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CLINICAL AND GENETIC SPECTRUM OF BARTTER SYNDROME TYPE 3

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1 **ABSTRACT**

2

3 **Background:** Bartter syndrome type 3 is a clinically heterogeneous hereditary salt-losing
4 tubulopathy. It is caused by mutations of *CLCNKB*, which encodes the ClC-Kb chloride
5 channel involved in NaCl reabsorption in the renal tubule. We report phenotype/genotype
6 correlation and follow-up for a large cohort of patients.

7 **Methods:** Genetic analyses were performed by direct sequencing and multiplex ligation-
8 dependent probe amplification; medical charts were analyzed retrospectively for 115 patients
9 with *CLCNKB* mutations. Functional analyses were performed in *Xenopus laevis* oocytes for
10 eight missense and two nonsense mutations.

11 **Results:** Sixty mutations, including 27 previously unreported mutations, were detected. The
12 phenotype was ante/neonatal Bartter Syndrome (polyhydramnios or diagnosis in the first
13 month of life) in 30% of cases, classic Bartter Syndrome (diagnosis during childhood,
14 hypercalciuria and/or polyuria) in 45% and Gitelman-like syndrome (fortuitous discovery of
15 hypokalemia with hypomagnesemia and/or hypocalciuria in childhood or adulthood) in 25%.
16 Nine of the 10 mutations expressed *in vitro* decreased or abolished chloride conductance.
17 Severe (large deletions, frameshift, nonsense, and essential splicing) and missense mutations
18 resulting in poor residual conductance were associated with younger age at diagnosis.
19 Electrolyte supplements and indomethacin were frequently used to induce catch-up growth
20 with few adverse effects. After a median follow-up of 8 years (1-41) in 77 patients, chronic
21 renal failure was detected in 19 patients (25%): One required hemodialysis and four
22 underwent renal transplantation.

23 **Conclusion:** We report the first genotype/phenotype correlation for Bartter syndrome type 3:
24 Complete loss-of-function mutations were associated with younger age at diagnosis. Chronic
25 kidney disease was observed in all phenotypes.

1 INTRODUCTION

2 Bartter syndromes (BS) and Gitelman syndrome (GS) are autosomal recessive salt-losing
3 tubulopathies caused by defective salt reabsorption. They are characterized by hypokalemia,
4 metabolic alkalosis, and secondary aldosteronism, with normal or low blood pressure^{1,2}. BS
5 are classified by phenotype (antenatal, classic) or genotype (types 1 to 5). Antenatal BS
6 (ABS) is the most severe form, characterized by polyhydramnios, premature birth, life-
7 threatening episodes of neonatal salt and water loss, hypercalciuria, and early-onset
8 nephrocalcinosis³. Classic BS (CBS) occurs in infancy or early childhood and is characterized
9 by marked salt wasting and hypokalemia, leading to polyuria, polydipsia, volume contraction,
10 muscle weakness, growth retardation and, sometimes, nephrocalcinosis⁴. BS types 1, 2, and 3
11 are caused by mutations of genes expressed in the thick ascending limb of Henle's loop
12 encoding the luminal Na⁺-K⁺-2Cl⁻ cotransporter (*SLC12A1*; OMIM #601678), the luminal K⁺
13 channel ROMK (*KCNJ1*; OMIM #241200), and the basolateral chloride channel ClC-Kb
14 (*CLCNKB*; OMIM #607364), respectively⁵⁻⁷. Loss-of-function mutations of *BSND*, encoding
15 barttin, an essential beta subunit for chloride channels, cause BS type 4a with sensorineural
16 deafness (OMIM #602522)⁸. Simultaneous mutations of *CLCNKB* and *CLCNKA* cause type
17 4b BS (OMIM #613090)⁹. Finally, severe gain-of-function mutations of the extracellular
18 Ca²⁺-sensing receptor gene can result in a Bartter-like syndrome (BS type 5, OMIM
19 #601199)^{10,11}. GS (OMIM #263800) is a milder disease frequently associating
20 hypomagnesemia and hypocalciuria. GS is often asymptomatic or associated with mild
21 symptoms such as muscle weakness, salt craving, paresthesia, and tetany. GS is related to
22 loss-of-function mutations of the *SLC12A3* gene encoding the apically expressed thiazide-
23 sensitive NaCl cotransporter (NCC) of the distal convoluted tubule (DCT)¹².
24 The first BS3 patients described had a clinical phenotype corresponding to CBS⁷.
25 Considerable phenotypic variability has since been described: *CLCNKB* mutations can also

