Gitelman-like syndrome caused by pathogenic variants in mtDNA

Daan Viering*[1], Karl-Peter Schlingmann*[2], Marguerite Hureaux*[3,4], Tom Nijenhuis*[5], Andrew Mallett[6,7], Melanie MY Chan[8], André van Beek[9], Albertien M van Eerde[10], Jean-Marie Coulibaly[11], Marion Vallet[12], Stéphane Decramer[13], Solenne Pelletier[14], Günter Klaus[15], Martin Kömhoff[16], Rolf Beetz[17], Chirag Patel[7], Mohan Shenoy[18], Eric J. Steenbergen[19], Glenn Anderson[20], Genomics England Research Consortium[21], Ernie MHF Bongers[22], Carsten Bergmann[23,24], Daan Panneman[25], Richard J. Rodenburg[25], Robert Kleta[8,26], Pascal Houillier[3,27,28], Martin Konrad[2], Rosa Vargas-Poussou*[3,4,27], Nine Knoers*[29], Detlef Bockenhauer*[8,30], Jeroen de Baaij*[1].

Affiliations:
[1] Radboud university medical center, Radboud Institute for Molecular Life Sciences, Department of Physiology, Nijmegen, the Netherlands.
[2] Department of General Pediatrics, University Children`s Hospital, Münster, Germany.
[3] Reference Center for Hereditary Kidney and Childhood Diseases (MARHEA), Paris, France.
[4] Department of Genetics, Assistance Publique Hôpitaux de Paris, Hôpital Européen Georges-Pompidou, Paris, France.
[5] Radboud university medical center, Radboud Institute for Molecular Life Sciences, Department of Nephrology, Nijmegen, the Netherlands.
[6] Department of Renal Medicine, Townsville University Hospital, Townsville, Australia.
[7] Queensland Conjoint Renal Genetics Service - Genetic Health Queensland, Royal Brisbane and Women's Hospital, Brisbane, Australia.
[8] Department of Renal Medicine, University College London, London, United Kingdom.
[9] Department of Endocrinology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.
[10] Genetics Department, University Medical Center Utrecht, Utrecht, the Netherlands
[11] Service of Nephrology, Yves Le Foll Hospital, Saint Brieuc, France.
[12] Department of Physiological Functional Investigations, CHU de Toulouse, Université Paul Sabatier, Toulouse, France.
[13] Pediatric Nephrology, Internal Medicine and Rheumatology, Southwest Renal Rares Diseases Centre (SORARE), University Children's Hospital, Toulouse, France.
[14] Department of Nephrology, University Hospital – Lyon Sud, France
[15] Kuratorium für Heimdialyse Pediatric Kidney Center, Marburg, Germany.
[16] University Children's Hospital, Philipps-University, Marburg, Germany.
[17] Johannes Gutenberg Universität Mainz, Zentrum für Kinder- und Jugendmedizin, Mainz, Germany.
[18] Department of Paediatric Nephrology, Royal Manchester Children's Hospital, Manchester, United Kingdom.
[19] Department of Pathology, Radboud university medical center, Nijmegen, the Netherlands.
[20] Department of Pathology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom.
[21] Genomics England, London, United Kingdom.
[22] Department of Human Genetics, Radboud university medical center, Nijmegen, the Netherlands.
[23] Limbach Genetics, Medizinische Genetik Mainz, Prof. Bergmann & Kollegen, Mainz, Germany
[24] Department of Medicine, Division of Nephrology, University Hospital Freiburg, Germany
[25] Radboud Center for Mitochondrial Medicine, Translational Metabolic Laboratory, Department of Pediatrics, Radboud university medical center, Nijmegen, the Netherlands
[26] Department of Paediatric Nephrology, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom.
[27] Centre de Recherche des Cordeliers, Sorbonne Université, INSERM, Université de Paris, CNRS, Paris, France
[28] Department of Physiology, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France.
[29] Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[30] Renal Unit, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom.

* These authors contributed equally to this work

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Corresponding author:

Jeroen H.F. de Baaij

Department of Physiology,

Radboud institute for molecular life sciences, Radboudumc

P.O. Box 9101, 6500HB, Nijmegen, The Netherlands

Phone: +31- 24 361 7347, Email: jeroen.debaaij@radboudumc.nl

Significance statement

Biallelic pathogenic variants in *SLC12A3*, encoding the thiazide-sensitive sodium-chloride co-transporter NCC, cause Gitelman syndrome (GS). Gitelman patients suffer from hypokalemic alkalosis, hypomagnesemia and salt wasting. A subset of GS-cases remains genetically unsolved. This paper describes the identification of pathogenic mitochondrial DNA (mtDNA) variants in the genes encoding the transfer RNAs for phenylalanine (*MT-TF*) and isoleucine (*MT-TI*) in thirteen families with a GS-like phenotype. Six families were additionally affected by progressive chronic kidney disease. Mitochondrial dysfunction was demonstrated in patient-derived fibroblasts and linked to defective sodium reabsorption by NCC *in vitro.* These findings advocate screening for mtDNA variants in unexplained GS patients and influence genetic counseling of affected families. Furthermore, they provide insight into the physiology of renal sodium handling.

Abstract

Background: Gitelman syndrome (GS) is the most frequent hereditary salt-losing tubulopathy characterized by hypokalemic alkalosis and hypomagnesemia. GS is caused by biallelic pathogenic variants in *SLC12A3,* encoding the Na^+-Cl^- cotransporter (NCC) expressed in the distal convoluted tubule. Pathogenic variants in *CLCNKB, HNF1B, FXYD2* or *KCNJ10* may result in renal phenocopies of GS, as they can lead to reduced NCC activity. Nevertheless, ±10% of patients with a GS phenotype remain genetically unsolved.

Methods: After identification of mitochondrial DNA (mtDNA) variants in three families with GS-like electrolyte abnormalities, 156 families were investigated for variants in *MT-TI* and *MT-TF*, encoding the transfer RNAs for phenylalanine and isoleucine. Mitochondrial respiratory chain function was assessed in patient fibroblasts. In NCC-expressing HEK293 cells, mitochondrial dysfunction was induced to assess the effect on thiazide-sensitive $^{22}Na^+$ transport.

Results: Genetic investigations revealed four mtDNA variants in 13 families: m.591C>T (*n*=7), m.616T>C (*n*=1), m.643A>G (*n*=1) (all in *MT-TF*) and m.4291T>C (*n*=4, in *MT-TI*). Variants were near homoplasmic in affected individuals. All variants were classified as pathogenic, except for m.643A>G which was classified as variant of uncertain significance. Importantly, affected members of six families with an *MT-TF* variant additionally suffered from progressive chronic kidney disease. Maximal mitochondrial respiratory capacity was reduced in patient fibroblasts, caused by dysfunction of oxidative phosphorylation complex IV. *In vitro* pharmacological inhibition of complex IV, mimicking the effect of the mtDNA variants, demonstrated an inhibitory effect on NCC phosphorylation and NCC-mediated sodium uptake.

Conclusion: Pathogenic mtDNA variants in *MT-TF* and *MT-TI* can cause a GS-like syndrome. Genetic investigation of mtDNA should be considered in patients with unexplained GS-like tubulopathies.

