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(54) Title: METHODS OF DETECTING AND TREATING GATM-RELATED RENAL DISORDERS

(57) Abstract: Methods for detecting a glycine amidinotransferase (GATM) mutation in a biological sample from a patient are disclosed. Also disclosed are related methods for diagnosing a renal disorder in a patient, treating a patient suffering from a renal disorder, and stabilizing renal function in a patient suffering from a renal disorder.



METHODS OF DETECTING AND TREATING GATM-RELATED RENAL DISORDERS

CROSS-REFERENCE TO RELATED APPLICATION

[0001] This application claims the benefit of U.S. Provisional Application No. 62/515,834, filed on June 6, 2017, the entire contents of which are incorporated by reference.

STATEMENT REGARDING
FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with Government support under project number HG000215 14, by the National Institutes of Health, National Human Genome Research Institute. The Government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED
ELECTRONICALLY

[0003] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 72,136 Byte ASCII (Text) file named "738580_ST25.txt," created on May 30, 2018.

BACKGROUND OF THE INVENTION

[0004] Renal disorders may compromise kidney function, decrease quality of life, and may result in renal failure or death. For example, Fanconi syndrome is a renal disorder that involves inadequate reabsorption in the proximal renal tubules. Despite advances in methods of detecting and treating renal disorders, there exists a need for additional methods of detecting and treating renal disorders.

BRIEF SUMMARY OF THE INVENTION

[0005] An embodiment of the invention provides a method for treating a patient suffering from a renal disorder and having at least one dominant heterozygous missense mutation in glycine amidinotransferase (GATM) protein associated with a renal disorder, the method

comprising administering an effective amount of one or both of creatine and a creatine substitute to the patient.

[0006] Another embodiment of the invention provides a method for stabilizing renal function in a patient suffering from a renal disorder and having at least one dominant heterozygous missense mutation in GATM protein associated with the renal disorder, the method comprising administering an effective amount of one or both of creatine and a creatine substitute to the patient.

[0007] Still another embodiment of the invention provides a method of diagnosing a renal disorder in a patient, the method comprising: obtaining a biological sample from the patient; assaying the biological sample to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of GATM genetic sequence and (ii) GATM protein in the biological sample; and diagnosing the patient with a renal disorder if the at least one dominant heterozygous missense mutation is detected in the biological sample.

[0008] Another embodiment of the invention provides a method of diagnosing renal disorder in a patient and stabilizing renal function in the patient, the method comprising: diagnosing a renal disorder in the patient by any of the methods described herein with respect to other aspects of the invention and stabilizing renal function in the patient by administering an effective amount of one or both of creatine and a creatine substitute to the diagnosed patient.

[0009] Still another embodiment of the invention provides a method for detecting a GATM mutation in a biological sample from a patient, the method comprising: obtaining a biological sample from the patient; and assaying the biological sample to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of GATM genetic sequence and (ii) GATM protein.

[0010] Yet another embodiment of the invention provides a method for treating a patient with one or both of creatine and a creatine substitute, the method comprising: determining whether the patient has the renal disorder by: assaying a biological sample obtained from the patient to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein in the biological sample to determine if the patient has the renal disorder; wherein the detection of the at least one dominant heterozygous missense mutation is indicative of the

renal disorder in the patient; and if the at least one dominant heterozygous missense mutation is detected in the patient, then administering one or both of creatine and a creatine substitute to the patient.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING(S)

[0011] Figure 1 depicts pedigree analysis charts for all members from the four families (A-D) studied. Squares indicate males and circles indicate females; a filled symbol indicates that the person is affected. Deceased individuals are drawn with a diagonal line through the symbol. An asterisk indicates that the person contributed to linkage and sequencing studies.

[0012] Figure 2 depicts a graph of a multipoint parametric linkage analysis for families A, B, and C for chromosome 15. The Y axis presents the logarithm to the base 10 of the linkage odds (LOD score), and the X axis presents the genetic distance in centiMorgans (cM). The dotted line represents an LOD score of zero, which indicates no increased or decreased likelihood of linkage to the renal disorder.

[0013] Figure 3 depicts sequencing electrophoretograms of genomic DNA from renal Fanconi and kidney failure affected patients and non-affected control individuals. The heterozygous mutations in GATM are indicated by an arrow in each electrophoretogram section containing a DNA sequence obtained from an affected patient. The electrophoretogram labeled WT-2 contains a DNA sequence corresponding to the region comprising c.994-c.1024 of a control individual; the nucleotide sequence is set forth in SEQ ID NO: 17. The electrophoretogram labeled c.1006A>G contains a DNA sequence corresponding to the region comprising the c.1006A>G mutation from an affected patient; the mutation is at position 13 of the nucleotide sequence set forth in SEQ ID NO: 18. The electrophoretogram labeled c.1007C>T contains a DNA sequence corresponding to the region comprising the c.1007C>T mutation from an affected patient; the mutation is at position 14 of the nucleotide sequence set forth in SEQ ID NO: 19. The electrophoretogram labeled c.1022C>T contains a DNA sequence corresponding to the region comprising the c.1022C>T mutation from an affected patient; the mutation is at position 35 of the nucleotide sequence set forth in SEQ ID NO: 20.

[0014] Figures 4A and 4B depict ¹H-NMR spectra of brain (grey matter) creatine content. The X axis presents the chemical shift in ppm, and the Y axis presents the grey and white

matter creatine levels in arbitrary units. Fig. 4A is an ^1H -NMR spectrum from a unaffected control individual. Fig. 4B is an ^1H -NMR spectrum from a renal Fanconi syndrome patient. The arrow indicates a normal total creatine peak in the control individual and in the renal Fanconi syndrome patient.

[0015] Figures 5A and 5B depict the normalized mRNA expression of *NLRP3* (A) or IL-18 (B) obtained by real-time PCR in LLC-PK1 cells overexpressing wild-type *GATM* or the T336A *GATM* mutant. Fig. 5A, normalized mRNA expression of *NLRP3*. Fig. 5B, normalized mRNA expression of IL-18.

[0016] Figures 6A and 6B depict the amino acid levels in urine (A) and plasma (B) from *Gatm*^{+/+} and *Gatm*^{-/-} mice. Fig. 6A depicts the urinary excretion amino acids in $\mu\text{mol/osmol}$. Fig. 6B depicts the plasma concentration of amino acids in μM .

[0017] Figures 6C and 6D depict the creatine, creatinine, and guanidinoacetate levels in plasma (C) and urine (D) from *Gatm*^{+/+} and *Gatm*^{-/-} mice. Fig. 6C depicts the plasma concentration of creatine, creatinine, and guanidinoacetate in μM .

[0018] Figure 7 depicts the O_2 consumption of intact (non-permeabilized) LLC-PK1 cells overexpressing wild-type GATM (n=12) or the GATM T336A mutant (n=11). Measurements were done using high-resolution respirometry, and the results are presented in $\text{pmol}/[\text{s}*\text{ml}*CS \text{ activity}]$.

[0019] Figures 8A and 8B depict the live cell imaging of mitochondrial membrane potential (as measured using tetramethylrhodamine ester “TMRM”) and reactive oxygen species (ROS) production. Fig. 8A depicts the percent TMRM mean intensity of LLC-PK1 cells overexpressing wild-type GATM induced with tetracycline “TET” (WT + TET), or overexpressing the GATM T336A mutant “MUT” (MUT TA + TET). The numbers (7) and (6) indicate the number of times the experiments were repeated. The results are presented as mean $\pm$ SEM. Fig. 8B depicts a graph of the time course of production of reactive oxygen species of the same cells as Fig. 8A, measured with the CELLROX DEEP RED mitochondrial-targeted superoxide probe (ThermoFisher Scientific, Waltham, MA). The X axis presents the time in minutes, and the Y axis presents the percentage of the increase in CELLROX fluorescent signal in 30 minutes. The “F/F” label indicates a fluorescence ratio that reflects the amount of reactive oxygen species. The results are presented as mean $\pm$ SEM.

[0020] Figures 9A and 9B depict the quantification of the intracellular IL-18 content in LLC-PK1 cells induced with tetracycline and overexpressing wild-type GATM or the GATM T336A mutant. Fig. 9A depicts the ELISA quantification of intracellular IL-18 content as pg IL-18/ μ g protein ($p=0.0012$). Fig. 9B depicts the Western blot quantification of intracellular IL-18 content. Western blot data were normalized to β -actin protein expression ($p=0.0095$).

[0021] Figures 10A and 10B depict the levels of GATM mRNA (A) and GATM protein (B) expression in wild-type mice fed creatine (creatine) in their water, or fed water without creatine (untreated). Fig. 10A depicts the amounts of GATM mRNA expression normalized to β -actin mRNA expression. Fig. 10B depicts the amounts of GATM protein normalized to β -actin protein expression.

DETAILED DESCRIPTION OF THE INVENTION

[0022] Glycine amidinotransferase (GATM) is an enzyme in the creatine biosynthetic pathway. GATM is involved in generating the immediate precursor to creatine. GATM is expressed in the kidney in the proximal tubule mitochondria. Human GATM is assigned Gene NCBI Gene ID No. 2628 and an Mendelian Inheritance in Man (MIM) No. 602360. The human GATM gene is found on chromosome 15 at 15q21.1. A wild type genomic GATM DNA sequence comprises SEQ ID NO: 21 (Genbank Accession No. NG_011674.2). Two (wild type) transcriptional variants include GATM mitochondrial isoform 1 precursor (Genbank Accession No. NM_001482.2; CCDS (coding sequence) CCDS10122.1) and GATM mitochondrial isoform 2 (Genbank Accession No. NM_001321015.1). CCDS10122.1 is set forth as SEQ ID NO: 22 (GATM mitochondrial isoform 1 precursor).

[0023] It has been discovered that one or more heterozygous missense mutations in GATM protein are associated with renal disorders. The heterozygous missense mutations in GATM trigger intramitochondrial fibrillary deposition of GATM and lead to elongated and abnormal mitochondria in the kidney. Without being bound to a particular theory or mechanism, it is believed that this renal proximal tubular mitochondrial pathology initiates a response from the inflammasome with subsequent development of kidney fibrosis.

[0024] Accordingly, an embodiment of the invention provides a method of diagnosing a renal disorder in a patient. The method may comprise obtaining a biological sample from the

patient. In an embodiment of the invention, the biological sample is a sample of blood, urine, kidney, or skin.

[0025] The method may further comprise assaying the biological sample to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein in the biological sample.