1 underlie the ABS, neonatal BS (NBS), and Gitelman-like (GLS) phenotypes¹³⁻¹⁵. This study
2 aimed to shed light on the phenotypic heterogeneity of BS3 by investigating phenotype-
3 genotype correlations in a very large French cohort and by evaluation of published results and
4 original data for *in vitro* expression.

5

6 **RESULTS**

7 **Population**

8 We retrospectively analyzed results for 115 patients (56 male and 59 female patients) from
9 111 families with *CLCNKB* mutations evaluated at the Genetics Department of Georges
10 Pompidou European Hospital over the last 15 years. A history of consanguinity was recorded
11 for 22 families; the geographic origin is shown in Supplementary Tables 1 to 3.

12 **Initial clinical presentation**

13 Thirty-four patients from 32 families (29.5%) presented with A/NBS, 51 patients from 49
14 families (44.5%) presented with CBS, and 30 patients from 30 families (26%) presented with
15 GLS.

16 **Mutations and Large Rearrangements**

17 Genetic status and mutation type were determined for each initial phenotype group (Table 1).
18 The detailed genotypes of each patient are summarized in Supplementary Tables 1 to 3. The
19 deletion of a single allele was excluded in patients with homozygous point mutations and no
20 consanguinity, and molecular abnormalities of the other genes implicated in GS and BS were
21 excluded in patients with only one heterozygous mutation. The breakpoints of large
22 rearrangements were not characterized; in consequence, we cannot exclude the possibility that
23 patients with homozygous deletions from non-consanguineous families harbored two different
24 deletions. Testing was carried out for both parents in 22 families and only the mother in seven

1 families. In all cases, parents were heterozygous for the homozygous mutation detected in the
2 proband or for one of the two mutations detected in compound heterozygous probands.

3 Sixty different mutations were detected: 55% missense, 13% frameshift, 12% nonsense, 10%
4 large deletions, and 10% splice-site mutations (Figure 1). Twenty-seven of these mutations
5 were previously unknown (Figure 2A and B, Supplementary Tables 1 to 3). Two of the three
6 splice-site mutations disrupt the obligatory consensus donor or acceptor splice site and were
7 considered pathogenic as likely to cause exon skipping and frameshift. The variant at position
8 -6 in the acceptor site of exon 14 is a known rare variant (rs369329893, allele frequency in
9 African populations of 0.02%) for which MaxEntScan predicts a 100% decrease in splice-site
10 score and SpliceSiteFinder predicts activation of an intronic cryptic acceptor site.
11 Unfortunately, no mRNA from this patient was available for analysis.

12 Two of the 13 previously unreported missense mutations were present in the same allele in
13 patient BR050 (p.Arg395Trp and p.Ala469Pro), and p.Gly465Arg was detected in the same
14 allele as the known mutation p.Pro124Leu in three patients (BR116-1, BR157-1, and GT657-
15 1). Eight of the 13 missense variations affected conserved amino acids and were predicted by
16 at least four out of five tools used for *in silico* analysis as potentially pathogenic. The
17 remaining five missense changes (p.Ser218Asn, p.Ala254Val, p.Arg395Trp, P.Ile447Thr, and
18 p.Ala469Pro) were classed as variations of unknown significance (VOUS) (Supplementary
19 Table 4). Among these changes, only the p.Arg395Trp has been described in databases
20 (rs34255952) with an allelic frequency of 2% in African Americans and has not been detected
21 in European Americans. Of the 33 missense mutations detected in our population, 13 were
22 previously shown to result in loss of function¹⁶. *In silico* predictions are presented in
23 Supplementary Table 5 for missense mutations for which *in vitro* analysis was not performed.