Introduction

Gitelman syndrome (GS) is a recessively inherited renal tubulopathy caused by pathogenic variants in *SLC12A3*, which encodes the thiazide-sensitive Na^+-Cl^--cotransporter (NCC). NCC mediates reabsorption of sodium and chloride in the distal convoluted tubule (DCT).(1) GS is characterized by distal tubular salt wasting with secondary hyperaldosteronism, hypochloremic metabolic alkalosis, hypokalemia, hypomagnesemia and hypocalciuria. Common clinical manifestations of GS include muscle cramps, paresthesias, nocturia, salt craving, muscle weakness and fatigue.(2)

GS may be phenocopied by a number of genetic as well as non-genetic conditions. Non-genetic causes include diuretic abuse, chronic laxative abuse and chronic vomiting. The most important genetic differential diagnosis to pathogenic variants in *SLC12A3* is the presence of biallelic pathogenic variants in *CLCNKB*, which encodes the distal tubular basolateral chloride channel ClC-Kb. Such variants can be found in approximately 3% of patients with a GS-like tubulopathy.(3) Additionally, pathogenic variants in *KCNJ10*, *FXYD2* and *HNF1B* may result in a similar biochemical phenotype, but typically cause additional symptoms such as sensorineural deafness, epilepsy, ataxia, intellectual disability, diabetes or renal cysts.(4-6) Still, 10% of patients with clinical characteristics of GS do not have a pathogenic variant in *SLC12A3* or other genes currently associated with a GS-like tubulopathy, suggesting that not all genetic causes for GS have been identified.(3)

Mitochondrial diseases form a heterogenous group of hereditary disorders characterized by mitochondrial dysfunction.(7) Interestingly, a small group of mitochondrial diseases has been associated with distal tubular dysfunction.(8-11) For instance, a large family carrying a variant in the mitochondrial transfer RNA (tRNA) gene for isoleucine (*MT-TI*) was affected by hypokalemia and hypomagnesemia in addition to arterial hypertension and hypercholesterolemia.(9) To date, all reports report extra-renal manifestations in addition to the GS-like electrolyte abnormalities.

In this study, we describe three large families with genetically unexplained GS. The presumed maternal inheritance pattern led to the identification of mtDNA variants in mitochondrial tRNAs for isoleucine and phenylalanine (encoded by *MT-TI* and *MT-TF*, respectively). We subsequently screened two

cohorts of patients with hypomagnesemia or a clinical diagnosis of GS, and identified ten more families with variants in *MT-TI* and *MT-TF*. We analyzed the clinical phenotype of these patients, characterized mitochondrial function in patient-derived fibroblasts, and assessed the effect of mitochondrial dysfunction on NCC-mediated sodium transport.

Methods

Inclusion and ethical approval

The maternal inheritance pattern in families 1, 2 and 3 prompted an analysis of the mitochondrial genome. The identification of three mtDNA candidate variants in *MT-TI* and *MT-TF* encouraged us to screen for variants in these two genes in additional families with unexplained hypomagnesemia or a clinical suspicion of GS (156 families). This led to the identification of variants in *MT-TI* or *MT-TF* in eight more families (families 4 to 12). Family 13 was known to have a pathogenic variant in *MT-TF* and has been published before as Pedigree III by Connor et al.(12).

The study was performed in accordance with the Declaration of Helsinki, informed consent was obtained from all patients before inclusion into the study. Where needed, ethical approval was provided by the institutional review board Arnhem-Nijmegen (study reference 2019-5749).

DNA sequencing

In family 1, the initial diagnosis was made on DNA isolated from cells in urine, amplification through long-template PCR and sequencing with the Ion Torrent PGM. The obtained mtDNA sequence was screened for rearrangements and mismatches. The presence of the variant was later confirmed in DNA isolated from blood by Sanger sequencing using the *MT-TI* sequencing primers listed in Supplementary Table 1. In family 2, DNA was isolated from whole blood. Exome enrichment was done with SureSelectXT Automated Target Enrichment and sequencing by a HiSeq4000 platform (Illumina) with 2x75 bp paired-end reads. Sequence reads were aligned to the Human Genome Reference Assembly GCRh37/hg19 using Burrows-Wheeler Alignment (BWA) version 0.7.12(13) and indexed using SAMtools version 1.6.(14) SNVs and indels were subsequently called by the Genome Analysis Toolkit (GATK) HaplotypeCaller version 3.4-46. The candidate variant was identified by targeted re-analysis of the mitochondrial DNA covered by the exome sequencing data. Family 3 underwent whole-genome 150bp paired-end sequencing using an Illumina HiSeq X platform as part of the 100,000 Genomes Project, and processed on the Illumina North Star Version 4 Whole Genome Sequencing Workflow

(NSV4, version 2.6.53.23), comprising the iSAAC Aligner (version 03.16.02.19) and Starling Small Variant Caller (version 2.4.7). Samples were aligned to the Homo Sapiens NCBI GRCh38 assembly. The candidate variant was identified after targeted re-analysis of mtDNA. In all three families, results were confirmed by Sanger sequencing in an extended set of family members for segregation (primers listed in Supplementary Table 1).

Families 4 to 9 were ascertained through screening for variants in *MT-TI* and *MT-TF* by Sanger sequencing of DNA obtained from whole blood. The variants in family 10 and 12 were identified by analysis of the complete mitochondrial genome with a long-range PCR followed by circular consensus sequencing on a Sequel (Pacific Biosystems). The variant in family 11 was identified by multi-gene panel analysis (Bioscientia). This multi-gene tubulopathy panel used Roche/Nimblegen enrichment and sequencing on an Illumina platform. The variant in family 13 was identified by sequencing of the mitochondrial genome.(12) In all families except for family 13, the diagnostic trajectory had included a screen for pathogenic variants in the coding regions of *SLC12A3*, *CLCNKB* and several other tubulopathy genes by a multi-gene panel or by exome sequencing.

To exclude other genetic causes of reduced glomerular filtration rate, two genome sequencing panels were analyzed in family 3 (panel names: 'unexplained kidney failure in young people' and 'tubulointerstitial kidney disease'(15)). In family 10, a >300 gene containing exome sequencing panel for kidney diseases was used. In family 13, targeted genetic analysis of *UMOD*, *HNF1B*, *REN* and *MUC1* was performed, including SNaPshot minisequencing of *MUC1* and MLPA for *HNF1B* (as described for family 6 in the study by Ekici et al.(16)).

Determination of heteroplasmy

Heteroplasmy levels were determined in fibroblasts and/or whole blood from nine families using single molecule Molecular Inversion Probes (smMIPs). A variant was considered homoplasmic if coverage at the variant position was at least 300 and the percentage of reads with the variant was above 99%. Detailed description can be found in the Supplementary Material.

Identification, selection and assessment of candidate variants

Very rare mtDNA variants (population frequency <0.1%) in families 1, 2 and 3 were considered candidate variants. Variant population frequencies were obtained from MITOMAP, HelixMTdb and gnomAD. Furthermore, MITOTIP and PON-mt-tRNA were used to predict pathogenicity of candidate variants. The secondary structures of the mitochondrial transfer RNAs (mt-tRNAs) for isoleucine (mt-tRNA^{Ile}) and phenylalanine (mt-tRNA^{Phe}) were modeled using rtools, CentroidHomFold. For conservation analysis of *MT-TI* and *MT-TF* (encoding mt-tRNA^{Ile} and mt-tRNA^{Phe}, respectively), we selected the species suggested by Yarham et al. (17) and aligned sequences using clustal O followed by manual curation.