[0026] In an embodiment, the method comprises obtaining genetic material from the biological sample. Obtaining genetic material from the biological sample may be carried out in any suitable manner known in the art. In an embodiment, the inventive method involves assaying genetic material obtained from a patient to detect a mutation in at least one copy of the GATM genetic sequence. The genetic material can be, for example, DNA (for example, genomic DNA or complementary DNA (cDNA) or RNA (e.g., messenger RNA (mRNA))). In a preferred embodiment of the invention, the method comprises assaying the biological sample to detect at least one dominant heterozygous missense mutation in the coding sequence of GATM mitochondrial isoform 1 precursor.

[0027] The genetic material may be obtained directly from the biological sample of the patient, or the genetic material can be copied or amplified from genetic material within cells in the patient's biological sample (e.g., via polymerase chain reaction (PCR), reverse transcription polymerase chain reaction (RT-PCR), or other suitable technique). For example, peripheral blood cells can be harvested from the patient's blood to obtain genetic material. To ensure that a sufficient quantity of genetic material is available for testing, genetic material may be amplified from cells obtained from the patient, and the amplified genetic material is assayed in accordance with the inventive method. Preferably, a PCR or RT-PCR strategy is employed using primers flanking all or a portion of the GATM gene, so as to amplify this sequence from the patient for the assay. While the method may comprise amplifying and assaying one copy of the GATM gene, preferably, the method comprises amplifying both copies of the GATM gene from the patient, so that both can be assayed in accordance with the inventive method.

[0028] However obtained, the method comprises assaying the genetic material to detect a mutation in the GATM gene (e.g., a mutation at least one of the two GATM alleles). Any test able to detect mutations appropriate to the type of genetic material (e.g., genomic DNA

(gDNA), cDNA, RNA) may be employed. The assaying may comprise obtaining the sequence of at least a portion of the GATM genetic sequence or obtaining the sequence of substantially all of the GATM genetic sequence. In an embodiment, the method may further comprise comparing the sequence of the genetic material of the patient to the sequence of the wild type GATM genetic sequence and identifying any differences between the sequence of the genetic material of the patient and the wild type GATM genetic sequence to detect any mutations. Examples of wild type GATM genetic sequences may include, for example, SEQ ID NO: 21 (wild type GATM genomic DNA) and SEQ ID NO: 22 (wild type GATM isoform 1 cDNA). Other examples of wild type GATM genetic sequences may include sequences set forth in Genbank Accession Nos. NC_000015.10, NC_018926.2, AC025580.8, AMYH02030357.1, CH471082.1, AI253042.1, AK098055.1, AK098393.1, AK223585.1, AK294995.1, AK298350.1, BC039389.1, CD365153.1, DB048816.1, and DB457418.1.

[0029] In an embodiment of the invention, the assaying comprises carrying out a PCR assay that specifically detects the mutation. Examples of PCR assays that specifically detect the mutation may include any one or more of (i) carrying out PCR using primers that amplify the mutated GATM genetic sequence but not the wild type GATM genetic sequence; (ii) carrying out PCR using primers that amplify the wild type GATM genetic sequence but not the mutated GATM genetic sequence; and (iii) carrying out PCR using primers that amplify the mutated GATM genetic sequence, the wild type GATM genetic sequence, and GATM pseudogenes, but the sequences of the PCR products make it possible to distinguish the mutated GATM genetic sequence from the wild type GATM genetic sequence as well as GATM pseudogenes. In an embodiment of the invention, the primers used in the PCR assay amplify the GATM genetic sequence (wild type or mutated) but not GATM pseudogenes.

[0030] In an embodiment of the invention, the assaying comprises sequencing the whole exome, the whole genome, or the whole transcriptome of the genetic material to detect the mutation. Sequencing may be carried out in any suitable manner known in the art. Examples of sequencing techniques that may be useful in the inventive methods include Sanger sequencing, Next Generation Sequencing (NGS) (also referred to as “massively parallel sequencing technology”) or Third Generation Sequencing. NGS refers to non-Sanger-based high-throughput DNA sequencing technologies. With NGS, millions or billions of DNA strands may be sequenced in parallel, yielding substantially more throughput and minimizing

the need for the fragment-cloning methods that are often used in Sanger sequencing of genomes. In NGS, nucleic acid templates may be randomly read in parallel along the entire genome by breaking the entire genome into small pieces. NGS may, advantageously, provide nucleic acid sequence information of a whole genome, exome, or transcriptome in very short time periods, e.g., within about 1 to about 2 weeks, preferably within about 1 to about 7 days, or most preferably, within less than about 24 hours. Multiple NGS platforms which are commercially available or which are described in the literature can be used in the context of the inventive methods, e.g., those described in Zhang et al., *J. Genet. Genomics*, 38(3): 95-109 (2011) and Voelkerding et al., *Clinical Chemistry*, 55: 641-658 (2009).

[0031] Non-limiting examples of NGS technologies and platforms include sequencing-by-synthesis (also known as “pyrosequencing”) (as implemented, e.g., using the GS-FLX 454 Genome Sequencer, 454 Life Sciences (Branford, CT), ILLUMINA SOLEXA Genome Analyzer (Illumina Inc., San Diego, CA), or the ILLUMINA HISEQ 2000 Genome Analyzer (Illumina), or as described in, e.g., Ronaghi et al., *Science*, 281(5375): 363-365 (1998)), sequencing-by-ligation (as implemented, e.g., using the SOLID platform (Life Technologies Corporation, Carlsbad, CA) or the POLONATOR G.007 platform (Dover Systems, Salem, NH)), single-molecule sequencing (as implemented, e.g., using the PACBIO RS system (Pacific Biosciences (Menlo Park, CA) or the HELISCOPE platform (Helicos Biosciences (Cambridge, MA))), nano-technology for single-molecule sequencing (as implemented, e.g., using the GRIDON platform of Oxford Nanopore Technologies (Oxford, UK), the hybridization-assisted nano-pore sequencing (HANS) platforms developed by Nabsys (Providence, RI), and the ligase-based DNA sequencing platform with DNA nanoball (DNB) technology referred to as probe-anchor ligation (cPAL)), electron microscopy-based technology for single-molecule sequencing, and ion semiconductor sequencing.

[0032] The GATM mutation may be any type of gene mutation. For example, the GATM mutation may be any one or more of a missense mutation, a nonsense mutation, an insertion, a deletion, a duplication, and a frameshift mutation. Preferably, the GATM mutation is a dominant heterozygous missense mutation. In an embodiment of the invention, the heterozygous GATM mutation is a mutation which does not reduce or eliminate the overall enzymatic function of GATM.

[0033] The GATM mutation may be located anywhere in the coding sequence of the GATM gene. In an embodiment of the invention, the dominant heterozygous missense mutation is at least one mutation selected from the group consisting of: (i) c.1006A>G, (ii) c.1007C>T, and (iii) c.1022C>T. The foregoing GATM genetic mutations (i)-(iii) are defined herein by reference to the wild type GATM mitochondrial isoform 1 precursor cDNA sequence of GATM (SEQ ID NO: 22). Thus, these GATM genetic mutations are described herein by reference to cDNA ("c."), followed by the particular position in the sequence at which the mutation is taking place, followed by the native nucleotide at that position, followed by the symbol ">," followed by the nucleotide with which the native nucleotide is being replaced.

[0034] The method may further comprise diagnosing the patient with a renal disorder if the at least one dominant heterozygous missense mutation is detected in the biological sample. In an embodiment of the invention, the renal disorder is one or both of Fanconi syndrome and glomerular failure. Individuals with one or more of the GATM mutations described herein may develop Fanconi syndrome early in life, which can be clinically and diagnostically missed, followed by renal glomerular failure in early, mid or late-adulthood. In an embodiment of the invention, the absence of a GATM mutation indicates that the patient does not have the renal disorder.

[0035] In an embodiment of the invention, the method comprises assaying the biological sample to detect a mutation in GATM protein. For example, the GATM protein can be purified from the biological sample (either partially or substantially) and assayed via immunohistological techniques (e.g., Western blotting, ELISA, immunoprecipitation, etc.) using one or more antibodies recognizing GATM protein having any of the mutations described herein (hereinafter referred to as "mutated GATM protein") but not wild type GATM protein. In this regard, the assaying may comprise contacting the sample with an antibody that specifically binds to mutated GATM protein and does not bind to wild type GATM protein, thereby forming a complex, and detecting the complex. Alternatively, or in conjunction, the GATM protein sample from the patient can be assayed using one or more antibodies recognizing wild type GATM protein but not mutated GATM protein. In this regard, the assaying may comprise contacting the sample with an antibody that specifically binds to wild type GATM protein and does not bind to mutated GATM protein, thereby forming a complex, and detecting the complex. In an embodiment, the wild type GATM

protein comprises the amino acid sequence of the wild type mitochondrial isoform 1 precursor GATM protein (Genbank Accession No. NP_001473 (SEQ ID NO: 23)). Other examples of wild type GATM protein sequences may include Genbank Accession Nos. EAW77306.1, EAW77307.1, EAW77308.1, BAD97305.1, BAG58060.1, BAG60595.1, AAH04141.1, AEE61156.1, and AAB29892.1.

[0036] In an embodiment, the heterozygous missense mutation is at least one mutation selected from the group consisting of: (i) p.T336A, (ii) p.T336I, and (iii) p.P341L. The GATM protein mutations (i) p.T336A, (ii) p.T336I, and (iii) p.P341L are defined herein by reference to the wild type isoform 1 amino acid sequence of GATM (SEQ ID NO: 23). Thus, these GATM protein mutations are described herein by reference to protein ("p."), followed by the native amino acid residue being replaced, followed by the particular position in the sequence at which the mutation is taking place, followed by the amino acid residue with which the native amino acid residue is being replaced. In a preferred embodiment of the invention, the method comprises assaying the biological sample to detect at least one dominant heterozygous missense mutation in the GATM mitochondrial isoform 1 precursor protein.

[0037] In an embodiment of the invention, assaying the biological sample to detect at least one dominant heterozygous missense mutation in GATM comprises carrying out one or more of polymerase chain reaction (PCR) amplification, reverse-transcriptase PCR analysis, single-strand conformation polymorphism analysis (SSCP), mismatch cleavage detection, heteroduplex analysis, Southern blot analysis, Western blot analysis, deoxyribonucleic acid sequencing, restriction fragment length polymorphism analysis, haplotype analysis, serotyping, and any combination thereof.

[0038] Another embodiment of the invention provides a method for detecting a GATM mutation in a biological sample from a patient. The method may comprise obtaining a biological sample from the patient. The biological sample may be as described herein with respect to other aspects of the invention. The method may further comprise assaying the biological sample to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein, as described herein with respect to other aspects of the invention. The mutation in GATM may be as described herein with respect to other aspects of the invention.