24 **Functional expression of CIC-Kb mutants in *Xenopus* oocytes**

1 We investigated the effect of two new missense mutations predicted to be pathogenic
2 (p.Gly345Ser, p.Ala510Thr), two new VOUS (p.Arg395Trp, p.Ala469Pro), four previously
3 described missense mutations (p.Gly296Asp, p.Ser297Arg, p.Gly424Arg, and p.Gly433Glu)
4 and two nonsense mutations (p.Trp391Ter and p.Arg595Ter) on chloride conductance in
5 *Xenopus* oocytes; p.Gly424Arg and p.Gly433Glu are located in α -helix N of ClC-Kb, which
6 is involved in the selectivity filter; p.Gly296Asp and p.Ser297Arg are located in the α -helix J,
7 which interacts with barttin; p.Ala510Thr is found in the α -helix Q involved in the dimer
8 interface, and Arg595Ter is present in the CBS1 domain involved in channel common gating
9 and trafficking. The p.Gly345Ser and p.Ala469Pro mutations affect α -helices K and O,
10 respectively, and the p.Trp391Ter and p.Arg395Trp mutants affect the L-M linker (Figure
11 2B). Nine of these 10 mutations significantly decreased or abolished normalized conductance
12 (Figure 3). The p.Trp391Ter, p.Gly296Asp, p.Gly424Arg, p.Gly433Glu, p.Ala469Pro, and
13 p.Arg595Ter mutations abolished conductance, whereas p.Ser297Arg, p.Gly345Ser and
14 p.Arg395Trp decreased conductance to 61%, 57%, and 65% of wild-type values, respectively
15 (significantly different from oocytes expressing WT ClC-Kb and non-injected oocytes). By
16 contrast, p.Ala510Thr had no influence on channel conductance. Finally, the
17 p.Arg395Trp/p.Ala469Pro double mutation decreased conductance to 37% of wild-type
18 values.

19 **Clinical Data at Diagnosis**

20 Table 2 summarizes clinical and biochemical characteristics at birth and at diagnosis. As
21 expected, gestational age (GA) at birth was significantly lower in the A/NBS group than in
22 the CBS and GLS groups, but similar between the CBS and GLS groups. Age at diagnosis
23 was significantly lower in the A/NBS group than in the other two groups and in the CBS
24 group than in the GLS group. Polyhydramnios was found in 29 patients with A/NBS (85%),
25 at mean GA of 28 weeks, and amniotic fluid had to be drained in four cases. Ten patients (5

1 A/NBS and 5 CBS) had birth weights below the 10th percentile, and four patients (2 A/NBS
2 and 2 CBS) had birth heights below the 10th percentile for gestational age at birth.

3 Plasma sodium and chloride concentrations were significantly lower and plasma renin and
4 magnesium concentrations were significantly higher in CBS and A/NBS patients than in GLS
5 patients; plasma potassium and total CO₂ concentrations were similar in all groups. Strong
6 hypochloremia is a known phenotypic hallmark of BS3¹⁷⁻¹⁹. We therefore compared the
7 relationship between plasma sodium and chloride concentrations between patients with BS
8 types 1 and 2 (*n*=21) and patients with BS3 (*n*=51). This curve was shifted downwards in BS3
9 patients, indicating that plasma chloride depletion could not be accounted for by
10 hyponatremia (Figure 4). No difference was observed in parameters at diagnosis when
11 confirmed homozygous patients are compared with compound heterozygous patients (data not
12 shown).

13 **Clinical and Biological Data during Follow-up**

14 Clinical manifestations during follow-up and treatment were recorded for 77 patients (Table
15 3). Median follow-up was eight years and was similar in the three groups. The main
16 treatments administered to these patients were NaCl and KCl supplementation and non-
17 steroidal anti-inflammatory drugs (NSAIDs, mainly indomethacin). The main adverse effects
18 were abdominal pain (*n*=5), weight gain (*n*=1), esophagitis (*n*=1), and diarrhea (*n*=1). One of
19 the main criteria of a successful treatment is a normal growth; in this cohort, 63 out of 77
20 patients (82%) had a height between -2 SD and +1 SD or a normal height as adults. Fourteen
21 patients had height below -2SD: five A/NBS patients including one with GH deficiency (IGF1
22 = 38 ng/ml before GH initiation at 15 years of age), eight CBS patients including two with
23 GH deficiency and two with chronic kidney disease (CKD), and one GLS patient with no
24 identified cause of failure-to-thrive.

