aphthous stomatitis, pharyngitis, cervical adenopathy).

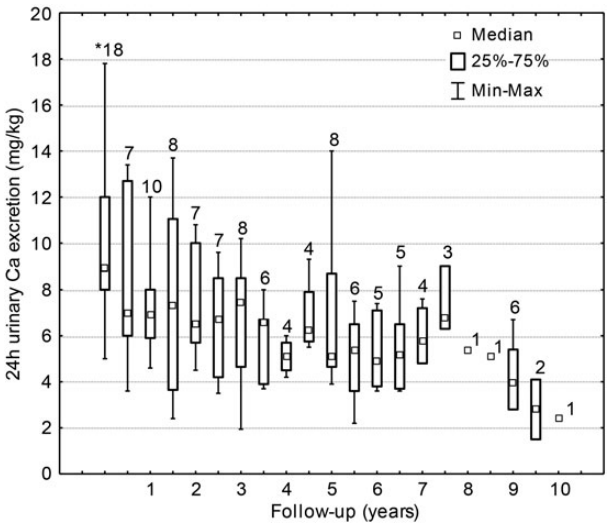

FIGURE 3: Follow-up of 24-h urinary calcium (Ca) excretion during hydrochlorothiazide treatment in 18 patients with FHHNC (*numbers of available results).

DISCUSSION

In this study, we have evaluated 25 paediatric patients from 18 families with FHHNC caused exclusively by *CLDN16* gene mutations.

The majority of previous reports come from other European countries, including Germany, Italy, Switzerland, Serbia, Macedonia, Czech Republic, France, Great Britain, Slovakia, Bulgaria and Portugal [3, 6, 8, 21, 24, 25], followed by Middle-Eastern (Saudi Arabia, Lebanon, Turkey) [3, 7, 21, 26, 27] and North African (Morocco, Algeria, Tunisia, Egypt) countries [3, 8, 21, 28]. Only a few cases were reported in USA [29], East Asia [30, 31] or South Asia [32, 33]. With rare exceptions, mutations in the *CLDN19* gene leading to FHHNC with ocular involvement were almost exclusively identified in Spanish and French populations [8, 9]. Although the true incidence of FHHNC was not estimated, the disorder seems to be one of the most frequent inherited tubulopathies and important genetic cause of NC and urolithiasis leading to CKD [34, 35]. Independently of Polish NEPHROCALCINOSIS registry FHHNC patients were also reported to the Polish Registry of Inherited Tubulopathies (POLtube). Based on this latter database, FHHNC with a frequency of 22.9% is currently the most common disorder among inherited tubulopathies diagnosed in Poland (unpublished). Since this finding is somewhat surprising in comparison to other European registries, we cannot exclude overrepresentation of FHHNC due to low detection rate of other, clinically less characteristic tubulopathies such as Gitelman syndrome or Dent disease.

The clinical course of FHHNC is variable. In our study, initial clinical symptoms of FHHNC develop in infancy, but they are usually mild and non-specific. Similar to other studies [1, 3], the patients present with polyuria/polydipsia due to impaired urinary concentrating ability, failure to thrive, abdominal pain, vomiting and occasionally with tetany related to hypomagnesaemia. The most common urine analysis finding was sterile leucocyturia, most likely reflective of FHHNC-related tubulointerstitial injury. Rarely, urinary tract infections or microscopic haematuria were found, partly explaining a significant delay of clinical diagnosis in some cases [3, 6–8]. However, an insufficient awareness for this rare disease is likely contributing to the delayed diagnosis.

Bilateral medullary NC is one of the hallmarks of FHHNC and a very important diagnostic clue [3, 6–8]. In children, this feature is also observed in rare diseases including distal renal tubular acidosis (dRTA), primary hyperoxaluria, idiopathic infantile hypercalcemia (IIH), Bartter syndrome, Dent's disease, Lowe syndrome, hypophoshataemic rickets with hypercalciuria (HHRH) and other conditions as idiopathic HC, vitamin D intoxication or NC in preterm infants [36, 37]. In our group, NC was found in all patients, even within the first months of life. In 96% of our patients, NC was severe, reaching grade 2b or 3 according to *Hoyer's* grading [19]. The precise mechanism of calcium accumulation in renal parenchyma in FHHNC is not fully understood, but it is probably related to HC, hypocitraturia and magnesium deficiency [1]. Less frequently, NC may also be accompanied by urolithiasis [3].