Pathogenicity of mitochondrial DNA variants was evaluated by using the criteria proposed by Wong et al.(18), similar to the ACGS criteria.(19)

Clinical data

Clinical data, including renal biopsies, were obtained as part of routine clinical care at the respective local centers. Electrolyte measurements were performed in serum samples in some centers, in plasma samples in others. For simplicity, we will henceforth refer to all as “serum” measurements. Reference values for the measurements presented were very similar across centers. Urinary calcium excretion was normalized to the upper limit of normal to enable comparison between children and adults (Supplementary Table 2). The estimated GFR (eGFR) was calculated using serum creatinine and the CKD-EPI (adults) or Schwartz formula (children). Hypertension was defined as a systolic blood pressure above 140 mmHg or a diastolic blood pressure above 90 mmHg. In family 12, genders were left out in some individuals and some unaffected siblings were added for pseudonymization purposes.

Thiazide tests were performed in three families according to previously described protocols (20, 21).

Fibroblasts

In families 3 and 4, fibroblasts were grown from a skin biopsy. In families 6 and 11, fibroblasts were obtained by nasal brush (Cytobrush Plus, Cooper Surgical, # 176291). Culture conditions are described in the Supplementary Material. In addition to one family control (unaffected relative on the paternal line in family 6, control 1), two control cell lines of unrelated individuals were included. Both had been shown to have normal mitochondrial function in earlier experiments. One fibroblast line was obtained commercially (ATCC® PCS-201-012™, lot # 61683453, from a 40-year-old woman, control 2), the other was derived from a skin biopsy done at the Radboudumc, Nijmegen (control 3).

OXPHOS activity measurements

Measurements of the activity of the mitochondrial oxidative phosphorylation (OXPHOS) complexes were performed per clinical routine as described previously (additional information in Supplementary Methods).(22)

Mitochondrial respiration by the Seahorse XFe96 Analyzer

Oxygen consumption rate (OCR) was measured in a Cell Mito Stress test by the Seahorse XFe96 Analyzer as described earlier.(23) Citrate synthase activity was measured in all wells after the stress test for normalization purposes (Supplementary Methods).

Seahorse XFe96 data analysis

Wave Desktop Software version 2.3 (Agilent) was used to read Seahorse data, remove background signal and to normalize for citrate synthase activity. However, use of this program for subsequent analysis has several disadvantages. Firstly, there is no automated way to exclude wells that did not respond to the Mito Stress test (which can occur if one of the drugs was not injected correctly). Secondly, the larger variation observed with larger OCR values (i.e. heteroscedasticity) violates the assumptions underlying many statistical tests, including ANOVA.(24) Lastly, inter-plate variation can be significant.(24) To improve the validity of the data, an R-script was developed to analyze the data

(Supplementary Methods). The code is publicly available on Github (https://github.com/DaanViering/Seahorse-analyzeR).

Effect of complex IV inhibition on NCC-mediated $^{22}Na^{+}$ uptake

HEK293 cells were transfected with either 0.5 µg DNA construct containing NCC (pCIneo-NCC-IRES-GFP) or 0.5 µg construct without NCC (pCIneo-IRES-GFP, hereafter indicated with mock). Two days after transfection, samples were put on hypotonic-low-chloride or isotonic buffer with or without 100 µmol/L thiazide, and with either 1 mmol/L potassium cyanide (KCN, experimental condition) or as a control 1 mmol/L potassium chloride (KCl). After half an hour incubation, samples were put on isotonic buffer containing both $^{22}Na^{+}$ and inhibitors of other sodium transporters and channels (i.e., amiloride 100 µmol/L, bumetanide 100 µmol/L and ouabain 1 mmol/L). After half an hour in the $^{22}Na^{+}$, cells were lysed and radioactivity measured on a liquid scintillation counter (Hidex 600SL). NCC expression was assessed by immunoblotting, following the same protocol as described below. Culturing and the $^{22}Na^{+}$ uptakes were done in triplicate; the complete experiment was performed four times (Supplementary Methods).

Effect of complex IV inhibition on NCC phosphorylation

Seeding of HEK293 cells and transfection were similar to what is described above for the $^{22}Na^{+}$ uptake experiments. Samples were subsequently put on hypotonic-low-chloride or isotonic buffer with or without 100 µmol/L hydrochlorothiazide, and with either 1 mmol/L potassium cyanide (KCN, experimental condition) or as a control 1 mmol/L potassium chloride (KCl). Culturing was done in duplicate; the complete experiment was performed three times. To investigate the effect of complex IV inhibition on other sodium transporters, its effect on the phosphorylation of the sodium-potassium-chloride cotransporter NKCC2 was assessed using an analogous protocol (Supplementary Methods).

Immunoblotting

SDS-PAGE immunoblotting was performed with the following primary antibodies: rabbit anti-NCC (1:2000, Millipore, #AB3553), rabbit anti-pT58-NCC (NCC phosphorylated at human position p.Thr60, 1:2000, kind gift from Robert Fenton (25)). Primary antibodies were targeted with the following secondary antibody: peroxidase anti-rabbit-IgG (1:10 000, Sigma Aldrich, #A4914). Imaged blots were subjected to densitometric analysis of band intensities (Supplementary Methods).

Statistical analyses

GraphPad Prism 8.4.3 was used for statistical analyses. For the Seahorse XFe96 experiments, Welch's ANOVA test was applied with the null hypothesis that maximal mitochondrial respiration was not different for any of the variants compared to the control fibroblasts. Correction for multiple testing was performed using Dunnett T3 testing. Additionally, we assessed for each patient whether the maximum mitochondrial respiratory capacity in their fibroblasts was significantly different from the fibroblasts of control 1, again using Welch's ANOVA test (for this, fibroblasts from 5.I.2 were excluded because only one measurement was available) with Dunnett T3 correction.

To assess the difference between the KCN and KCl conditions during the $^{22}Na^{+}$ absorption, we used an unpaired t-test. To assess the difference between the KCN and KCl conditions in the immunoblotting experiments, we used multiple t-tests with Holm-Sidak correction for multiple testing. Statistical significance was defined as $p < 0.05$ unless stated otherwise.

Results

Identification of four mtDNA variants in MT-TI *and* MT-TF

Three large families with an unexplained GS-like electrolyte constellation showed pedigrees compatible with maternal inheritance (families 1, 2 and 3 in Figure 1). No pathogenic variants in *SLC12A3* and *CLCNKB* were found in any of these families, nor in other known tubulopathy genes.(26) Analysis of mtDNA revealed three candidate pathogenic variants: m.4291T>C in the mitochondrial tRNA for isoleucine (*MT-TI*) in family 1, and two variants in the mitochondrial tRNA for phenylalanine (*MT-TF*) in families 2 and 3 (m.591C>T and m.643A>G, respectively). The variants co-segregated with the GS phenotype in all three families. Individuals were shown to carry the variant at (near) homoplasmy (97-100% of reads carried the variant in all three families). We subsequently screened for variants in *MT-TF* and *MT-TI* in 156 additional families and individual patients with an unexplained GS phenotype or unexplained hypomagnesemia. This screening identified three more families/individual patients with the m.4291T>C variant and 6 more families/individual patients with the m.591C>T variant. Lastly, a family with the m.616T>C variant in *MT-TF* (family 13, described previously as 'Pedigree III' in (12)), was also shown to have GS-like electrolyte abnormalities.

Heteroplasmy levels in blood and fibroblasts ranged from 97% to 100% (homoplasmic) in all tested patients (Table 1 and Supplementary Table 3). Pedigrees can be found in Figure 1.