1 Abnormalities in psychomotor and neurological development included psychomotor
2 retardation in four A/NBS and four CBS patients (Table 3). Five CBS patients required
3 psychiatric follow-up (hyperactivity, anorexia, or eating disorders).

4 Irregular heart rate or ECG abnormalities were documented in six patients: one A/NBS patient
5 had premature ventricular beats with prolonged QT interval, three CBS patients had a right
6 bundle branch block or U wave, and one GLS patient presented torsade de pointe attacks.
7 None of the patients in this cohort had high blood pressure.

8 Fourteen patients developed nephrolithiasis or nephrocalcinosis during follow-up. None of the
9 patients required shock-wave lithotripsy. Other renal and urological abnormalities diagnosed
10 in eight A/NBS and nine CBS patients are detailed in Table 3. Proteinuria data were available
11 for 43 patients, nine of whom displayed glomerular proteinuria over 50 mg/dl.

12 Nineteen (10 female and 9 male patients) of the 77 patients (25%) presented CKD (Table 4).
13 Ten patients presented stage 2 CKD: four A/NBS, four CBS, and two GLS patients. Two
14 patients reached stage 3 CKD (1 A/NBS and 1 GLS). One CBS patient reached stage 4 CKD.
15 Six patients reached stage 5 CKD (3 A/NBS and 3 CBS patients), at a mean age of 25 years (6
16 - 49). Renal biopsies were performed in five out of 19 patients with CKD. Four patients with
17 stage 5 CKD had diffuse glomerular and tubulointerstitial lesions with enlarged glomeruli
18 presenting focal lesions of segmental glomerulosclerosis/hyalinosis (FSGS). One patient with
19 stage 2 CKD had minimal glomerular and tubular alterations (Table 4 and Supplementary
20 Table 6). Patients with CKD were older than patients without CKD; they did not differ in
21 terms of birth weight, AINS treatment, urologic or renal abnormalities, or hypokalemia
22 severity (Table 6).

23 The last eGFR follow-up data for 30 patients with BS1, 34 patients with BS2, and 11 patients
24 with BS4a were compared with eGFR for the 77 patients with BS3. These groups had similar
25 age distribution, and eGFR decreased with age (Supplementary Figure 1). In BS1 and BS4

1 patients, eGFR decrease was more severe and there were higher proportions of patients with
2 CKD 3 to 5 than in BS2 and BS3 (Supplementary Figures 1 and 2).

3 **Genotype/Phenotype Correlation**

4 Large deletions were more frequent in patients with earlier onset, and severe phenotypes and
5 missense mutations were more common in the GLS phenotype (Table 1). Similar results were
6 obtained if other potentially severe mutations (frameshift, nonsense, and essential splicing)
7 were considered with large deletions: Severe mutated alleles were more frequent in A/NBS
8 and CBS (74 and 66% respectively) patients than in GLS patients (42%). Further, missense
9 mutations were more frequent in patients with less severe phenotypes: 58% in GLS patients,
10 versus 34% and 26% in CBS and A/NBS patients, respectively.

11 We classified mutations into two groups: complete loss-of-function (CL) and partial loss-of-
12 function (PL) groups (Table 5). We included p.Trp610Ter, the only C-terminus-truncating
13 mutation expressed *in vitro* and yielding a residual current²⁰. Each mutated allele was
14 classified separately, independently of the initial phenotype, and only patients for whom both
15 alleles could be classified were analyzed ($n=85$) (Supplementary Tables 1 to 3). CIC-Kb
16 functions as homodimer. The residual activity of the CL/PL genotypes may therefore
17 correspond to homodimers of PL mutants, with similar consequences to PL/PL genotype. We
18 therefore analyzed CL/PL genotypes together with PL/PL genotypes. With this classification,
19 56 patients had a CL/CL genotype, and 29 patients had a CL/PL or PL/PL genotype. CL/CL
20 genotypes were associated with a significantly younger age at diagnosis than CL/PL and
21 PL/PL genotypes. No difference was observed for the other biological parameters analyzed
22 (Table 5).