In FHHNC, both HC and hypomagnesaemia are caused by impaired paracellular reabsorption of calcium and magnesium in the thick ascending limb of Henle's loop due to molecular defects in tight junctions proteins, claudin-16 and -19 [2, 4]. However, the exact mechanism leading to renal calcium and magnesium loss is still a matter of debate [38].

Although hypomagnesaemia is one of the key features of FHHNC [1, 3, 7, 8], it was absent in 34% of our patients at initial examination and, in some patients, even during follow-up. This phenomenon was also observed in other patients with FHHNC and it was not related to the decreased GFR [6, 29]. In contrast, at diagnosis, all our patients showed increased urinary FEMg%. Therefore, to avoid a misdiagnosis, we recommend a calculation of FEMg% in all cases of NC with HC, especially in CKD stage 1-2.

Secondary hyperparathyroidism was initially diagnosed in 76% of our patients, similar to other studies [3]. This was usually explained by HC but the exact mechanism seems to be more complex. In accordance to previous reports [1, 8, 39], we have found that in patients with FHHNC and renal failure, sPTH levels are higher than expected for the stage of CKD.

At the time of diagnosis, we found a high number of patients with mild hyperuricaemia (56%) and hypocitraturia (60%). Increased sUA level could be explained as a result of deterioration in GFR, however, one patient (F14.1) showed hyperuricaemia despite of normal renal function. Therefore other disturbances of UA metabolism may be implicated.

Citrate is a well-known crystallization inhibitor of calcium-oxalate and calcium-phosphate salts in urine. Thus, decreased uCit excretion is an important risk factor for urolithiasis and NC [40]. This feature was found in the majority of patients with FHHNC [3, 39], but its mechanism is not clear. It could be related to an acidification defect as seen in incomplete distal renal tubular acidosis, which is frequently associated with FHHNC [3, 41]. Unfortunately, urinary acidification capacity was not routinely tested in our study cohort.

The major long-term complication of FHHNC is the development of progressive renal failure. At the time of diagnosis, eGFR was already decreased in 60% our patients with a median of 86 mL/min/1.73 m^2 for children >2 years, reaching CKD stage 3 in 4% of them.

The pathogenesis of CKD in FHHNC is not fully understood [3, 8]. It was previously explained by chronic tubulointerstitial nephropathy attributed to HC and NC [1]. However, other disorders associated with NC (except of primary hyperoxaluria) rarely lead to renal failure in childhood. In addition, no clear correlation between CKD progression and the degree of NC in FHHNC was found [21]. Therefore, other mechanisms related to specific type of *CLDN16* mutations possibly resulting in abnormal nephron development have been suggested [21, 42].

In our cohort, nine different mutations of the *CLDN16* gene were detected. In accordance with a previous case series of Central and Eastern European countries [3], the most frequent mutation was a missense variant Leu151Phe, present in 72% of cases. This mutation previously has been characterized as a widespread founder mutation by extended haplotype analysis of the CLDN16 gene locus. Weber *et al.* [43] could demonstrate that the Leu151Phe mutation is embedded in a shared haplotype excluding the alternative possibility of a mutation hotspot. Its high frequency in FHHNC renders molecular screening feasible and inexpensive. In this context, it is also important to point out that the functional characterization of this Leu151Phe mutation revealed a significant residual function of claudin-16 which is associated with less rapid decline of GFR when compared with loss-of-function mutations [21].

To date, there are no published guidelines for the management of patients with FHHNC. Similar to other studies [1, 6–8, 39], the majority of our patients were treated symptomatically with various combinations of thiazides (alone or in combination with amiloride), magnesium preparations and potassium citrate. Additionally, a high fluid intake and dietary salt restriction were advised. Thiazide diuretics are widely and successfully administered to reduce urinary calcium excretion. The pharmacological effect is based on extracellular volume depletion indirectly enhancing passive paracellular calcium reabsorption in the proximal tubule [44]. In contrast to idiopathic HC [45], many patients with FHHNC seem not to respond to this therapy [1, 8]. However, the hypocalciuric effect of HCT in FHHNC was shown in longitudinal study [39] and then confirmed by short-term prospective clinical trial [46]. In line with these observations, the vast majority of our patients treated with HCT demonstrated a significant lowering of calciuria and almost 50% of them became normocalciuric at last observation. However, the efficacy of HCT treatment on the natural course of FHHNC is still not known, especially with regard to the decline of GFR. In this study, we could not demonstrate a significant influence on GFR. Even if no proven side effects of HCT therapy were reported in this study, in one patient (F14.1) HCT treatment seemed to decrease eGFR and therefore it was discontinued. In this context, it is also important to point out that treatment with HCT that blocks the reabsorption of NaCl in the distal convoluted tubule (DCT) bears the potential risk of counteracting adaptive processes in this part of the nephron which aim at the increased conservation of NaCl primarily lost in the TAL in the context of the primary disease. Therefore, FHHNC could be at a higher risk of acute dehydration in the context of intercurrent illness such as prolonged fever or gastroenteritis.