Assessment of variant pathogenicity

The m.591C>T variant, m.616T>C and m.643A>G variants are all located in the *MT-TF* gene encoding mt-tRNA^{Phe} (Figure 2A). The m.4291T>C variant is positioned in mt-tRNA^{Ile}, encoded by the *MT-TI* gene (Figure 2B). In the MITOMAP Genbank, HelixMTdb and gnomAD population databases (together comprising 304,824 individuals), homoplasmic occurrences have been observed for the m.643A>G variant (3 homoplasmic occurrences, 1 heteroplasmic occurrence), but not for the other variants (Supplementary Table 4). Furthermore, finding the m.591C>T variant in six out of 156 screened families is unlikely to have occurred by chance *(*corrected *p*-value $6.6 * 10^{-16}$). Finding the m.4291T>C variant

in three out of 156 screened families is also unlikely to have occurred by chance (corrected *p*-value 1.7 $*10^{-9}$). Evolutionary conservation ranged from well-conserved (m.4291T>C and m.616T>C, conserved to fruit flies) to poorly conserved (m.591C>T, conserved only to chimpanzees) as shown in Figure 2C-D. Computational evidence was conflicting on pathogenicity of the variants (Supplementary Table 4). Application of the criteria by Wong et al.(18) resulted in classification of the m.591C>T, m.616T>C, m.4291T>C variants as pathogenic and the m.643A>G variant as a variant of uncertain significance (Supplementary Table 5). Variant classifications were submitted to ClinVar.

Clinical phenotype

To better characterize the clinical phenotype associated with the four variants in *MT-TI* and *MT-TF*, phenotypical data from all patients were collected (Table 1, Figure 3 and Supplementary Table 3). Ten index patients presented with hypomagnesemia-related symptoms, such as tetany, tremor, paresthesia, muscle fatigue, joint complaints (chondrocalcinosis) or cerebral seizures at the initial visit. In two other index patients, hypokalemia or hypomagnesemia were discovered as an incidental finding. Of the investigated individuals on the maternal lineage of each family, 31 out of 36 had hypomagnesemia (86%). A significant degree of variation in serum magnesium was present among individuals, with patient 5.II.1 having an immeasurably low serum magnesium (<0.1 mmol/l); she receives supplementation of magnesium with a subcutaneous pump system. A high fractional magnesium excretion (FEMg) in 21 of 25 patients with available data (average 6.9%, range 2.8 – 12%) implicated renal magnesium wasting as the cause of the hypomagnesemia. The average serum potassium level was at the lower border of normal (3.5 mmol/L) and hypokalemia was present in 26 out of 41 family members on the maternal lineage (63%).

Activation of the renin-angiotensin-aldosterone system is common in GS. In four of eight individuals in whom renin levels were measured, renin was elevated (families 6, 7 and 8). Additionally, aldosterone levels were elevated in two of them. Furthermore, five individuals from families 5, 6 and 9 reported salt and/or spicy food craving.

Increased renal echogenicity was observed in three patients, of which two had CKD (6.II.1, 3.III.1 and 13.II.3; Supplementary Table 3). Renal ultrasound was unremarkable in three other patients (1.III.6, 6.II.2, 9.II.1 and 13.III.2). One or more renal cysts were present in two patients (10.II.3 and 10.I.2).

Patients with GS have a markedly blunted response to thiazide diuretics.(21) Thiazide tests were performed in five patients (1.III.6, 4.I.2, 4.II.2, 12.III.3 and 5.II.6). 50 mg hydrochlorothiazide induced a maximal increase of fractional chloride excretion (maximal ΔFECl) of 4.39%, 2.52%, 2.38%, 2.35% and 0.16% respectively (Supplementary Figure 1). Whereas the first four patients with the m.4291T>C variant (1.III.6, 4.I.2, 4.II.2, 12.III.3) demonstrate a relatively preserved response to hydrochlorothiazide, the response was completely blunted in individual 5.II.6 with the m.591C>T variant (cut-off value used for the diagnosis of GS is 2.3%).(21)

Hypertension was present in 8 of 27 individuals (30%), which is comparable to the general adult population.(27, 28)

Chronic kidney disease in several families with MT-TF variants

The GFR is usually normal in patients with GS.(29) In contrast, a high prevalence of reduced estimated GFR (eGFR < 90 mL/min/1.73m^2) was observed in six families (families 3, 5, 6, 9, 10 and 13; Table 1, Figure 1, Supplementary Table 3). Interestingly, affected members of all these families carried a variant in *MT-TF*. Elaborate screening for other genetic causes of reduced GFR by different gene panels was negative in tested families (families 3, 10 and 13).

In family 3 (m.643A>G), eGFR was impaired in four individuals (median eGFR 34 mL/min/1.73m^2, ranging between 30-55). The only individual in this family with a currently normal eGFR (100 mL/min/1.73m^2) was a 2-year-old girl (3.III.2). The older sibling (3.III.1) developed end-stage kidney disease necessitating kidney transplantation at the age of 9 years. We observed mild albuminuria (8-22.7 mg/mmol creatinine) in three patients (3.II.2, 3.III.1 and 3.III.2). In family 5, a mild decrease in eGFR was observed in three individuals (5.II.1, 5.III.5 and 5.III.6, eGFR between 60 and 90 mL/min/1.73m^2). In family 6, individual 6.I.2 has reached CKD stage 4 (eGFR between 15-30

mL/min/1.73m^2) at the time of study, and she reported having a sister diagnosed with CKD and early onset diabetes. Based on a family history in family 9, three individuals were affected by CKD, of whom 9.III.3 is on hemodialysis and 9.III.4 has received her second kidney transplantation. Individuals 10.I.2 and 10.II.3 had an eGFR of 24 and 50 mL/min/1.73m^2, respectively. Lastly, family 13 (m.616T>C), was diagnosed initially with autosomal dominant tubulointerstitial kidney disease(12) based on a KDIGO consensus report.(30) Data was available for ten individuals in the maternal lineage. Eight had a decreased eGFR, of whom two are included in this study based on their electrolyte abnormalities. Four of these eight individuals currently receive kidney replacement therapy.

Kidney biopsies have been taken in families 3, 10 and 13. The kidney biopsy performed in patient 3.III.1 to investigate CKD showed localized cortical scarring with tubular atrophy, glomerulosclerosis, interstitial fibrosis and a chronic interstitial infiltrate. After identification of the m.643A>G variant in this individual, electron microscopy was performed and showed abnormal mitochondria, especially in the distal tubule (Figure 4 A-C). Notably, proximal convoluted tubules demonstrated well developed apical microvilli and normal mitochondria. Other cells, including blood vessel smooth muscle and endothelial cells, showed no apparent mitochondrial irregularity. The kidney biopsy of patient 10.II.3 contained sclerosed glomeruli (20%) together with interstitial fibrosis and tubular atrophy (10%). Examination of the distal tubule by electron microscopy showed evidently abnormal mitochondria (Figure 4 D-E). In this patient, mitochondria appeared abnormal in both proximal tubule and distal tubule, although mitochondria in proximal tubules had better cristae structures than those in the distal tubule (Supplementary Figure 2 A-B). In this patient, electron microscopy also showed subtle signs resembling those normally seen in chronic thrombotic microangiopathy. Kidney biopsies in family 13 showed isolated interstitial fibrosis with tubular atrophy. Unfortunately, biopsies and images could not be retrieved for this family as they were taken several decades ago.