[0039] GATM is involved in the biosynthesis of creatine. The biosynthesis of creatine is regulated by negative feedback of the creatine product. Without being bound to a particular theory or mechanism, it is believed that mutated GATM protein ultimately leads to renal disorder(s). It is also believed that the administration of one or both of creatine and a creatine substitute to the patient may suppress the production of mutated GATM protein. Suppression of the endogenous production of mutated GATM protein may, in turn, treat or prevent renal disorder(s).

[0040] Accordingly, another embodiment of the invention provides a method of diagnosing and treating a renal disorder in a patient. The method may comprise diagnosing a renal disorder in the patient using any of the methods described herein with respect to other aspects of the invention. The method may further comprise treating the renal disorder in the patient by administering an effective amount of one or both of creatine and a creatine substitute to the diagnosed patient. The creatine or creatine substitute may be, for example, cyclocreatine. Creatine substitutes include structural analogues of creatine which prevent or reduce the endogenous production of mutated GATM protein and which may be produced, e.g., by medicinal chemistry. In general, creatine substitutes may not be metabolizable and may have beneficial chemical properties including, for example, improved solubility, increased biochemical activity, superior pharmacokinetics, etc.

[0041] Another embodiment of the invention provides a method for treating a patient suffering from a renal disorder and having at least one dominant heterozygous missense mutation in GATM protein associated with a renal disorder. The mutation in GATM protein may be as described herein with respect to other aspects of the invention. The method may comprise administering an effective amount of one or both of creatine and a creatine substitute to the patient.

[0042] Still another embodiment of the invention provides a method for stabilizing renal function in a patient suffering from a renal disorder and having at least one dominant heterozygous missense mutation in GATM protein associated with the renal disorder. The mutation in GATM protein may be as described herein with respect to other aspects of the invention. The method may comprise administering an effective amount of one or both of creatine and a creatine substitute to the patient. Renal function refers to the excretory and blood purification or filtration function of the kidney. Renal function may be evaluated by

measuring the glomerular filtration rate (GFR) (the flow rate of filtered fluid through the kidney) or the creatinine clearance rate (CCr) (the volume of blood plasma that is cleared of creatinine per unit time). Renal function may be stabilized if the renal function following administration of one or both of creatine and creatine substitute is not worse (e.g., is the same as or is better than) than the renal function prior to administration of the same compound(s).

[0043] Another embodiment of the invention provides a method of diagnosing a renal disorder in a patient and stabilizing renal function in the patient. The method may comprise diagnosing a renal disorder in the patient using any of the methods described herein with respect to other aspects of the invention. The method may further comprise stabilizing renal function in the patient by administering an effective amount of one or both of creatine and a creatine substitute to the diagnosed patient.

[0044] Many assays for determining an effective amount of one or both of creatine and a creatine substitute are known in the art. For purposes of the invention, an assay, which comprises comparing the extent to which there is an improvement in one or both of GFR and CCr upon administration of a given dose of one or both of creatine and a creatine substitute among a set of mammals of which is each given a different dose of one or both of creatine and a creatine substitute, could be used to determine a starting dose to be administered to a mammal. In an embodiment of the invention, the dose of one or both of creatine and a creatine substitute is sufficient to prevent or reduce the endogenous production of mutated GATM protein. Exemplary dosages of one or both of creatine and a creatine substitute include, but are not limited to, about 0.5 mg/kg/day to about 2000 mg/kg/day, about 1.0 mg/kg/day to about 1500 mg/kg/day, about 1.5 mg/kg/day to about 1000 mg/kg/day, about 2.0 mg/kg/day to about 500 mg/kg/day, about 2.5 mg/kg/day to about 400 mg/kg/day, about 3.0 mg/kg/day to about 300 mg/kg/day, about 3.5 mg/kg/day to about 200 mg/kg/day, about 4.0 mg/kg/day to about 100 mg/kg/day, about 4.5 mg/kg/day to about 50 mg/kg/day, about 5.0 mg/kg/day to about 25 mg/kg/day, about 5.5 mg/kg/day to about 10 mg/kg/day, about 10 mg/kg/day to about 2000 mg/day, about 50 mg/kg/day to about 1500 mg/kg/day, about 150 mg/kg/day to about 1000 mg/kg/day, about 200 mg/kg/day to about 500 mg/kg/day, about 250 mg/kg/day to about 400 mg/kg/day, or about 300 mg/kg/day to about 400 mg/kg/day (“kg” in each of the foregoing exemplary dosage ranges refers to the body weight of the patient in kg).

[0045] In an embodiment, the invention provides a method for treating a patient with one or both of creatine and a creatine substitute, the method comprising: determining whether the patient has the renal disorder by: assaying a biological sample obtained from the patient to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein in the biological sample to determine if the patient has the renal disorder; wherein the detection of the at least one dominant heterozygous missense mutation is indicative of the renal disorder in the patient; and if the at least one dominant heterozygous missense mutation is detected in the patient, then administering one or both of creatine and a creatine substitute to the patient.

[0046] The route of administration of the creatine and/or creatine substitute is not limited. Examples of suitable routes of administration include oral, topical, parenteral, subcutaneous, intravenous, intramuscular, and interperitoneal. Preferably, the one or both of creatine and a creatine substitute are administered orally.

[0047] The inventive methods may, advantageously, make it possible to diagnose and treat renal disorders before or after kidney failure occurs and before or after treatment with kidney dialysis becomes necessary. In an embodiment of the invention, the patient may have received kidney dialysis at one time because of acute renal failure but no longer requires kidney dialysis. In an embodiment of the invention, the method may make it possible to diagnose and treat renal disorders before kidney failure occurs and before treatment with kidney dialysis becomes necessary. Accordingly, in an embodiment of the invention, the method does not comprise treating the patient with kidney dialysis. The inventive methods may, advantageously, prevent or delay the onset of renal failure, e.g., glomerular failure. In another embodiment of the invention, the patient has not been treated with kidney dialysis and the method does not comprise treating the patient with kidney dialysis. The inventive methods may, advantageously, prevent or delay the onset of renal failure, e.g., glomerular failure.

[0048] The terms “treat” or “prevent,” as well as words stemming therefrom, as used herein, does not necessarily imply 100% or complete treatment or prevention. Rather, there are varying degrees of treatment or prevention of which one of ordinary skill in the art recognizes as having a potential benefit or therapeutic effect. In this respect, the inventive methods can provide any amount of any level of treatment or prevention of renal disorder in a

subject. Furthermore, the treatment or prevention provided by the inventive method can include treatment or prevention of one or more conditions or symptoms of the renal disorder being treated. Also, for purposes herein, "prevention" can encompass delaying the onset of the disorder, or a symptom or condition thereof.

[0049] The patient referred to in the inventive methods can be any mammal. As used herein, the term "mammal" refers to any mammal, including, but not limited to, mammals of the order Rodentia, such as mice and hamsters, and mammals of the order Logomorpha, such as rabbits. It is preferred that the mammals are from the order Carnivora, including Felines (cats) and Canines (dogs). It is more preferred that the mammals are from the order Artiodactyla, including Bovines (cows) and Swines (pigs) or of the order Perssodactyla, including Equines (horses). It is most preferred that the mammals are of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). An especially preferred mammal is the human.

[0050] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

EXAMPLES

[0051] Members of four families with autosomal dominant renal Fanconi syndrome and glomerular failure were characterized clinically and genetically. Genome-wide linkage analysis, sequencing, and expression studies were performed in kidney biopsies and renal cells. Knockout mouse studies and evaluations of mitochondrial morphology and function were also performed. Structural studies examined the effects of recognized mutations. The following materials and methods were employed in the experiments described in Examples 1 through 8, below.

Patients

[0052] Members of one family were admitted to the National Institutes of Health (NIH) Clinical Center and enrolled in clinical protocols for rare diseases. Three further families were evaluated in Cambridge, Oxford, and London. All participating individuals or their parents gave written, informed consent. All investigations, including genetic studies, were

approved by the respective Institutional Review Boards and conducted according to the principles of the Declaration of Helsinki.

[0053] The diagnosis of renal Fanconi syndrome and kidney failure was established by routine laboratory investigations of urine and blood samples. Clinical details about three of these four families have been published (Dent et al., *Ann. Eugen.*, 16: 60-87 (1951); Luder, *Arch. Dis. Child.*, 30: 160-164 (1955); Sheldon et al., *Arch. Dis. Child.*, 36: 90-95 (1961); Smith et al., *Q. J. Med.*, 45: 387-400 (1976); Brenton et al., *J. Inherit. Metab. Dis.*, 4: 211-215 (1981); Patrick et al., *Clin. Nephrol.*, 16: 289-292 (1981); Long et al., *Yale J. Biol. Med.*, 63: 15-28 (1990); and Harrison et al., *Clin. Nephrol.*, 35: 148-150 (1991)). Kidney samples were acquired from two affected individuals. One patient was biopsied at age 21, another patient died at the age of 65 due to end stage renal failure and the kidney was studied at autopsy. Electron microscopic studies and histological stainings were performed using established procedures (Klootwijk et al., *N. Engl. J. Med.*, 370: 129-138 (2014)). One affected and one unaffected adult underwent brain ¹H-NMR spectroscopy utilizing standard diagnostic procedures (Joncquel-Chevalier et al., *Biochimie*, 119:146-165 (2015)).

Genetic studies

[0054] The Fanconi syndrome and glomerular failure locus was previously linked to a region on chromosome 15q in an extended US family (Lichter-Konecki et al., *Am. J. Hum. Genet.*, 68: 264-268 (2001)). To prove linkage to the same locus, linkage studies were performed in other families showing the same trait. To this end, DNA was isolated from whole blood using standard procedures and was genotyped with 2000 highly polymorphic STS markers by deCODE Genetics (Iceland) for families A, B, C, or with commercially available SNP chips (AFFYMETRIX diagnostic reagents; Santa Clara, CA) for family D (Fig. 1). Multipoint parametric linkage analysis was performed using established procedures for families B-D (Klootwijk et al., *N. Engl. J. Med.* 370: 129-138 (2014)). Initial gene discovery was performed by targeted capture and next generation sequencing (The Eastern Sequence and Informatics Hub, University of Cambridge, UK) in five affected individuals from three of the families in the study. Recognized sequence variants within the linked region were examined for segregation in all available affected and unaffected family members and confirmed by Sanger sequencing. Observed novel sequence variants were

assessed for uniqueness and evolutionary conservation in various species. Public databases (dbSNP, 1000 Genomes, exome database) were also interrogated.

Renal proximal tubular cell model and kidney imaging

[0055] A permanently transfected, inducible renal proximal tubular cell line derived from LLC-PK1 cells was created for each patient *GATM* mutation using recombinant technology. LLC-PK1 cells are an established model and reliably express many properties of the renal proximal tubule (Klootwijk et al., *N. Engl. J. Med.*, 370: 129-138 (2014); Kleta et al., *Pflügers Arch.*, 429: 370-377 (1995)). For *in-situ* immunostaining, subcellular studies, metabolic studies, expression studies and electron microscopy, cells and tissues were prepared and investigated using established procedures. Specifically, the renal and intracellular localizations of GATM were studied. Changes in expression of relevant genes were studied using established real-time polymerase chain reaction (PCR) technology.