23 **DISCUSSION**

24 Bartter syndromes are phenotypically and genotypically heterogeneous. Phenotype/genotype
25 correlations highlighting the link between particular traits and genetic types (*i.e.* transitory

1 hyperkalemia in BS2, severe hypochloremia in BS3, and hearing loss in BS4) have been
2 identified in previous studies^{17,19}. BS3 is particularly heterogeneous in terms of clinical
3 presentation, accounting for the diverse initial diagnoses attributed (A/NBS, CBS, or GLS).
4 We investigated the basis of this variability in a cohort of 115 patients harboring *CLCNKB*
5 mutations, and studied the phenotype/genotype correlation based on clinical presentation and
6 follow-up as well as on *in vitro* functional studies of missense mutants.

7 More than 54 mutations of this gene have been reported in free access HGMD
8 (www.hgmd.cf.ac.uk) and scientific publications^{4,7,17, 19, 21-25}. They include a high frequency
9 of large rearrangements favored by the close location of the homologous *CLCNKA*. We
10 detected 60 different mutations, 27 of which had not been previously reported (13 missense, 5
11 frameshift, 3 nonsense, 3 splice-site mutations, and 3 large deletions). Thirteen of these
12 mutations (frameshift, nonsense, splice-site mutations, and large deletions) were predicted to
13 result in the production of unstable mRNAs or truncated or absent proteins. Eight of the 13
14 previously unknown missense mutations were predicted to be pathogenic *in silico*
15 (Supplementary Table 4). Three out of other five, classified as VOUS, were expressed in *X.*
16 *laevis* oocytes (p.Arg395Trp, p.Ala469Pro, and p.Gly345Ser) as were two previously
17 described mutations (p.Gly424Arg and p.Gly433Glu) detected as the only heterozygous
18 mutation in two patients. All these mutations significantly decreased chloride conductance.
19 The p.Ala510Thr, predicted *in silico* as pathogenic had a chloride conductance similar to that
20 of the wild-type channel. The molecular abnormality of patient heterozygous for this variant
21 thus remains unidentified.

22 In this large BS3 cohort, we confirmed the phenotypic variability, consisting of about 30%
23 A/NBS, 45% CBS, and 25% GLS (Table 2). In order to determine if the type of mutation
24 influences the phenotype, we first correlated them with initial clinical presentation. Large
25 deletions and severe mutations were associated with all clinical presentations, but were more

1 frequent in A/NBS and CBS. Next, eighty-five patients with two mutated alleles were
2 analyzed for phenotype-genotype correlations, taking into account the type of mutation and *in*
3 *vitro* expression results, regardless of initial clinical presentation. Patients with complete loss-
4 of-function (CL/CL) were significantly younger at diagnosis than patients harboring one or
5 two alleles with a partial loss-of-function (CL/PL or PL/PL), suggesting that the type of
6 mutation may influences the clinical presentation of BS3.

7 Surprisingly, the milder GLS phenotype did occur in patients harboring severe mutations or
8 deletions, suggesting that the phenotype severity is not only driven by *CLCNKB* allelic
9 variability. Phenotypic heterogeneity of BS3 has been attributed to distribution of the ClC-Kb
10 channel along the nephron and to possible compensatory function of the ClC-Ka channel.
11 ClC-Kb channel is expressed in the thick ascending limb (TAL), distal convoluted tubule
12 (DCT), and collecting duct, where it transfers chloride (Cl⁻) ions to the basolateral side²⁶.
13 Impaired ClC-Kb function in the TAL results in lower levels of Cl⁻ exit, NaCl reabsorption
14 through the Na-K-2Cl cotransporter, and divalent cation reabsorption, accounting for the
15 Bartter phenotype. Defective basolateral Cl⁻ exit in the DCT decreases NaCl reabsorption via
16 the Na-Cl cotransporter (NCC), accounting for the GLS phenotype in other patients. Two
17 recent studies in which the mouse *Clcnk2* gene (corresponding to *CLCNKB* in humans) was
18 disrupted confirmed that ClC-K2 is the principal chloride channel in all three nephron
19 segments and that TAL impairment is not compensated by ClC-K1 (corresponding to the
20 human ClC-Ka channel, which is also expressed in the TAL)^{27,28}. Nevertheless, it cannot be
21 excluded that allelic variants of genes encoding KCl cotransporters or other chloride channels
22 may also compensate for renal sodium loss accounting for phenotypic variability^{27,29}.