Apparent absence of extra-renal disease

In contrast to patients with other mitochondrial diseases, serum lactate was normal in all patients who were tested. Furthermore, no signs of proximal tubular disease were seen, as urinary amino acid analyses were unremarkable in the 7 patients tested (families 3 and 6). Proteinuria was absent in the tested healthy individuals (seven patients from families 2, 4, 5, 7, 11 and 12), but was present in 10.II.3. Newcastle Mitochondrial Disease Adult Scale (NMDAS) scores were low and would concur with calling the two patients 'asymptomatic' (4.I.2 and 4.II.1). No obvious abnormalities were seen on cholesterol levels (total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL), Supplementary Table 3).

Mitochondrial function in fibroblasts

To confirm that the mtDNA variants are indeed associated with mitochondrial dysfunction, we isolated fibroblasts from eight patients and assessed mitochondrial function. The Mito Stress Test on a Seahorse XFe96 (Figure 5A) showed decreased maximal mitochondrial respiration in fibroblasts from patients compared to controls (Figure 5B). The average decreases for the respective mutants were: a 17% decrease for m.643A>G ($p = 0.046$), a 51% decrease for m.4291T>C ($p = 0.02$) and a 51% decrease for m.591C>T (not significant, $p = 0.22$). Welch's ANOVA showed a significant difference between all individuals ($p < 0.0001$), with a Dunnett T3 test showing that the maximal respiratory capacity in fibroblasts of 6.II.1, 4.I.2, 1.II.2 and 11.II.1 differed significantly from maximal respiratory capacity in control 1 (corresponding to the unaffected father 6.I.1; Figure 5C).

We hypothesized that the defect in mitochondrial respiration would be caused by dysfunction of one or more OXPHOS complexes, and measured their activity. Patient fibroblasts showed an average 67% reduction in the activity of OXPHOS complex IV (also known as cytochrome C oxidase) compared to reference values of healthy individuals (Figure 6D). The individual with the greatest reduction in maximal mitochondrial respiration also showed the largest impairment in complex IV activity (6.I.2). OXPHOS complex I activity was low to borderline normal in patients 6.I.2, 6.II.2 and 11.II.1 (Figure 6A). Activity of OXPHOS complexes II, III and V was within the reference range in all patients, except for

patients 6.I.2 and 3.III.1 (Figure 6B, C and E). Citrate synthase activity was within the reference ranges in all patients, except for individuals 4.I.2 and 4.II.2 (m.4291T>C) who had elevated activity (11 and 23 % above the upper boundary of normal, Figure 6F).

Complex IV does not have a particularly high content of phenylalanine and isoleucine, as can be seen in Supplementary Table 6.

Complex IV inhibition reduces NCC-mediated sodium absorption in HEK293-cells

Due to the clinical similarities with GS and the abnormal mitochondria in the distal tubule observed with electron microscopy, we hypothesized that inhibition of OXPHOS complex IV would reduce NCC-mediated sodium uptake. Indeed, inhibition of complex IV with the specific inhibitor(31, 32) potassium cyanide (KCN) reduced thiazide sensitive $^{22}Na^{+}$ absorption in NCC-transfected HEK293 cells by 45% (p = 0.001, Figure 7A, Supplementary Figure 3). Even when adjusting for the observation that KCN induced an increase in $^{22}Na^{+}$ uptake in mock-transfected cells and hydrochlorothiazide-treated cells, KCN still reduced $^{22}Na^{+}$ uptake by 10% (Figure 7B). Furthermore, KCN blunted the response on NCC-phosphorylation that is normally observed after 30 minutes of incubation in hypotonic-low-chloride buffer (adjusted p = 0.00006, Figure 7C-E, Supplementary Figure 4). A similar effect was observed of KCN on NKCC2-phosphorylation in hypotonic-low-chloride buffer (p = 0.007, Supplementary Figure 5 and 6).

Discussion

To date, approximately 10% of cases with clinical characteristics of GS remain genetically unsolved.(3) Here, we show that variants in two mitochondrial tRNAs can lead to a GS-like syndrome, even in the apparent absence of other manifestations of mitochondrial disease. Thirteen families are described with hypokalemia and hypomagnesemia caused by renal magnesium wasting together with elevated renin levels. Nine families carry a variant in *MT-TF*, which encodes the mitochondrial tRNA for phenylalanine. Four families carry a variant in *MT-TI*, encoding the tRNA for isoleucine. The variants in *MT-TF* were associated with the development of CKD in 19 individuals on the maternal lineage of six families. Electron microscopy of kidney biopsies from two individuals with a pathogenic variant in *MT-TF* and CKD demonstrated tubulointerstitial kidney disease and abnormal mitochondria in the distal tubule. Cells in the DCT have the largest number of mitochondria per unit length of the nephron and would therefore be sensitive to mitochondrial dysfunction.(33) In line with these findings, patient-derived fibroblasts were found to exhibit a disturbed mitochondrial oxidative phosphorylation, putatively caused by a significant impairment of complex IV that was observed in patient mitochondria. In HEK293 cells, pharmacological inhibition of complex IV was shown to result in a reduction in NCC-mediated sodium reabsorption. We propose that the mitochondrial variants result in reduced NCC activity (Figure 8).

In total, we identified three different variants in *MT-TF* in nine families, of which m.591C>T has not been described before, and describe the m.4291T>C variant in *MT-TI* in three other families. Heteroplasmy levels ranged from 97% to 100% (homoplasmy) in all affected individuals, suggesting that a large proportion of mtDNA copies needs to be affected before a clinically overt phenotype manifests itself.

The four mtDNA variants described here were associated with hypomagnesemia, hypokalemia and activation of renin production, hallmarks of GS.(9) Two other symptoms of GS, hypocalciuria and metabolic alkalosis were only ascertained in a subset of patients. Interestingly, none of the 13 families

had clinically overt extra-renal manifestations of mitochondrial dysfunction at the moment of this study. This is in contrast to what is normally observed with pathogenic variants in mtDNA or nuclear encoded mitochondrial genes.(8-10, 34) Systematic evaluation of symptoms associated with mitochondrial dysfunction should be performed to definitively exclude the presence of rare or subclinical extra-renal manifestations. Yet, specific mtDNA variants have been described that can result in diseases that affect only a single organ, such as in Leber hereditary optic neuropathy and nonsyndromic hearing loss.(7) Our results show that mitochondrial tRNA variants can cause a maternally inherited GS-like syndrome. Moreover, mitochondrial tRNA variants may explain other familial and sporadic individual cases, as systematic screening of our cohort identified pathogenic mtDNA variants in 9 out of 156 families with characteristics of GS.

Genetic heterogeneity and pleiotropy are common phenomena in mitochondrial disorders. For example, the MELAS phenotype (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) can be caused by pathogenic variants in the mitochondrial tRNA for leucine (*MT-TL1*), phenylalanine (*MT-TF*) and histidine (*MT-TH*).(35-37) On the other hand, different pathogenic variants in one mitochondrial tRNA can lead to different clinical manifestations. For instance, variants in *MT-TI* and *MT-TF* have already been associated with cardiomyopathy,(38) Leigh syndrome(39), nonsyndromic hearing loss,(40) chronic progressive external ophthalmoplegia,(41) as well as MELAS(35).