Gatm knockout mice

[0056] *Gatm* knockout mice have been generated to study the biochemical function of this mitochondrial protein (Choe et al., *Hum. Mol. Genet.* 22: 110-123 (2013)). Mutant mice were viable, without detectable gross phenotypic defects, but dystrophic. Urinary metabolites were assessed in knockout and control mice using established analytic procedures.

Structural studies

[0057] To test the hypothesis of mutation-mediated aggregation of GATM, molecular dynamics simulations on the wild-type monomer and on the four mutants were performed. Modelling of possible protein-protein interaction surfaces was performed starting with the structure of the wild-type monomer (pdb: 1JDW), and using the GRAMM-X Protein-Protein Docking Web Server at the Vakser laboratory at the Center for Computational Biology at the University of Kansas (Lawrence, KS, USA).

Formation of stable, inducible cell lines

[0058] Tetracycline-inducible stably transfected LLC-PK1 cells were obtained using the FLP-IN T-REX system (Invitrogen, Paisley, UK) containing the constructs pFRT/lacZeo and

pcDNA/5/TR (JCRB0060, Health Science Research Resources Bank, Tokyo, Japan). These cell lines and derived cells were maintained in 1 g/L glucose DMEM medium (PAA laboratories, E15-005, Yeovil, UK) with 8% fetal bovine serum (PAA laboratories, A15-109, Yeovil, UK), 2 mM L-glutamine (PAA laboratories, M11-006, Yeovil, UK), and 100 IU/ml penicillin/100 µg/ml streptomycin (PAA laboratories, P11-010, Yeovil, UK).

[0059] The human *GATM* coding region was obtained from a TRUECLONE full-length cDNA clone (sc321307, NM_001482.1; OrigGene Technologies; Rockville, MD, USA). The full length *GATM* cDNA was subcloned into the *NotI* site of the pcDNA5FRT/TO vector (ThermoFisher Scientific, Waltham, MA, USA). The patients' mutations, c.1006A>G (p.T336A), c.1007C>T (p.T336I), and c.1022C>T (p.P341L), were inserted using a QUIKCHANGE II site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The oligonucleotide pairs used for this purpose are listed in Table 1, below:

TABLE 1
Primer Nucleotide Sequences

Mutation	Nucleotide Sequence	SEQ ID NO:
(c.1006A>G)	5'-TCAAGAAAGCAGGATGGG <u>G</u> CTATCATTACTCCTC-3'	1
	5'-GAGGAGTAATGATAG <u>CCC</u> ATCCTGCTTTCTTGA-3'	2
(c.1007C>T)	5'-TCAAGAAAGCAGGATGGGA <u>T</u> TATCATTACTCCTC-3'	3
	5'-GAGGAGTAATGATA <u>A</u> TCCATCCTGCTTTCTTGA-3'	4
(c.1022C>T)	5'-TCAAGAAAGCAGGATGGGA <u>T</u> TATCATTACTCCTCC-3'	5
	5'-GGAGGAGTAATGATA <u>A</u> TCCATCCTGCTTTCTTGA-3'	6

[0060] The full length wild-type and mutant cDNA clones were all sequence-verified. The fixed and immunolabeled cells were imaged with a LSM700 confocal microscope using ZEN 2012 SP1 software (Zeiss, UK).

[0061] The stable transfected Frt-1 cells containing the pcDNA/5/TR construct were cotransfected at a ratio of 9:1 (w/w) circularized pOG44:pcDNA5/FRT/TO/GATM, pOG44:pcDNA5/FRT/TO/T336AGATM, pOG44:pcDNA5/FRT/TO/T336IGATM, or pOG44:pcDNA5/FRT/TO/P341LGATM. Transfection was performed using LIPOFECTAMINE 2000 reagent (ThermoFisher Scientific, 11668027, UK) according to the

manufacturer's protocol. The transfected cells were incubated for four weeks in selection medium containing 500 µg/ml hygromycin-B. Several clones were obtained for cells transfected with these constructs. Incorporation of the constructs in the genomic DNA was demonstrated by PCR analysis, and subsequently by sequence analysis of the full coding region of human *GATM*. Induced expression of human *GATM* was achieved using 1 µg/ml tetracycline (Applichem/VWR, A1685, Lutterworth, UK) for each of the clones. To this end, real-time PCR, Western analysis, and immunohistochemical analysis were utilized. For all functional analyses described, a single clone was used. Upon induction with 1 µg/ml tetracycline, these clones showed equal expression of human *GATM*.

RNA isolation

[0062] Inducible LLC-PK1 cells were seeded in cell medium (RPMI medium 1640 with 11.1 mM glucose (Gibco Cell Culture Systems - Invitrogen, Karlsruhe, Germany), 10% FCS, 5 IU penicillin G, 50 µg/ml streptomycin, and 5 mM sodium hexanoate). To induce the expression system of the cells, tetracycline (1 µg/ml) was added to the medium. Cells were cultured and induced for three weeks until ribonucleic acid (RNA) was isolated. Medium was changed every other day.

[0063] Total RNA from inducible LLC-PK1 cells was isolated using an RNEASY MICRO KIT, a column based kit optimized for the purification from small amounts of tissue (Qiagen, Hilden, Germany). Total RNA was prepared following the manufacturer's instructions. The RNA concentration was quantified using a NANODROP ND-1000 photometer (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Quality of the RNA used for real-time RT-PCR was tested by agarose electrophoresis.

Quantitative real-time RT-PCR

[0064] Reverse transcription with M-MLV-RT (Promega GmbH, Mannheim, Germany) and random primers (Fermentas GmbH, St. Leon-Rot, Germany) was done using 1 µg total RNA to generate single-stranded cDNA. Relevant contamination with genomic DNA was excluded by negative control reactions without the reverse transcriptase enzyme (RT). Real-time PCR of cDNA samples was performed using a LIGHTCYCLER 480, a rapid high-throughput, plate-based real-time PCR amplification and detection instrument (Roche, Basel,

Switzerland) using specific and, wherever applicable, intron-spanning primers, and a SYBR GREEN fluorescent chemicals mastermix (Roche, Basel, Switzerland). Target gene expression levels were quantified relative to beta-actin expression under consideration of PCR efficiencies calculated on the basis of standard dilution curves. The specificity of PCR amplifications was verified by agarose electrophoresis and melting curve analysis. Primers specific for murine *Gatm* (GenBank accession No. NM_025961.5), porcine *IL-18* (GenBank accession No. NM_213997), porcine *NLRP3* (GenBank accession No. NM_001256770), porcine β -actin (GenBank accession No. ENSSTCT00000008324), and murine β -actin (GenBank accession No. NC_000085.6) were obtained from Life Technologies GmbH (Darmstadt, Germany). Table 2, below, lists the gene name, the annealing temperature used for the expression studies, and the sequences of each primer.

TABLE 2

PCR primer sequences and PCR conditions for expression studies

Gene	Primer	Sequence (5'>3')	Annealing temp.	SEQ ID NO
murine <i>Gatm</i>	sense	TTTTCAAGAAAGCAGGATGGA	57°C	7
	antisense	TGACATCCAGAGGGGATGAT		8
Porcine <i>IL-18</i>	sense	AGCTGAAAACGATGAAGACCTG	61°C	9
	antisense	AAACACGGCTTGATGTCCCT		10
porcine <i>NLRP3</i>	sense	CCTGCAAAAACCTGGGGTTGG	60°C	11
	antisense	CTCCAAGAGCATTTCCTCCGT		12
Porcine β -actin	sense	CCCATCTACGAGGGGTACG	60°C	13
	antisense	CGCTCCGTCAGGATCTTC		14
Murine β -actin	sense	GACAGGATGCAGAAGGAGATTACTG	57°C	15
	antisense	CCACCGATCCACACAGAGTACTT		16

Immunofluorescence on inducible LLC-PK1 cells

[0065] Inducible LLC-PK1 cells were grown on glass coverslips and induced with 1 μ g/ml tetracycline for at least 24 hours. For mitochondrial staining, MITOTRACKER ORANGE CMTMROS (Invitrogen, Karlsruhe, Germany) was added (1:5000) to the medium and incubated at 37°C, 5% CO₂, for 30 minutes. After washing with Ringer's solution, cells were fixated for 15 minutes with 3% paraformaldehyde in PBS (phosphate buffered saline pH 7.4). Cells were rinsed twice with PBS and afterwards incubated in PBS containing 0.1%

SDS for 5 minutes (to unmask the epitopes) followed by two 5 minute washes with PBS. Primary antibodies were applied for 1 hour. Primary and secondary antibodies were diluted in PBS with 0.04% TRITON X-100 surfactant (Sigma, Taufkirchen, Germany). After washing twice with PBS, for 5 minutes each, the cells were incubated for 1 hour with the secondary antibody and 2'-(4-Ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-1H,1'H-2,5'-bibenzimidazole trihydrochloride (HOE 33342) to stain the nuclei. After a final washing step (2 times for 5 minutes each in PBS), the glass coverslips with the cells were mounted on slides with fluorescent-free GLYCERGEL mounting medium (DakoCytomation, Hamburg, Germany). A list of the primary and secondary antibodies, as well as further dyes, used is provided in Table 3, below.