In our patients, the effect of magnesium supplementation on serum Mg level was rather disappointing. During follow-up, no significant increase in median sMg level was found and only 20% of patients showed normomagnesemia at the last observation. Inability to compensate urinary Mg losses by oral magnesium administration in patients with FHHNC was also reported by others [1, 8, 39]. It could be theoretically aggravated by a magnesiuric effect of thiazides [47]. Therefore, according to some historical reports, concomitant treatment with amiloride might be beneficial [48, 49]. Moreover, in a recent clinical trial in patients with Gitelman syndrome, another magnesium-losing tubular disorder, the Mg excretion significantly decreased in adult patients treated with amiloride [50]. However, the exact mechanism leading to conservation

of Mg^{2+} of this potassium-sparing diuretic is unclear. Because only a part of our patients was temporarily treated with hydrochlorothiazide/amiloride formulation, we were not able to assess its effect. The symptomatic treatment of our patients did not significantly decrease median serum PTH level. However, during follow-up, despite of significant deterioration of eGFR, median serum PTH did not increase and at the last observation, 36% of patients showed even lower values than initially. The influence of treatment was particularly evident in patients F1.1 and F2.1. In the former, starting of therapy at the age of 14 years due to delayed diagnosis of FHHNC led to the very significant lowering of serum PTH [22], whereas in the latter one, a self-discontinuation of treatment resulted in critical hyperparathyroidism with serum PTH level of 3977 pg/mL and high-grade bilateral slipped capital femoral epiphysis [23]. Unfortunately, in other short- and long-term follow-up studies, the course of serum PTH in patients with FHHNC was not investigated [39, 46].

Although the majority of our patients received potassium citrate, the urinary citrate excretion was not commonly measured due to limited laboratory availability. Thus, a follow-up of this parameter was impossible. However, as other authors [39], we would advocate this treatment as prevention from urolithiasis and progression of NC in patients with confirmed hypocitraturia.

In our patients, despite of treatment, a slow progression of CKD was observed. Last median eGFR in children who started follow-up at the age >2 years was significantly lower than that at initial presentation, 69 mL/min/1.73 m^2 and 86 mL/min/1.73 m^2, respectively. At the end of follow-up, 8% of patients reached 3rd whereas only one patient developed 4th (F18.1) and one 5th (F2.1) grade of CKD. Thus, the progression of CKD in our patients was slower than observed in other studies showing ESRD in 30% of patients at 15 years and 50% of patients at 20 years of age [21].

In summary, this study represents one of the largest case series of FHHNC caused by *CLDN16* mutations and likely is the largest cohort reported from one country so far. We confirm the high incidence of the missense Leu151Phe mutation, an important founder mutation in Central Europe. Although the clinical picture of FHHNC in Polish patients is generally comparable with previous descriptions, the results provide some specific insights, mainly into diagnostic and therapeutic considerations. We have found that normomagnesaemia does not exclude FHHNC and calculation of FEMg% in all cases of NC with HC is critical to confirm the diagnosis in the setting of normomagnesaemia. Our data demonstrate the efficacy of thiazides in reducing calcium excretion in the majority of patients, although our observations are limited by the retrospective nature of data. Of note, with respect to the concomittant salt loss observed in FHHNC, the potential benefits of thiazides need to be seen in the context of possible side effects, especially the increased risk of acute dehydration because of the primary gene defect which impairs salt reabsorption in the TAL. FHHNC is a rare, slowly progressive kidney disease and well-designed multicentre prospective studies are clearly needed to better evaluate the available treatment strategies.

CONFLICT OF INTEREST STATEMENT

None declared.

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Received for publication: 12.8.2014; Accepted in revised form: 6.11.2014