The specific base pair affected by the variant thus appears to be important, as demonstrated by the finding of multiple families with the m.591C>T and m.4291T>C variants in our study. Interestingly, variants in *MT-TF* have previously been associated with renal phenotypes, especially tubulointerstitial kidney disease with progressive kidney failure.(12, 42-45) Indeed, also in our cohort, CKD was observed in affected members of two families with the m.643A>G and m.616T>C variants and members of four of the seven families with the m.591C>T variant (all in *MT-TF*). In contrast, CKD is not a hallmark of classical GS, nor did any of the patients with the m.4291T>C variant (affecting *MT-TI*) have CKD. Thus,

the different mtDNA variants might confer a varying predisposition to tubulointerstitial kidney disease and loss of GFR.

The clinical similarity with GS and the finding of abnormal mitochondria in the distal tubule on a renal biopsy suggested that the mitochondrial dysfunction reported here is responsible for a defect in sodium and magnesium reabsorption in this tubular segment. The electrolyte abnormalities in GS are caused by loss of function of NCC, the electroneutral sodium-chloride cotransporter expressed apically in the DCT. Notably, other genetic diseases have further highlighted the link between reduced NCC function and GS-like electrolyte abnormalities.(46-48) For instance, pathogenic variants in three DCT-localized proteins, namely ClC-Kb (*CLCNKB*), Kir4.1 (*KCNJ10*) and Kir5.1 (*KCNJ16*), give rise to very similar electrolyte abnormalities as observed in GS.(49-51) Kir4.1 is essential for K^+ extrusion at the basolateral membrane and for recycling K^+ imported by the Na^+-K^+-ATPase.(49) Loss-of-function of Kir4.1 thus reduces basolateral negative membrane potential.(52) This inhibits Cl^- extrusion through ClC-Kb, a basolateral chloride channel encoded by *CLCNKB*.(53) A subsequent rise in intracellular chloride is sensed by WNK4 and reduces their activation of SPAK/OSR1.(54, 55) Finally, this will lower NCC phosphorylation and consequently NCC-mediated sodium transport.(56)

Here, we provide evidence that pathogenic variants in *MT-TI* and *MT-TF* also impair NCC function. Patient-derived fibroblasts showed impairment of mitochondrial function, especially of oxidative phosphorylation complex IV. Given the difficulty of introducing mtDNA mutations in a cell model, we decided to take a pharmacological approach at inhibiting complex IV. This indeed resulted in lower NCC phosphorylation and lower NCC-mediated sodium transport.

Reabsorption of sodium is a process with a high energy demand, and even more so in the DCT because of the smaller osmotic gradient.(57) To accomplish this, the DCT is dependent on aerobic energy production.(58-60) Consequently, mitochondrial dysfunction here might lead to diminished function of the Na^+-K^+-ATPase, which needs ATP to maintain basolateral membrane potential; a prerequisite for sodium and magnesium transport.(61, 62) Supporting this mechanism, pathogenic variants in *FXYD2*,

ATP1A1 and *HNF1B* are also known to cause a GS-like phenotype.(5, 63-66) *FXYD2* and *ATP1A1* encode two different subunits of the Na^+-K^+-ATPase, while HNF1B has been shown to regulate *FXYD2* and *KCNJ16* mRNA expression.(67, 68)

Whereas one patient showed a blunted response to the administration of hydrochlorothiazide, three patients demonstrated a larger increase in maximal ***Δ***FECl than observed in patients with GS, suggesting residual NCC function in these patients. This is in line with other GS-like tubulopathies, such as those caused by pathogenic variants in *CLCNKB* or *HNF1B*. In these patients also, thiazide tests did not show the same level of reduction in thiazide response as has been established for GS.(21, 69, 70)

The penetrance of hypocalciuria is lower in our patients than in GS. Interestingly, urinary calcium excretion in *CLCNKB* patients, *HNF1B* patients and *FXYD2* patients do not seem to be as low as in GS, either.(65, 66, 71) Metabolic alkalosis seems to be more pronounced in classic GS, too.(65, 66) For these diseases, it is thought that the phenotypic differences arise from an additional dysfunction of other segments, e.g., thick ascending limb dysfunction in *CLCNKB,* in addition to the effects in the DCT. We cannot exclude that *MT-TI/MT-TF* associated GS-like syndrome also affects the connecting tubule(72, 73) or the thick ascending limb. Indeed, our *in vitro* data demonstrate reduced NKCC2 phosphorylation.

The m.4291T>C variant has been reported before in a large family with a 50% penetrance of hypertension, hypercholesterolemia, and hypomagnesemia(9) and in a family with congenital cataract.(74) In the 13 families with a mtDNA variant reported here, no individuals were affected by congenital cataract. Hypercholesterolemia and hypertension were not more frequent than in the general population, although this conclusion might not be generalizable to all four variants. Incomplete penetrance of clinically significant hypomagnesemia was noticed in a few cases too, even with (near) homoplasmic presence of the variant, although we cannot exclude the presence of subclinical symptoms. Systematic analysis of a larger number of patients and families will be required to identify subclinical symptoms and determine the penetrance of additional disease manifestations in the

different variants. The m.616T>C has been reported before in three families,(12, 44, 45) but none of these had reported GS-like electrolyte abnormalities. However, it should be noted that these symptoms could have been missed initially, as in family 13, or could develop later in life.(12)
The m.643A>G variant has been reported only once before,(18) and was classified in this study as a variant of uncertain significance towards the pathogenic side of the spectrum.(19) Future studies might provide decisive evidence on its pathogenicity.

Current next-generation sequencing analyses usually do not report mitochondrial variants. Not all exome sequencing kits cover the mitochondrial genome well, and if they provide adequate coverage, pipelines often focus on variants in or near protein coding sequences in nuclear DNA. Nevertheless, identification of pathogenic near homoplasmic mtDNA variants has important clinical and genetic implications. First of all, clinicians should be aware of the combination of GS-like electrolyte abnormalities and progressive CKD in patients with *MT-TF* variants. Secondly, our findings stress that clinicians treating patients with mitochondrial disorders should appreciate the possibility of electrolyte abnormalities, as hypomagnesemia might sometimes explain part of the muscle complaints. Furthermore, finding mtDNA variants in patients with unexplained GS has important consequences for genetic counseling, given the different inheritance pattern. Thus, specific testing for pathogenic variants in the mitochondrial genome, or including the mitochondrial genome in analyses of next-generation sequencing approaches, is warranted. Lastly, the fact that the variant was found in (near) homoplasmic state in all families suggests that genetic testing can be performed on DNA isolated from blood (in contrast to many other mitochondrial disorders, where affected tissue is needed to avoid false negative results).

In conclusion, *MT-TI* and *MT-TF* variants can cause a Gitelman-like syndrome. In all patients evaluated for a genetic cause of hypomagnesemia or hypokalemia, clinicians should consider screening *MT-TI*

and *MT-TF* for pathogenic variants by next generation sequencing or specific mtDNA testing. Importantly, pathogenic variants in *MT-TF* also confer a significant risk for the development of CKD.