TABLE 3

Antibodies and conditions for imaging studies

Antibody/Dye (Usage)	Species	Dilution	Manufacturer
anti-GATM (IF, WB)	rabbit	1:200 (IF) 1:2000 (WB)	Proteintech, Manchester, UK
anti- α -SMA (IF)	mouse	1:600	Abcam, Cambridge, UK
anti- β -actin (WB)	rabbit	1:5000	Sigma-Aldrich, Taufkirchen, Germany
anti-IL-18 (IF, WB)	goat	1:15 (IF) 1:500 (WB)	R&D systems, Minneapolis, USA
ALEXA FLUOR 555 anti rabbit (IF)	donkey	1:400	Invitrogen, Karlsruhe, Germany
ALEXA FLUOR 488 anti mouse (IF)	donkey	1:400	Invitrogen, Karlsruhe, Germany
anti rabbit IgG-HRP (WB)	donkey	1:5000	Santa Cruz, Heidelberg, Germany
Mitotracker (IF) (Stock: 1 mM)		1:5000	Invitrogen, Karlsruhe, Germany
HOE33342 (IF) (Stock: 5×10^{-4} M)		1:1000	Invitrogen, Karlsruhe, Germany

(IF: immunofluorescence, WB: Western blot)

Tissue preparation and immunofluorescence for cryosections

[0066] Mice were anesthetized using isoflurane, and were euthanized by replacement of the blood with 0.9% NaCl solution containing 10 IU /mL heparin through a catheter placed into the abdominal aorta. Afterwards, dead mice were perfused with a “fixative solution” containing 3% paraformaldehyde, 3.4% sucrose, 90 mM NaCl, 15 mM K_2HPO_4 , 1 mM EGTA, and 2 mM $MgCl_2$, pH 7.4. Kidneys were removed and incubated overnight at 4°C in

the “fixative solution” with 17% sucrose and only 1% paraformaldehyde. Then, kidneys were frozen in 2-methylbutane (-35°C) and stored at -80°C until use. Cryosections (5 µm) were mounted on poly-L-lysine slides (Kindler, Freiburg, Germany). Prior to incubation with the primary antibodies, sections were incubated in 0.1% SDS for 5 minutes to unmask epitopes, washed 2 times for 5 minutes each in PBS, followed by blocking with 5% bovine albumin for 10 minutes. Primary and secondary antibodies were diluted in phosphate buffered saline (PBS), pH 7.4, with 0.04% TRITON X-100 surfactant (Sigma, Taufkirchen, Germany). Primary antibodies were applied overnight at 4°C. After washing 2 times for 5 minutes each with PBS, sections were incubated for 1 hour with the secondary antibodies and HOE33342 to stain the nuclei. The slides were then washed 2 times for 5 minutes each in PBS, and mounted with fluorescent-free GLYCERGEL mounting medium. A list of the primary and secondary antibodies, as well as dyes used is provided in Table 3, above.

Immunofluorescence on paraffin sections

[0067] Human kidney samples were embedded in paraffin and 5 µm slices were mounted on poly-L-lysine slides (Kindler, Freiburg, Germany). To remove paraffin from the tissues, slides were put into xylol twice for 10 minutes each and afterwards rehydrated in an alcohol series (99% isopropanol, 95% EtOH, 80% EtOH, 70% EtOH) for 10 minutes each. Slices were then rinsed with PBS for 5 minutes, and heated at 95°C in citrate buffer (pH 6.0) for 15 minutes to unmask epitopes. The rest of the protocol was performed according to the protocol for cryosections (above). A list of the primary and secondary antibodies, as well as dyes used is provided in Table 3, above.

Masson-Goldner staining on paraffin sections

[0068] For Masson-Goldner staining, paraffin was removed from the sections as described above. Afterwards, slices were incubated in WEIGERT'S IRON HEMATOXYLIN staining solution for 2 minutes, rinsed in running tap water for 2 minutes, and then briefly put into 0.1% HCl-alcohol solution. Subsequently, the slices were rinsed in running tap water for 10 minutes, incubated in 0.5% phosphotungstic acid for 15 seconds, and washed three times for 5 minutes each in distilled water, followed by staining in FUCHSINE acid and PONCEAU for 10 minutes. Next, slides were incubated 3 times each for 1 minute in 1%

glacial ethanoic acid, 20 seconds in orange G-solution, 2 minutes in 1% glacial ethanoic acid, 4 minutes in LIGHT GREEN histology stain solution, and rinsed twice in 2% glacial ethanoic acid. Afterwards, slices were dehydrated by immersing once for 20 seconds in 96% isopropanol, twice for 5 minutes each in 99% isopropanol, and twice for 10 minutes each in xylol. The slides were mounted with xylol-containing DEPEX mounting medium.

Quantification of intracellular IL-18 in inducible LLC-PK1 cells

[0069] Quantification of intracellular IL-18 protein content in induced LLC-PK1 cells was performed using an ELISA Kit from PromoKine (PK-EL-62816P, Heidelberg, Germany) recognizing porcine IL-18. The ELISA was performed according to the manufacturer's protocol. In addition, Western blots were used according to the method described below. Antibodies used are shown in Table 3, above.

Determination of protein concentration with Bradford assay

[0070] Frozen cell pellets were used to determine protein concentration of inducible LLC-PK1 cells. Cell pellets were resuspended in sample buffer containing DTT, 86% glycerol, 10 % SDS, 0.5 M tris(hydroxymethyl)aminomethane (TRIS) pH 6.8, 0.5 M MgCl₂, and 25 U/μl benzonase. For quantification, different bovine serum albumin (BSA) solutions of known concentration were prepared. All samples were diluted in BRADFORD reagent (1:100) and transferred to 96-well plates. After incubating for 15 minutes in the dark, the absorption at 595 nm was measured using a microplate reader (Bio-Rad, Hercules, USA). Protein concentrations were determined on the basis of the standard linear slope from the BSA standards.

Respirometric measurements in inducible LLC-PK1 cells

[0071] Inducible LLC-PK1 cells for respirometric measurements were cultured and induced with tetracycline (1 μg/ml) for 7 days before respiratory measurements were performed. Medium was changed every other day. LLC-PK1 cells overexpressing wild-type GATM (n=12) or overexpressing GATM containing the T336A mutation (n=11) were measured.

[0072] Activity of the respiratory system was analyzed in a two-channel titration injection OXYGRAPH-2K respirometer (Oroboros Instruments, Innsbruck, Austria) at 37°C. Cells were harvested, resuspended in mitochondrial medium MiR05 and transferred to the OXYGRAPH chambers. After a stabilization phase of 30 minutes, ROUTINE respiration of intact cells was measured. Specific activity (pmol/s) of the mitochondrial marker citrate synthase (CS) was measured photometrically at 412 nm using an established protocol as published before (Klootwijk et al., *N. Engl. J. Med.*, 370: 129-138 (2014)). Respiration rates were calculated as the time derivative of oxygen concentration (pmol/(s*ml)) and normalized to citrate synthase (CS) activity (pmol/(s*ml*CS activity)).

Live cell imaging of mitochondrial membrane potential and reactive oxygen species production

[0073] Cells (3.5×10^5 LLC-PK1) were seeded on a 30-mm cover glass coated with poly-L-lysine. When confluent, cells were incubated with 1 µg/ml tetracycline for 48 hours to induce expression of wild-type or mutant T336A GATM. Mitochondrial membrane potential was assessed by loading the cells with 50 nM tetramethylrhodamine ester (TMRM) for 1 hour at 37°C in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered solution at pH 7.4 containing 138 mM NaCl, 5.6 mM KCl, 1.2 mM NaH₂PO₄, 2.6 mM CaCl₂, 1.2 mM MgCl₂·6H₂O, 10 mM Glucose, 4.2 mM NaHCO₃, 10 mM HEPES. Ten µM VERAPAMIL was added to prevent dye extrusion by multi-drug resistance mechanisms. Reactive oxygen species (ROS) production was measured by incubating the cells with 5 µM CELLROX Deep Red fluorescent chemical reagent (Life Technologies) for 30 minutes at 37°C. The rate of increase in fluorescence signal was taken as proportional to the rate of ROS production. Cells were imaged at 37°C using a LEICA SP5 automated inverted confocal laser scanning microscope and a 63x objective. TMRM was excited at 561 nm and emission was detected between 566 nm and 629 nm. CELLROX was excited at 633 nm and light was collected between 638 nm and 737 nm. Images were acquired every 10 minutes to minimize laser-induced photo-toxicity for a maximal duration of 40 minutes. Thirty fields were acquired per experiment using the Matrix screener LEICA software. Images were analyzed using IMAGEJ software. IMAGEJ is an open source, Java-based, image processing program

designed for scientific multidimensional studies. TMRM mitochondrial signals were isolated by setting a threshold level to remove the background cytosolic fluorescence.

Structural studies for mutated GATM

[0074] Publicly available GATM crystal structures (i.e. 1JDW subunit A) were utilized to simulate the effect of all mutations identified. In order to test the hypothesis of mutation-mediated destabilization of the region around B4, molecular dynamics simulations were performed on the wild-type GATM monomer and on the three GATM mutants, starting from the coordinates of 1JDW subunit A. Each simulation lasted for 40 nanoseconds.

¹H-NMR creatine spectroscopy

[0075] Nuclear magnetic resonance (¹H-NMR) spectroscopy of body fluids shows the majority of proton-containing compounds and, therefore, can provide an overall view of metabolism. ¹H-NMR spectrum provides a characteristic ‘fingerprint’ of almost all proton-containing metabolites. In these spectra, the spectral parameters chemical shift, spin-spin coupling, and signal intensity are valuable for body fluid analysis. The peak area or signal intensity of a resonance in a ¹H-NMR spectrum is proportional to the number of protons contributing to the signal when appropriate experimental conditions are used. Since the peak area is proportional to the number of protons contributing to the signal, it is also proportional to the concentration of the molecule concerned. Therefore, it is possible to use ¹H-NMR spectroscopy for metabolite quantification. The sensitivity of the technique is in the low micromolar range for most metabolites. It is well suited to detect creatine in urine, plasma, and cerebrospinal fluid as well as *in-vivo* within the brain (overview and details taken from “Handbook of ¹H-NMR spectroscopy in inborn errors of metabolism: body fluid NMR spectroscopy and in vivo MR spectroscopy / Engelke U. et al., Heilbronn: SPS Verlagsgesellschaft, 2007). In the experiments described here, ¹H-NMR spectra were acquired on an ACHIEVA 3T MR scanner (Philips, Best, The Netherlands) using a PRESS sequence (TR=2000 ms, TE=35 ms, 128 averages, 15x15x15 mm voxel) and processed in LCModel (Provencher, Magn Reson Med 30, 672 (1993)), referencing to the unsuppressed water.

Creatine supplementation

[0076] Male, wild-type C57Bl/6 mice (n=8, age 10 weeks, The Jackson Laboratory, Bar Harbor, ME, USA) were randomly assigned to either a group nourished with a diet of bread and tap water (low creatine, high carbohydrate diet) or a group nourished with a diet of bread and an additional supplement of 1% (w/w) creatine (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in the drinking water. After sacrificing the mice, kidneys were removed, divided into halves, then immediately frozen in liquid nitrogen and stored at -80°C until further experimentation.

[0077] Frozen mouse kidneys were pulverized in liquid nitrogen using a mortar, and RNA was isolated using RNEASY MINI Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. RNA was quantified using the NANODROP 2000c spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany), and its quality was assessed by imaging on 1% agarose gel. RNA was then transcribed to cDNA using a Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's recommendations. Real-time PCR was performed as described above using cDNA, SYBR GREEN fluorescent mix (Roche Diagnostics GmbH, Mannheim, Germany) and primers for mouse Gatm or β -actin (listed in Table 2, above). For real-time detection as well as analyses of the samples a LIGHTCYCLER 480 real time PCR platform (Roche Diagnostics, Rotkreuz, Switzerland) was employed according to the manufacturer's recommendations. Quality of the amplicon was further investigated by imaging on a 3% agarose gel.