23 Next generation sequencing (NGS) approaches allow parallel analysis of several genes, which
24 is particularly useful in diseases with genetic heterogeneity, such as ABS, or in diseases with
25 phenotypic variability such as the BS type 3. A genetic confirmation is important for the

1 follow-up as well as to improve our knowledge of the natural history of these syndromes. In
2 consequence we recommend the use of NGS panels to diagnosis confirmation. NGS could
3 also be useful to determine whether additional genes are involved in the observed clinical
4 variability. *In vitro* studies of additional mutant proteins can contribute in the future to
5 improve our understanding of the phenotype/genotype correlation and of the precise
6 pathogenic mechanism of mutants. These studies have the potential interest to define targeted
7 therapeutic approaches such as channel openers or pharmacological chaperones^{30,31}.

8 Despite missing data for some phenotypic criteria due to the retrospective nature of this study,
9 several patterns emerged from our analysis. First, growth retardation was common but
10 frequently improved with treatment. Fourteen patients presented with persistent growth
11 retardation; two of these patients had CKD, a common cause of growth retardation due to a
12 combination of abnormalities of the growth hormone axis, vitamin D deficiency,
13 hyperparathyroidism, inadequate nutrition, and drug toxicity and three patients presented with
14 growth hormone (GH) deficiency³². BS and potassium deficiency have already been reported
15 to be associated with GH deficiency³³⁻³⁵. One previous study showed that GH and IGF1 did
16 not stimulate longitudinal growth unless hypokalemia was corrected³⁶. In two patients with
17 hypokalemia (median=2.5 mmol/L), growth improved after GH supplementation but
18 remained below -2SD.

19 Second, hypochloremia is a hallmark of BS3: An analysis of the data available at diagnosis
20 showed that hypochloremia was more severe in A/NBS and CBS patients than in GLS
21 patients. ClC-Kb is expressed not only in the diluting segment but also in the intercalated cells
22 of the collecting duct (CD). Defects in this segment may impair chloride exit and
23 transepithelial chloride reabsorption through the pendrin Cl/HCO₃ exchanger, potentially
24 accounting for the stronger chloride depletion in BS3 patients than in BS1 or BS2 patients¹⁹.
25 We found a downward shift of the relationship between plasma chloride and sodium

1 concentrations consistent with a defect in adaptation to chloride depletion in BS3 as compared
2 to BS1 and BS2 patients. These results are consistent with the phenotype of mice with *Clnk2*
3 disruption²⁷ and suggest that sodium and potassium supplementation should be provided as
4 chloride salts in BS3 patients.

5 Third, nineteen patients presented CKD, and seven of these patients also had proteinuria
6 (Table 4). Five patients underwent renal biopsy, which revealed diffuse glomerular and
7 tubulointerstitial lesions with enlarged glomeruli in four cases, suggesting compensatory
8 hypertrophy to nephron reduction (Table 4 and Supplementary Table 6). Six patients
9 diagnosed before the age of 8 years displayed progression to ESDR at a median age of 24
10 years, associated in four cases with FSGS. Proteinuria, a low glomerular filtration rate and
11 FSGS have been reported in BS and GS patients^{22,37-39}. It has been suggested that FSGS is a
12 secondary lesion due to adaptation to salt loss, resulting in chronic stimulation of the renin-
13 angiotensin system^{37,39,40}. In the present study FSGS occurred in late stage of CKD suggesting
14 a large contribution of nephron reduction. We failed to identify other risk factors of CKD
15 progression including birth weight, age at diagnosis, long-term NSAID treatment, persistent
16 hypokalemia, and other renal abnormalities (Table 6). CKD has been described in other types
17 of BS^{19,22}. In our BS cohort, CKD was observed in all BS types but the proportion of patients
18 with preserved renal function (i.e., eGFR > 90 ml/min/1.73 m²) was higher in patients with
19 BS types 2 and 3 and the proportion of patients with moderate to severe kidney disease (i.e.,
20 eGFR < 60 ml/min/1.73 m²) in patients with BS types 1 and 4 suggesting that the laterBS
21 subtypes 1 and 4 are associated with more severe renal prognosis. The mechanism of CKD
22 development is probably multifactorial, and its elucidation will require prospective studies.