Author contributions

Patient inclusion: Günter Klaus, Jean-Marie Coulibaly, Marion Vallet, Solenne Pelletier, Stéphane Decramer, Martin Kömhoff, Rolf Beetz, Mohan Shenoy, Karl-Peter Schlingmann, Detlef Bockenhauer, Rosa Vargas-Poussou, Pascal Houillier, Martin Konrad, Robert Kleta, André van Beek, Tom Nijenhuis, Andrew Mallett, Chirag Patel. Genetic investigations: Karl-Peter Schlingmann, Jeroen de Baaij, Detlef Bockenhauer, Melanie Chan, Rosa Vargas-Poussou, Carsten Bergmann, Nine Knoers, André van Beek, Ernie Bongers, Albertien van Eerde, Tom Nijenhuis, Andrew Mallett, Marguerite Hureaux, Daan Viering, Genomics England Research Consortium. Histological investigations: Glenn Anderson, Eric Steenbergen. Cell experiments: Daan Viering, Jeroen de Baaij, Daan Panneman. Supervision: Karl-Peter Schlingmann, Jeroen de Baaij, Detlef Bockenhauer, Rosa Vargas-Poussou, René Bindels, Richard Rodenburg, Nine Knoers, Tom Nijenhuis, Robert Kleta. Drafting manuscript: Jeroen de Baaij, Daan Viering. Revision and approval of manuscript: all authors.

Supplementary material

1. Supplementary Methods
2. Description of Supplementary Tables
    a. Supplementary Table 1: primer and smMIP sequences
    b. Supplementary Table 2: Age-specific reference for urinary calcium:creatinine ratio
    c. Supplementary Table 3: Full Clinical data
    d. Supplementary Table 4: Additional variant information
    e. Supplementary Table 5: Criteria for pathogenicity in each of the variants
    f. Supplementary Table 6: Number of phenylalanine and isoleucine residues in the mitochondrial genes
3. Supplementary Figure 1: Thiazide test
4. Supplementary Figure 2: Electron microscopy of proximal tubular cells
5. Supplementary Figure 3: Control immunoblots $^{22}Na^{+}$ uptake experiments

6. Supplementary Figure 4: Full immunoblot NCC phosphorylation experiments
7. Supplementary Figure 5: NKCC2 phosphorylation with complex IV inhibition
8. Supplementary Figure 6: Full immunoblot NKCC2 phosphorylation experiments

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Disclosures

Nothing to disclose.

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TABLES

Table 1: Summary of clinical data

Patient Reference range	*Sex*	*Age^ (years)*	*Variant*	*Gene*	*Heteroplasmy (%) in blood | fibroblasts*	*Serum magnesium (mmol/L)* 0.7-1.1	*FEMg* <4.0 §	*Serum potassium (mmol/L)* 3.6-5.2	*eGFR (mL/min/ 1.73m2)* >90	*Blood pressure (mmHg)* <140/90	*Remarks*
1.III.6	F	39	m.4291T>C	MT-TI	100 |	0.40	6.7	3.6	82	140/80	Primary hyperparathyroidism
1.III.2	F		m.4291T>C	MT-TI	99 |						
1.IV.4	M	36	m.4291T>C	MT-TI		0.67	2.9	3.7	>90		
1.IV.5	M	34	m.4291T>C	MT-TI	97 |	0.56	8.3	3.4	>90		
2.II.6	F		m.591C>T	MT-TF	98 |	0.86*		3.3*	65	122/80	
2.III.1	F	5	m.591C>T	MT-TF		0.73		3.2	>90		
2.III.2	M	10	m.591C>T	MT-TF		0.55	9.0	3.0	>90	125/71	
2.III.4	M	8	m.591C>T	MT-TF		0.71*	13	3.6	>90	151/89	
2.III.5	F		m.591C>T	MT-TF	99 |	0.48	7.8	3.0	>90	114/65	
3.I.2	F	50	m.643A>G	MT-TF		0.70	7.9	3.6	35		
3.II.2	F	22	m.643A>G	MT-TF		0.52	4.9	3.3	32	120/73	Albuminuria
3.II.4	F	21	m.643A>G	MT-TF		0.66	4.2	3.1	55		
3.II.5	F	14	m.643A>G	MT-TF				2.9			
3.III.1	F	2	m.643A>G	MT-TF		0.76*	8.2	4.1	30	98/54	Albuminuria and elevated RBP. Renal biopsy performed
3.III.2	F	1	m.643A>G	MT-TF		0.48	8.5	3.0	>90		Albuminuria and elevated RBP
3.III.3	F		m.643A>G	MT-TF		0.61		4.3			
4.I.2	F		m.4291T>C	MT-TI	97 | 100	0.52	4.3	3.4	>90	125/78	
4.II.2	F	15	m.4291T>C	MT-TI	100 | 100	0.42*	5.3	4.2	>90	113/72	
5.III.6	F	3	m.591C>T	MT-TF		0.10	10.1	3.6	75	123/67	Requires subcutaneous magnesium supplementation. Salt craving
5.II.1	M	70	m.591C>T	MT-TF		0.40		4.9	65		
5.III.2	F	34	m.591C>T	MT-TF		0.40	6.4	3.1	>90	149/91	
5.III.4	F	32	m.591C>T	MT-TF		0.73	2.8	3.7	>90	157/98	
5.III.5	M	42	m.591C>T	MT-TF		0.30	12.1	2.4	77	197/105	
5.IV.1	F		m.591C>T	MT-TF		0.70		3.9	>90	133/80	
5.IV.2	M		m.591C>T	MT-TF		0.80	3	3.9	>90	149/63	

6.I.2	F		m.591C>T	MT-TF					26		Salt and spicy food craving
6.II.1	M	12	m.591C>T	MT-TF		0.44*	9.5	3.1	>90	120/80	Salt and spicy food craving. High renin and aldosterone.
6.II.2	F	10	m.591C>T	MT-TF		0.63*	8.1	3.3*	>90	110/70	Salt and spicy food craving. High renin and aldosterone.
7.I.2	F	33	m.591C>T	MT-TF		0.56	5.5	2.8	>90	128/82	High renin
7.II.1	F	8	m.591C>T	MT-TF		0.74		3.3	>90	101/66	
8.II.1	F	18	m.591C>T	MT-TF	100 |	0.54*		2.7*		113/65	High renin
9.III.1	F	40	m.591C>T	MT-TF		0.51		4.0*	>90	100/50	Orthostatic hypotension, m. Winiwater-Buerger, migraine, Wolff-Parkinson-White syndrome.
10.II.3	F	39	m.591C>T	MT-TF	100 |	0.59*	5	3.2	50	122/70	Transient mild thrombopenia. Osteopenia.
10.I.2	F		m.591C>T	MT-TF		0.68	4	4.5	24		Primary hyperparathyroidism. Mild thrombopenia. CVA (2x). BP controlled with three antihypertensives. Osteopenia.
11.II.1	F		m.4291T>C	MT-TI	100 |	0.52	7.5	3.4*	>90	142/95	
12.II.1	M	46	m.4291T>C	MT-TI		0.56	8.3	4.5	>90		
13.II.3	M	27	m.616T>C	MT-TF		0.54*		4.0	39	120/60	
13.III.2	M	21	m.616T>C	MT-TF		0.60*		3.8*	56		

Summary of clinical data of patients with causative mtDNA variants in MT-TI or MT-TF. If values were outside measurement limits, the value was set equal to the measurement limit. If multiple measurements were available, the first measurement was taken in case of serum magnesium, serum potassium and FEMg, while the last available measurement was taken in case of eGFR. eGFR was calculated with CKD-EPI, except for individuals below the age of 19, in which case the Schwartz formula was used.