[0078] Frozen mouse kidneys were weighed, then pulverized in liquid nitrogen using a mortar, and proteins eluted by incubation in 3 μ l/mg kidney tissue of a lysis-buffer containing RIPA-buffer (1% Igepal CA-630, 0.5% sodium-desoxycholate, 0.1% SDS in PBS), 200 mM PMSF (1/200 v/v) and EDTA-free PROTEASE INHIBITOR COCKTAIL SET III (1/100 v/v, Calbiochem, San Diego, CA) for 20 minutes on ice. Samples were centrifuged at 14.800 rpm for 10 minutes at 4°C and supernatants stored at -20°C until further analysis. The protein content of the samples was quantified in 96-well plates (Nuncleon Delta Surface, NUNC A/S, Roskilde, Denmark) according to Bradford using BIO-RAD protein assay (Bio-Rad Laboratories GmbH, Muenchen, Germany) and BIO-RAD 550 Microplate Reader (Bio-Rad) according to the manufacturer's recommendations. Equal amounts of proteins in LAEMMLI-buffer (Bio-Rad) including 2-mercaptoethanol 1/20 (v/v, Merck Schuchard,

Hohenbrunn, Germany) were cooked for 5 minutes, separated by 10% SDS-PAGE and transferred to an AMERSHAM HYBOND PVDF blotting membrane (GE Healthcare Life Sciences; Little Chalfont, UK). The membrane was blocked by incubation with 5% fat-free dry milk-powder (w/v, AppliChem GmbH, Darmstadt, Germany) in PBS-Tween for 1 hour at room temperature followed by incubation with primary antibody polyclonal rabbit anti-GATM (1/2000 v/v, Proteintech, Manchester, UK) in 1.5% BSA in PBS-Tween at 4°C overnight. The secondary antibody, donkey anti-rabbit IgG-horse radish peroxidase (HRP, 1/5000, v/v, Santa Cruz Biotechnology) in 1% PBS-Tween, was incubated for 1 hour at room temperature and protein bands developed using Western Blot LUMINOL Reagent (Santa Cruz Biotechnology, Heidelberg, Germany) and visualized a FUSION FX7 chemiluminescence and fluorescence camera (Vilber Lourmat, Marne-la-Vallee, France). The membranes were stripped from bound antibodies by applying RESTORE WESTERN BLOT STRIPPING Buffer (ThermoScientific, Rockford, IL) for 15 minutes at room temperature, washed, and blocked as described above. The house-keeping protein was detected using as primary antibody rabbit anti-mouse beta-actin (1/5000 v/v, Sigma-Aldrich, Taufkirchen, Germany) in 1.5% BSA-PBS-TWEEN non-ionic surfactant at 4°C overnight, and as secondary antibody donkey anti-rabbit IgG bound to horse radish peroxidase (HRP; 1/5000, v/v, Santa Cruz Biotechnology, Heidelberg, Germany) in 1% PBS-TWEEN for 1 hour at room temperature. Quantification of Gatm and beta-actin was performed using IMAGEJ software.

Electron microscopy and immunogold labeling

[0079] Kidney specimens from the Fanconi patients were originally embedded in paraffin. For electron microscopy, paraffin was removed from the samples according to the following protocol. Samples were treated with xylol (2 x 15 minutes, 3 x 30 minutes), 100% ethanol (5 x 15 minutes), 95% ethanol (15 minutes), 90% ethanol (15 minutes), 70% ethanol (15 minutes), 50% ethanol (15 minutes), distilled water (2 x 15 minutes). After post fixation with 4% glutaraldehyde solution (in 0.1 M Na-cacodylate pH 7.4 for 60 minutes), samples were finally rinsed with 0.1 M Na-cacodylate pH 7.4 (2 x 15 minutes).

[0080] Inducible LLC-PK1 cells were grown on cover slips and induced with tetracycline (1 µg/ml) for 3 days. Before embedding, cells were fixed using a 2% glutaraldehyde solution

in 0.1 M Na-cacodylate pH 7.4 for about 1 hour. Post fixation and dehydration of the samples was carried out using the following protocol: rinsing in 0.1 M Na-cacodylate pH 7.4 (3 x 20 minutes), 1% OsO₄ in 0.1 M Na-cacodylate pH 7.4 (2 h), 0.1 M Na-cacodylate pH 7.4 cacodylic acid (3 x 20 minutes), 50% ethanol (15 minutes), 70% ethanol (15 minutes), 90% ethanol (15 minutes), 96% ethanol (15 minutes), 100% ethanol (20 minutes), acetone (3 x 15 minutes). Finally, samples were embedded in EPON embedding media, and polymerized at 60°C for 48 hours using standard protocols.

[0081] For immunogold labeling, freshly prepared ultrathin sections (70 nm; ULTRACUT UC6; Leica, Wetzlar, Germany) were mounted on grids and treated according to the following protocol: 0.1% glycine in PBS (5 minutes), 1% BSA in glycine solution (5 minutes), primary antibody against GATM in 0.1% BSA in PBS for 1.5 hours, 0.1% BSA in PBS (5 x 2 minutes), secondary antibody (goat anti rabbit 6 nm Gold; Aurion, Wageningen, Netherlands) in 0.1% BSA in PBS for 1.5 hours, 0.1% BSA in PBS (5 x 2 minutes), PBS (2 x 2 minutes), 2% glutaraldehyde in PBS (5 minutes), PBS (2 x 2 minutes), distilled water (3 x 2 minutes). To improve contrast, specimens were stained with 2% uranyl acetate (UAc) for 30 minutes.

[0082] Ultrathin sections were imaged using either an EM902 (Zeiss, Oberkochen, Germany) or a JEM-2100F (JEOL, Tokyo, Japan) transmission electron microscope, operated at 80keV (902) or 200 keV (JEM). Digital micrographs were recorded using a CCD 2k camera (Troendle, Moorenweis, Germany) or a CMOS 4k F416 camera (TVIPS, Gauting, Germany).

Analysis of plasma and urine of GATM^{+/+} and GATM^{-/-} mice

[0083] Spot urine of 9 - 12 week old GATM^{+/+} (n=6) and GATM^{-/-} (n=6) mice was collected two times per mouse on two different days. After the last sample was taken, mice were sacrificed and blood was collected. Metabolites of the creatine synthesis pathway (creatinine, creatine, guanidinoacetate), and amino acids were measured in urine and plasma as described below. All urine values were normalized to urinary osmolality. For measurements, urine samples were diluted 1:4.

[0084] Amino acids were derivatized with propylchloroformate/propanol and measured using Liquid chromatography–mass spectrometry/mass spectrometry (LC-MS/MS) with

electrospray ionization in positive mode and detection by multiple reaction monitoring (MRM) as described in van der Goot et al., *PNAS*, 109: 14912-14917 (2012). Five microliter of diluted urine or plasma were used for derivatization. Quantification was based on calibration curves using ^{13}C , ^{15}N -labeled amino acids as internal standards.

[0085] Creatinine, creatine, and guanidinoacetate were analyzed by LC-MS/MS with electrospray ionization (ESI) in positive mode. An AGILENT 1200 SL high performance liquid chromatograph (HPLC) hyphenated to a 4000 QTrap mass spectrometer (AB Sciex; Darmstadt, Germany), working with a TURBOV electrospray ion source (AB Sciex) was used. HPLC separation was performed on an ATLANTIS T3 reversed-phase column (150 mm $\times$ 2.1 mm i.d., 3 μm , Waters, Eschborn, Germany) with mobile phases A (0.1% formic acid in water, v/v) and B (0.1% formic acid in acetonitrile, v/v) employing a flow rate of 350 $\mu\text{l}/\text{minute}$. The following gradient was used with deviating parameters for the positive mode ionization run given in brackets: 0–10 minutes linear increase from 0% to 100% B, hold for 2 minutes (0 minutes) and return to 0% B at 12.1 minutes (10.1 minutes) and equilibrate for 7 minutes. The column was kept at 25°C. An injection volume of 10 μl was used. Analytes were detected in multiple reaction monitoring (MRM) mode. For the analysis in positive mode ionization, two time segments were programmed detecting different analytes listed in Table 4, below. This table presents the parameters used for LC-MS analysis (DP = declustering potential; P1 = Period 1; P2 = Period 2). Quantification was based on calibration curves using the stable isotope-labeled analogs as internal standards. Creatinine-d3 was used as an internal standard for creatine and guanidinoacetate because they eluted close to each other.

TABLE 4

Parameters for LC-MS/MS analysis

Analyte	Ionization mode	Q1 (m/z)	Q3 (m/z)	Dwell time (ms)	DP
Creatinine	positive –P1	114.0	86.0	150	26
Creatinine d3	positive –P1	117.0	89.0	150	26
Creatine	positive –P1	132.2	90.0	150	21
Guanidinoacetate	positive –P1	118.1	43.0	150	36

[0086] Ten microliters internal standard (IS1) solution, containing creatinine-d3 at a concentration of 100 μ M, were added to 10 μ l pre-diluted urine and further diluted with water to a final volume of 100 μ l. A plasma aliquot of 25 μ l was spiked with 5 μ l IS1 solution and 10 μ l surrogate solution containing [U- 13 C]glucose, [U- 13 C]lactic acid, and [U- 13 C]pyruvic acid at a concentration of 1 mM. Proteins were precipitated with 150 μ l cold methanol, and samples were centrifuged for 5 minutes at 4°C and 11200 g. The supernatant was collected and dried using a COMBIDANCER vacuum evaporator (Hettich AG, Bäch, Switzerland). The residue was redissolved in 100 μ l water and subjected to analysis.

EXAMPLE 1

[0087] This example provides a summary of the clinical and laboratory analyses findings in members from four individual families exhibiting an autosomal dominant disorder including renal Fanconi syndrome in childhood followed by progressive glomerular dysfunction later in life.

[0088] All affected individuals studied exhibited an autosomal dominant disorder consisting of renal Fanconi syndrome in childhood followed by progressive glomerular dysfunction later in life. The individuals belong to four different families for which a pedigree analysis chart is shown in Fig. 1. This chart shows a *de novo* “appearance” of dominant renal Fanconi syndrome and kidney failure in family A. Renal tubular losses appeared clinically mild, without causing debilitating rickets or bone deformities. Typical laboratory findings included glucosuria, hyperphosphaturia, generalized hyperaminoaciduria, low molecular weight proteinuria, and metabolic acidosis. As an example, at age 18 months, the youngest affected child studied exhibited laboratory findings typical of renal Fanconi syndrome but no glomerular compromise. Table 5, below lists the laboratory findings from an affected and genotyped 18 month old child before start of treatment, illustrating the presence of renal Fanconi syndrome. The table lists the tests measured, the results for each test, and the age appropriate reference range for each test. Normal plasma creatinine and normal parathyroid hormone (PTH) levels were obtained. Typical signs of renal Fanconi syndrome, i.e. glucosuria, “mild” hyperphosphaturia and hypophosphatemia, generalized hyperaminoaciduria, low molecular weight proteinuria, metabolic acidosis, and slightly elevated alkaline phosphatase (marked by an asterisk (*) in Table 5) were observed.