23 Case reports are rare for BS patients undergoing renal transplantation. The post-
24 transplantation period was uneventful in our four patients, with the complete disappearance of
25 BS and no recurrence of FSGS, as previously described⁴¹⁻⁴³.

1 In conclusion, BS3 syndrome, which is caused by *CLCNKB* mutations, is highly variable
2 phenotypically. We show, for the first time, that there is a correlation between severe
3 mutations and a significantly younger age at diagnosis, suggesting that milder defects of ClC-
4 Kb function may account for some of this variability. We also confirmed the severe chloride
5 depletion previously observed in BS3 patients and report that 25% of cases suffer from CKD.
6 Long-term prospective follow-up of this cohort will identify other severity parameters
7 involved in this genotype/phenotype correlation and will allow us to evaluate whether early
8 diagnosis and treatment have an influence on the evolution to CKD.

9

10 **CONCISE METHODS**

11 **Patients**

12 The study included 115 patients (from 111 families) with *CLCNKB* mutations referred to the
13 Genetics Department of Georges Pompidou European Hospital (Paris, France) from January
14 2001 to December 2014 for genetic analysis after the diagnosis of BS or GS. The study was
15 approved by the "Comité de Protection des Personnes, Paris-Île de France XI (Ref. 09069)"
16 and informed consent for genetic studies was obtained from each proband or from their
17 parents for minors. Genetic investigations were performed after the clinical and biological
18 diagnosis of salt-losing tubulopathy. Patients with a history of polyhydramnios or clinical
19 manifestations in the first month of life were considered to have A/NBS. Patients diagnosed
20 during childhood, with hypercalciuria and/or polyuria, were considered to have CBS, and
21 children, adolescents, or adults for whom hypokalemia and hypomagnesemia and/or
22 hypocalciuria were discovered fortuitously were considered to have GLS. Genetic
23 investigations were extended to both parents in 22 families and to the mother only in another
24 seven families. Twenty-three patients from this cohort have been described before^{19,23,25}
25 (Supplementary Tables 1 to 3).

1

2

3 **Detection of point mutations**

4 DNA was extracted with a salt-based method or with blood DNA midi kits (Qiagen, Venlo,
5 the Netherlands). *CLCNKB* exons and flanking intron sequences were amplified by PCR,
6 sequenced with BigDye Terminator v3.1 cycle sequencing kits and run on an ABI Prism
7 3730XL DNA Analyzer (Perkin Elmer Applied Biosystems®, Foster City, CA, USA), as
8 previously described (16). DNA mutations were identified with Sequencher software, by
9 comparison with the reference sequence for *CLCNKB*: NM_000085.4. Each mutation was
10 confirmed by sequencing a second independent PCR product.

11 **Detection of large rearrangements**

12 Large rearrangements were detected by quantitative multiplex PCR of short fluorescent
13 fragments (QMPSF) before June 2010 and by multiplex ligation-dependent probe
14 amplification (MLPA) thereafter. We adapted the QMPSF method for the detection of large
15 deletions of *CLCNKB*⁴⁴. The procedure is described in detail in the Supplementary Materials
16 and the primers used, covering all exons, are listed in Supplementary Table 7. For MLPA, we
17 used the SALSA® MLPA® P266-B1 *CLCNKB* Kit (MRC Holland, Amsterdam, the
18 Netherlands). The P136 Kit contains 29 probes: probes for 14 of the 20 exons of *CLCNKB*
19 (exons 4, 7, 9, 12, 16 and 20 are not represented), four probes for upstream genes (*PRM2*,
20 *CASP9* and the homologous *CLCNKA* gene), and 11 reference probes. The procedure is
21 described in detail in the Supplementary Materials.