*FEMg, fractional magnesium excretion; eGFR, estimated glomerular filtration rate. * with supplementation of magnesium or potassium. § Upper limit of normal for FEMg applies to hypomagnesemic individuals only and is based on (75). ^ Age at presentation.*

For conversion of serum magnesium (mmol/L) to (mg/dL) multiply by 2.43.

*FEMg is calculated by: serum creat * urinary Mg / (serum Mg * urinary creat) *100 %*.

FIGURE LEGENDS

Figure 1: Pedigrees

Pedigrees of the thirteen affected families. Black filling denotes tubulopathy. Probands are denoted with arrows, chronic kidney disease (any stage) is denoted by gray filling. Percentages indicate heteroplasmy level of the variant in blood. E+ indicates the presence of the variant as confirmed by genetic testing, E- the exclusion of the variant.

Figure 2: *in silico* prediction analysis of variants

In silico *prediction analysis of variants in the mitochondrial tRNAs for phenylalanine and isoleucine (mt-tRNA^{Phe} and mt-tRNA^{Ile}, respectively). (A-B) CentroidHomFold predictions of secondary structure of the two tRNAs. The grayscale indicates pseudo base-pairing probabilities; light shading represents a low probability and dark shading a high probability. Bold letters indicate anticodons. AA indicates amino acid binding position. (**A**) Predicted secondary structure of mt-tRNA^{Phe}, the locations of the patient variants m.591C>T, m.616T>C and m.643A>G are indicated. (**B**) Predicted secondary structure of mt-tRNA^{Ile}, the locations of the patient variant m.4291T>C is indicated. (**C**) MT-TF and (**D**) MT-TI nucleotide sequences in a standard set of species.(17) Fully conserved residues are indicated by stars (aligned with clustal O).*

Figure 3: Electrolyte measurements

*(**A**-**G**) Serum and urinary electrolyte values in patients with the different pathogenic variants. Dotted lines represent upper and lower limit of normal. For the fractional excretion of magnesium (FEMg) and urinary calcium excretion, lower limits of normal were not available, therefore only the upper limit of normal is depicted in panels B and D. Upper limit of normal for FEMg applies to hypomagnesemic individuals only and are based on (75). Black circles (●): without supplementation, open circles (○): with supplementation, gray circle in panel D: a child (individual 3.III.2), upper limit of normal for this age is*

*2.2 mmol/mmol Ca^{2+}/creatinine. FEMg is calculated by: serum creatinine * urinary magnesium / (serum magnesium * urinary creatinine) *100%.*

Figure 4: Renal biopsies

(A-C) Transmission electron microscopy of the renal biopsy of patient 3.III.1. (D-E) Transmission electron microscopy of a percutaneous renal biopsy of patient 10.II.3. (A) Representative image of a perpendicular cross-section of the distal tubule, with a large number of abnormally shaped and sized mitochondria (two examples indicated with white arrows). Cristae profiles appear distorted, including some mitochondria with no discernable cristae. Nanotunneling visible (three examples indicated with white arrowheads). Magnification x1000. (B) Close-up of atypical giant mitochondrium of over 3 µm in length (same as indicated by the left arrow in panel A). Note the large size and compartmentilization. Magnification x6000. (C) Close-up of atypical mitochondria (not in panel A). Note the concentric cristae (onion-like appearance). Magnification x6000. (D) Representative image of a perpendicular cross-section of the distal tubule, enlarged mitochondria are visible. (E) A close-up of panel D shows an almost complete lack of cristae structure in most mitochondria.

Figure 5: Mitochondrial oxygen consumption

*Mitochondrial function assessed by the Seahorse XFe96 analyzer. (**A**) Representative OCR plot of a Mito Stress test of fibroblasts from three patients with the m.4291T>C variant and three controls (n = 6 wells for each measurement point). Error bars denote + or – standard deviation. (**B**) Average maximal mitochondrial respiration for the different mtDNA variants. Each point represents the average of all independent experiments for one individual (n = 1-9, depending on the individual as can be seen in panel C). (**C**) Average maximal mitochondrial respiration for each individual. Each point represents the average of all replicate-wells on one Seahorse plate (n = 6). (**B-C**) Means are represented by horizontal bars, error bars denote the 95% confidence interval, a one-way ANOVA with Dunnett T3 was used to calculate significance.*

OCR is in pmol O_2/min/(mU/mL citrate synthase). AA, antimycin A; CS, citrate synthase activity; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; *OCR, oxygen consumption rate; RC, respiratory capacity; Rot, rotenone; *,* p *< 0.05; **,* p *< 0.005; ***,* p *< 0.0005.*

Figure 6: Activity of oxidative phosphorylation complexes

(A-E) Activities of the five OXPHOS complex (CI-CV), and (F) citrate synthase (CS) activity. All measurements were performed in isolated mitochondria from patient-derived fibroblasts. Thick dotted lines represent the reference range from our center, thin dotted lines represent the mean of control individuals. CI to CV, oxidative phosphorylation complex I to V; CS, citrate synthase activity.

Figure 7: NCC-mediated sodium uptake with complex IV inhibition

*(A-B) $^{22}Na^+$ uptake in HEK293 cells transfected with NCC or mock, with or without inhibition of OXPHOS complex IV with potassium cyanide. Potassium cyanide (KCN) 1 mmol/L or potassium chloride (KCl) 1 mmol/L (control) were added during both pre-incubation and the uptake period as indicated, the same applies to hydrochlorothiazide (HCTZ) 100 µmol/L. Bars represent mean with standard deviation. (**A**) shows the hydrochlorothiazide sensitive $^{22}Na^+$ uptake of NCC-transfected cells over a period of 30 minutes. Data in A is based on B. Significance was assessed with an unpaired t-test. (**B**) $^{22}Na^+$ uptake in 30 minutes after preincubation with hypotonic-low-chloride buffer or isotonic buffer. Cells were transfected with NCC or mock and treated with KCl or KCN (*n *= 4 of triplicates in each experiment). (**C**) Representative immunoblots showing NCC and phosphorylated NCC after a 30-minute incubation in hypotonic-low-chloride or isotonic buffer, with either KCN or KCl treatment. The mock condition has been incubated in hypotonic-low-chloride buffer as well. (**D-E**) Densitometry analysis of pNCC band intensity, and pNCC/tNCC ratio (*n *= 3 of duplicates in each experiment). Significance was assessed with unpaired t-tests and corrected for multiple testing. KCN, potassium cyanide; KCl, potassium chloride; HCTZ, hydrochlorothiazide; NCC, Na^+-Cl^--cotransporter; pNCC, NCC phosphorylated at Thr60. **,* p *< 0.005; ****,* p *< 0.00005.*

Figure 8: Induction of Gitelman-like syndrome by pathogenic mtDNA variants, proposed mechanism

*Proposed mechanism of Gitelman-like syndrome induced by pathogenic mitochondrial DNA variants in the genes encoding the isoleucine and phenylalanine tRNAs (*MT-TI *and* MT-TF*, respectively). The m.4291T>C, m.591C>T, m.616T>C and m.643A>G variants lead to complex IV dysfunction and reduced maximal respiration. This leads to a decrease in the phosphorylation of NCC and sodium transport. Reduced sodium transport in the distal convoluted tubule leads to reduced magnesium transport in the distal convoluted tubule and increased sodium transport in the collecting duct. Increased sodium reabsorption in the collecting duct leads to increased potassium excretion through ROMK (not shown here). CIV, oxidative phosphorylation complex IV; NCC,* Na^+-Cl^- *cotransporter.*