TABLE 5

Test	Result	Reference Range
Sodium	143 mmol/L	(133 – 146)
Potassium	3.6 mmol/L	(3.5 - 5.5)
Chloride	108 mmol/L	(100 - 108)
Total CO ₂ *	17 mmol/L	(20 - 30)
Urea	5.3 mmol/L	(2.5 - 6.0)
Creatinine	22 µmol/L	(16 - 33)
Calcium	2.47 mmol/L	(2.17 - 2.44)
Magnesium	0.95 mmol/L	(0.66 - 1.00)
Phosphate*	1.16 mmol/L	(1.2 - 2.1)
Albumin	49 g/L	(34 - 42)
Alkaline Phosphatase (ALP)*	413 U/L	(145 - 320)
Alanine Transaminase (ALT)	30 U/L	(5 - 45)
Total bilirubin	5 µmol/L	(<18)
Intact PTH	2.5 pmol/L	(0.7 - 5.6)
Urine Glucose Stix result*	positive +++	(negative)
Urine Glucose	8.0 mmol/L	not applicable (N/A)
Urine Phosphate	8.86 mmol/L	N/A
Urine Creatinine	0.5 mmol/L	N/A
Urine Phosphate/ Creatinine ratio*	17.72	(1.2 - 14)
Tubular Reabsorption of Phosphate*	66 %	(70 - 100)
TmP/GFR*	0.77 mmol/L	(1.10 - 2.70)
Urine Glycine/ Creatinine ratio*	5090 µmol/mmol	(250 - 626)
Urine Serine/Creatinine ratio*	2355 µmol/mmol	(20 - 100)
Urine Threonine/ Creatinine ratio*	2129 µmol/mmol	(10 - 45)
Urine Proline/Creatinine ratio*	1864 µmol/mmol	(0 - 3)
Urine Leucine/Creatinine ratio*	320 µmol/mmol	(3 - 10)
Urine Isoleucine/Creatinine ratio*	141 µmol/mmol	(2 - 10)
Urine Valine/Creatinine ratio*	738 µmol/mmol	(2 - 8)
Urine Alanine/ Creatinine ratio*	3092 µmol/mmol	(30 - 80)
Urine Glutamine/ Creatinine ratio*	4159 µmol/mmol	(30 - 120)
Urine Arginine/ Creatinine ratio*	166 µmol/mmol	(2 - 10)

Test	Result	Reference Range
Urine Ornithine/ Creatinine ratio*	230 umol/mmol	(2 – 10)
Urine Lysine/Creatinine ratio*	1614 umol/mmol	(5 - 30)
Urine Cystine/ Creatinine ratio*	226 umol/mmol	(5 - 25)
Urine Methionine/ Creatinine ratio*	46 umol/mmol	(3 - 15)
Urine Taurine/Creatinine ratio	184 umol/mmol	(30 - 105)
Urine Phenylalanine/Creat ratio	345 umol/mmol	(5 - 15)
Urine Tyrosine/Creatinine ratio	716 umol/mmol	(5 - 15)
Urine Tryptophan/Creatinine ratio	193 umol/mmol	(1 - 5)
Urine Histidine/Creatinine ratio	1909 umol/mmol	(100 - 300)
Urine Aspartate/Creatinine ratio	82 umol/mmol	(10 - 35)
Urine Glutamate/Creatinine ratio	86 umol/mmol	(0 - 3)
Urine Albumin/Creatinine Ratio	54.3 mg/mmol	(1.5 - 8.7)
Urine NAG/Creatinine ratio	392 Units/mmol	(2 - 22)
Urine Retinol Binding Protein/Creatinine ratio	10420 ug/mmol	(2.7 - 103)

[0089] Urinary guanidinoacetate excretion was normal in the individuals studied. No extra-renal clinical findings were noted.

EXAMPLE 2

[0090] This example demonstrates that heterozygous missense mutations in *GATM* are present in individuals with Fanconi syndrome and glomerular failure.

[0091] Next generation sequencing of all genes within the linked locus showed mutations in all five affected individuals sequenced in a single gene only, i.e. *GATM*. Subsequent Sanger sequencing of all clinically affected individuals in all families showed one heterozygous missense mutation in *GATM* in each. The results of a multipoint parametric linkage analysis for families A, B, and C for chromosome 15 are shown in Fig. 2. A significant linkage (LOD score >3) was identified in the region of 40 - 60 cM. A homology alignment was performed with *GATM* amino acids 319-342, the mutations-bearing non-catalytical region of *GATM*, from 19 vertebrate species. Amino acids T336 and P341 appeared conserved in all 19 species. These are the amino acids where the mutations were identified. Three different, previously not described, heterozygous missense mutations in *GATM* (c.1006A>G (p.T336A), c.1007C>T (p.T336I), c.1022C>T (p.P341L)) were

identified in the four families studied. The regions from electrophoretograms of renal Fanconi and kidney failure patients and wild-type control showing these mutations are shown in Fig. 3. In each family one variant segregated with the disorder and was fully penetrant. None of the unaffected family members carried any of these respective mutations. Family A in Fig. 1 showed a *de novo* heterozygous mutation in affected offspring confirmed by haplotype analysis, which was passed on to the next generation.

EXAMPLE 3

[0092] This example demonstrates that patients suffering from renal Fanconi syndrome have normal amounts of creatine. Because GATM synthesizes the immediate precursor of creatine, these data provide evidence that GATM is catalytically active in these patients.

[0093] The patients studied have a disorder due to monoallelic missense mutations in *GATM*, but autosomal recessive *GATM* disease is characterized by an inability to produce creatine, leading to severe neurological findings beginning in early childhood. Brain ¹H-NMR spectroscopy can make the diagnosis of cerebral creatine deficiency syndrome due to biallelic *GATM* mutations. As seen in Fig. 4A and 4B, a normal total creatine peak (indicated by an arrow on the figures) was identified in a healthy control volunteer (Fig. 4A) and in one of the adults suffering from renal Fanconi syndrome (Fig. 4B). These results also provide evidence for GATM's catalytical (i.e. enzymatic) competence. Quantification in the volunteer showed a total creatine of 5.8 mM and 5.0 mM in grey and white matter, respectively. The renal Fanconi syndrome patient showed 6.1 mM and 4.4 mM in grey and white matter, respectively.

EXAMPLE 4

[0094] This example demonstrates that in Fanconi syndrome and glomerular failure, the proximal tubules may be very rare in the patient's kidneys. This example also demonstrates that the kidneys of renal Fanconi syndrome and glomerular failure patients show enlarged mitochondria filled with filaments.

[0095] Patient biopsies showed the morphological correlate of fibrosis on light microscopy and on electron microscopy. In Masson-Goldner staining of the patient's post mortem kidney specimen, the connective tissue stained green. Renal fibrosis was prominent

in the medulla and papilla. The cortex appeared shrunken and contained very few proximal tubules. Most glomeruli appeared atrophic and fibrotic. Using immunofluorescence of the same specimen, a GATM positive proximal tubule (PT) appeared surrounded by several layers of myofibroblasts, which were visualized using anti- α -smooth muscle actin. The Bowman capsule of the glomerulus also stained positive for myofibroblasts, suggesting that the kidney damage is not restricted to proximal tubules during the final stage of the disease. While the cortex of a normal kidney is composed of 70% proximal tubules, in this patient's kidney, they were very rare.

[0096] In electron microscopy pictures, the patient's specimen showed the highly fibrotic terminal kidney morphology of the disease. Most tubules showed an extremely thick basal membrane containing myofibroblasts. Unusual filament-like, crystalline structures could be observed within the tubular lumen and the epithelial cells. Fibrosis was also present within the glomeruli.

[0097] In normal human kidney, strong GATM staining was detected predominantly in early segments of the proximal tubule. Using immunofluorescence, in human kidney, as in mouse kidney, GATM expression was localized in early proximal tubules. At higher magnification, GATM expression showed a typical mitochondrial staining pattern at the basolateral side of the proximal tubule. Electron microscopy and immunogold staining of renal proximal tubules of a patient's kidney biopsy revealed drastically enlarged and elongated mitochondria containing GATM aggregates. Immunogold staining of a proximal tubular cell with grossly enlarged mitochondrion from a patient's biopsy showed the proximal tubule brush border membrane and unusual filaments within an enlarged mitochondrion. Packed linear aggregates with gold particles attached within the mitochondrion were visible, indicating GATM constituting these intramitochondrial "filaments."

[0098] Using electron microscopy, in a kidney biopsy from a renal Fanconi syndrome patient, giant mitochondria were observed within proximal tubular cells. Higher magnification of a giant mitochondrion showed the mitochondrion filled with filaments. As will be discussed below, filaments were also observed in the LLC-PK1 cell model.

EXAMPLE 5

[0099] This example demonstrates that *Gatm* knockout mice do not suffer from renal Fanconi syndrome.

[0100] Previously, *Gatm* knockout mice were generated to study the biochemical function of this mitochondrial protein. Mutant mice were viable, without detectable gross phenotypic defects, but dystrophic. Urinary metabolites were assessed in knockout and control mice using established analytic procedures.

[0101] The *Gatm*^{-/-} mice were analyzed to determine whether mitochondrial *GATM* haploinsufficiency (i.e. decreased GATM activity) was likely to have caused the families' renal Fanconi syndrome. In normal mouse kidney, GATM staining was strong in the early proximal tubule, and was not detected in the kidney of *Gatm*^{-/-} mice. Using immunofluorescence, GATM expression was present at the early proximal tubule, at the urinary pole of the glomerulus, and decreased towards the late segments of the proximal tubule. F-actin was used as a marker for the proximal tubular brush border. Non-proximal nephron segments, including the glomeruli, did not show any GATM expression.

[0102] Results of urine and plasma analysis of *Gatm*^{+/+} and *Gatm*^{-/-} mice are shown in Fig. 6A to Fig. 6D. As seen in Fig. 6A, urinary excretion of amino acids was not different between *Gatm*^{+/+} and *Gatm*^{-/-} mice (n=6/6), which indicates that knockout animals do not suffer from renal Fanconi syndrome. As seen in Fig. 6B, the concentrations of most amino acids in plasma were similar between the two groups. As expected, plasma and urine concentrations of creatine and creatinine were very low in *Gatm*^{-/-} mice, since the endogenous creatine synthesis pathway is disturbed (see Fig. 6C and Fig. 6D). The residual creatine and creatinine amounts result from creatine absorption from food. Interestingly, the concentration of guanidinoacetate, the direct product of GATM activity, was normal in plasma whereas the urinary excretion was decreased in knockout mice. No aminoaciduria was observed in these mice indicating that lack of GATM did not significantly affect renal proximal tubular function.