22 **Bioinformatic analysis of mutations**

23 The software used to interpret variants is described in the Supplementary Methods.

24 **Functional expression in *Xenopus laevis***

1 Voltage clamp experiments were performed as previously described in *Xenopus laevis*
2 oocytes (25, 27). We injected 10 ng ClC-Kb cRNA and 5 ng barttin cRNA into defolliculated
3 oocytes, which were then incubated in modified Barth's solution at 16°C. Two-electrode
4 voltage-clamp experiments were performed at room temperature with TURBO TEC-10CX
5 (npi electronic GmbH, Tamm, Germany) and PClamp 8 software (Axon Instruments, Union
6 City, CA, USA), two to three days after injection. Conductance at +60 mV ($G_{+60\text{ mV}}$) was
7 calculated by dividing the current at +60 mV by the difference in current between +60 mV
8 and the reversal potential.

9 **Statistics**

10 Clinical data were analyzed with GraphPad Prism Software (La Jolla, CA, USA) and SPSS
11 software, release 20.0.0 (SPSS, Chicago, IL, USA). Kruskal-Wallis tests were used to
12 compare the three groups (A/NBS, CBS, and GS). In cases of statistical significance, Mann-
13 Whitney *U* tests were used to compare the groups in pairs. Dichotomous variables were
14 compared using the chi-squared test or Fisher's exact test as appropriate. Variables for which
15 more than 50% of the data were missing in one group were excluded from the analysis.
16 Clinical manifestations were analyzed by descriptive methods, using the number of subjects
17 for each group. Data for *in vitro X. laevis* studies were analyzed by ANOVA and Holm-Sidak
18 tests, with Sigma Stat software.

19

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18

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20

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- 30

1

2 **Figure Legends**

3 **Figure 1:** Mutations of *CLCNKB* detected in Bartter type 3, and classified by type ($n=60$).

4 **Figure 2: Locations of novel *CLCNKB* mutations and of mutations expressed *in vitro*.** A.

5 *CLCNKB* gene structure, showing the newly discovered large deletions and splicing

6 mutations. B. Schematic topological model of the ClC-Kb protein: the lower part of the model

7 corresponds to the intracellular region, and the upper part is extracellular. Each rectangle

8 represents one of the 18 α -helices and the two cystathionine- β -synthase (CBS) domains. The

9 α -helices involved in the selectivity filter, those interacting with Barttin, and those located at

10 the dimer interface are shown in blue, green, and pink, respectively. Previously unknown

11 missense (●) and nonsense (★) mutations are shown in red; previously described mutations

12 are shown in blue (●); mutations expressed *in vitro* are underlined.

13 **Figure 3: Functional studies of selected ClC-Kb mutants (n=10).** Conductance at +60 mV

14 for non-injected oocytes (NI) and for oocytes into which mutant ClC-Kb cRNA was injected,

15 normalized with respect to the mean value for wild-type (WT) ClC-Kb and expressed as the

16 mean \pm SEM. The mutants were exposed to a solution at pH 7.4 containing 10 mM Ca^{2+}

17 (panel A) or to a solution at pH 9.0 containing 20 mM Ca^{2+} (panel B). As ClC-Kb current

18 increases at high external Ca^{2+} concentration or high pH, these solutions were chosen to

19 obtain a submaximal current. \$, $P < 0.05$ for the difference between NI or mutant ClC-Kb and

20 WT; *, $P < 0.05$ for the difference between WT or mutant ClC-Kb and NI. Number of

21 measurements for panel A: NI ($n=63$), WT ($n=109$), p.Trp391Ter ($n=12$), p.Arg395Trp

22 ($n=16$), p.Arg395Trp-p.Ala469Pro ($n=20$), p.Gly424Arg ($n=16$), p.Ala469Pro ($n= 10$). For

23 panel B: NI ($n=9$), WT ($n=16$), p.Gly122Val ($n=6$), p.Gly296Asp ($n=5$), p.Ser297arg ($n= 7$),

24 p.Gly345Ser ($n=8$), p.Gly433Glu ($n=8$), p.Ala510Thr ($n=8$) and p.Arg595Ter ($n=3$).

25 **Figure 4:** Correlation between plasma sodium and plasma chloride concentrations in patients

- 1 with BS type 1 and 2 (black symbol, grey area) and in patients with BS type 3 (white
- 2 symbols, orange area).