[0103] Without being bound to a particular theory or mechanism; it is believed that mutant GATM proteins within mitochondria trigger a pathological cascade inside and outside the proximal tubules, resulting in the renal Fanconi syndrome patients' signs and symptoms. *Gatm* knockout mice exhibited normal mitochondria, suggesting that the heterozygous *GATM*

mutations in the patients specifically damage mitochondria, particularly those of kidney cells. Electron microscopy of proximal tubular mitochondria in *Gatm*^{+/+} mice showed that mitochondria are mainly localized at the basolateral side of the proximal tubular cells. In *Gatm*^{-/-} mice, the mitochondria were also mainly localized at the basolateral side of the proximal tubular cells. The morphology and the number of mitochondria in *Gatm*^{-/-} mice were indistinguishable from those in *Gatm*^{+/+} mice. Also, no intra-mitochondrial filaments could be observed in either *Gatm*^{-/-} mice or *Gatm*^{+/+} mice.

EXAMPLE 6

[0104] This example demonstrates that mutant *GATM*-transfected proximal tubular cells showed significantly less capacity for oxidative phosphorylation in comparison with controls, potentially explaining an impairment of transepithelial transport. The results in this example also strongly suggest that components of the inflammasome became activated by mitochondrial *GATM* aggregates, providing a potential pathogenic link between heterozygous *GATM* mutations, kidney fibrosis and renal failure.

[0105] LLC-PK1 cells permanently transfected with normal and mutated *GATM* were employed as a cellular model to study cell morphology. The control (wild-type) and muted *GATM* localized to mitochondria. Immunofluorescence and electron microscopy revealed morphologic abnormalities in the mitochondria of LLC-PK1 cells transfected with mutant *GATM* but not in those transfected with wild-type *GATM*. Cells were prepared as published in Klootwijk et al. (*N. Engl. J. Med.* 370: 129-138 (2014)). To investigate the subcellular localization of human wild-type *GATM* and mutated *GATM* (p.T336A, p.T336I and p.P341L), immunofluorescence experiments on inducible LLC-PK1_{WT} and LLC-PK1_{MUT} cells were performed.

[0106] RNA isolated from cells carrying a *GATM* mutation showed significantly elevated expression of *NLRP3* and Interleukin 18 (*IL-18*) compared with RNA from control cells. As depicted in Fig. 5A, real-time PCR in LLC-PK1 cells overexpressing wild-type *GATM* or the T336A mutant showed a significant up-regulation of *NLRP3* (p=0.037), an important component of the inflammasome. As depicted in Fig. 5B, real-time PCR in LLC-PK1 cells overexpressing wildtype *GATM* or the T336A mutant showed a significant increase of IL-18

($p=0.0041$), an inflammatory cytokine, which is activated by the inflammasome complex. In both figures, the values were normalized to β -actin mRNA expression.

[0107] Immunofluorescence studies of LLC-PK1 cells stably transfected with wild-type GATM, showed mitochondria with normal morphology. GATM appeared localized within mitochondria. Immunofluorescence studies of LLC-PK1 cells stably transfected with mutant GATM T336A showed mitochondria having grossly abnormal shapes. GATM was localized within mitochondria. Immunogold staining of LLC-PK1 cells expressing mutant GATM T336A showed intramitochondrial gold particles attached to linear long aggregates. These results indicate that GATM constitute these intramitochondrial “filaments.”

[0108] For cell function studies, a renal proximal tubule cell line, LLC-PK1, stably transfected with wild-type or mutant *GATM* was used. Light microscopic immunofluorescence showed normal mitochondrial morphology in cells transfected with wild-type *GATM*, but abnormal and elongated mitochondria in the cells transfected with the T336A *GATM* mutant. Overexpression of wild-type GATM in inducible LLC-PK1 cells showed the mitochondrial localization of the protein. Overexpression of the T336A mutant led to formation of very long and spindle-like mitochondria. Similar findings were present in cells transfected with *GATM* mutants T336I and P341L. Mock transfected LLC-PK1 cells showed little to no endogenous GATM expression. Overexpression of each GATM mutant led to the appearance of giant mitochondria within the cell. Electron microscopy and immunogold examination of cells transfected with *GATM* mutants showed drastically enlarged and elongated mitochondria containing GATM aggregates. Within the mitochondrial matrix of LLC-PK1 cells overexpressing the T336A mutant long filaments were visible, which were distinct from mitochondrial membranes. Overexpression of the P341L mutant in LLC-PK1 cells showed filaments that cross through the mitochondrion, appearing to prevent mitochondrial division.

[0109] Mutant *GATM*-transfected proximal tubular cells showed significantly less capacity for oxidative phosphorylation in comparison with controls, potentially explaining an impairment of transepithelial transport. Fig. 7 depicts a graph of the oxidative phosphorylation measurements in LLC-PK1 cells. Basic respiration of intact (non-permeabilized) LLC-PK1 cells overexpressing wild-type GATM ($n=12$) and the T336A mutant ($n=11$) were measured using high-resolution respirometry. As seen in Fig. 7, cells

overexpressing the mutant protein consumed significantly less oxygen under resting conditions ($p=0.00028$).

[0110] Whether oxidative stress was contributing to this phenotype was also investigated. While the mitochondrial membrane potential was not different in cells transfected with the T336A mutant *GATM* compared with wild-type *GATM*, reactive oxygen species (ROS) were increased in cells carrying a *GATM* mutation. As shown in Fig. 8A, mitochondrial membrane potential in induced T336A *GATM* cells (MUT TA + TET) was not different from induced wild-type cells (WT + TET), as measured using TMRM. A time course of production of reactive oxygen species of the same cells for which the mitochondrial membrane potential (as measured using TMRM) was imaged was measured with the CELLROX DEEP RED mitochondrial-targeted superoxide probe. As seen in Fig. 8B, the reactive oxygen species production is faster in T336A *GATM* cells than in induced wild-type cells.

[0111] IL-18 protein was also elevated as measured by immunofluorescence, ELISA, and Western blotting. LLC-PK1 cells overexpressing wild-type *GATM* ($n=3$) and the T336A mutant ($n=3$) were induced with tetracycline for 2 weeks. In the following week, cells received medium without tetracycline to prevent a possible inhibition of caspase-1. After 3 weeks, total cells were lysed and intracellular content of IL-18 was quantified using ELISA and Western blot. As seen in Fig. 9A and Fig. 9B, overexpression of the T336A mutant led to significant intracellular accumulation of IL-18 (ELISA $p=0.0012$; Western blot $p=0.0095$). These findings strongly suggest that components of the inflammasome became activated by mitochondrial *GATM* aggregates, providing a potential pathogenic link between heterozygous *GATM* mutations, kidney fibrosis and renal failure.

EXAMPLE 7

[0112] This example demonstrates that the three disease-related mutations identified herein involve two amino acids strictly conserved through evolution, i.e., Pro341, and Thr336. These two amino acids are located on the surface around sheet B4, which is located opposite to the B2 surface involved in forming the physiological wild-type dimer.

[0113] Seeking a structural explanation for the pathogenicity of the renal Fanconi syndrome patients' specific *GATM* mutations, structural studies were performed on the *GATM* protein. *GATM* is composed of 423 amino acids. Ten X-ray crystallographic

structures are deposited in the protein data bank. The protein structure is built around a central core formed by five antiparallel beta sheets (B1-B5) disposed around a fivefold axis of symmetry. Such structure is potentially prone to protein-protein aggregation due to the presence of the 5-solvent exposed beta sheets. In addition to the domain of five-fold symmetry, the GATM protein hosts one additional domain composed of four alpha helices and a beta hairpin involved in formation of the wild-type dimer. Assembly of this dimer involves the two facing beta sheets (B2) disposed in parallel with an angle of approximately 45° with respect to each other, harboring the critical catalytic enzymatic activity domain.

[0114] The three disease-related mutations identified involve two amino acids strictly conserved through evolution, i.e., Pro341 and Thr336. Both are located on the surface around sheet B4, which is located opposite to the B2 surface involved in forming the physiological wild-type dimer. Thus, these mutations could destabilize the proper folding of the B4 surface. Structural simulations of these three GATM mutants predict increased mobility of the same region for each of them, predisposing the mutated B4 region to form additional interactions. Specifically, the presence of two opposite dimerization surfaces, B2 and B4, would support the formation of linear polymers, with each GATM monomer linked to two protein partners by two dimerization interfaces, i.e. the existing “physiological” B2-B2 and the “*de novo*” pathological “B4-B4” interface.

[0115] Publicly available GATM crystal structures (i.e. 1JDW subunit A) were utilized to simulate the effect of all mutations identified. In order to test the hypothesis of mutation-mediated destabilization of the region around B4, molecular dynamics simulations were performed on the wild-type GATM monomer and on the three GATM mutants, starting from the coordinates of 1JDW subunit A. Each simulation lasted for 40 nanoseconds. At the end of the simulations (after 40 nanoseconds) superposing all the mutants on the wild-type protein resulted in C α root mean square displacement (r.m.s.d.) from 1.12 to 1.47 Å. This displacement may be said to be from 1.17 to 1.72 Å if the superposition is limited to the region around the B4 surface. The results are shown in Table 6, below. The lower differences with respect to the wild-type in the T336A mutant could be related to the possibility that this mutation may require more time (with respect to the 40 nanoseconds of the simulation) to propagate its effect.

TABLE 6

Superposition of the mutants on the WT protein at the end of the simulation.

Mutant	mutants' superposition on WT; r.m.s.d. [Å]	mutant B4 region in comparison to WT (amino acids 290-350); r.m.s.d. [Å]
T336A	1.12	1.17
T336I	1.42	1.72
P341L	1.47	1.55

[0116] Using GATM molecular dynamics simulations, the root mean square displacement (r.m.s.d.) of the mutants during the simulation time suggested elevated mobility in three zones which displayed the higher mobility during the simulation: (1) amino acids 296-302; (2) 322-332; and (3) 342-350. Region 3 showed increased mobility for all mutations. While the latter zone is highly mobile also in the wild-type protein, the other two zones display higher motility (HM) only for the mutants with different patterns: HM in zones 2,3: P341L; HM in zones 1,2,3 T336A; and HM in zones 1,3 T336I. Despite the differences, every mutant is characterized by an enhanced motility at least in one additional zone with respect to the wild-type protein. The HM of the mutants is coherent with the hypothesis of the mutation induced structural rearrangements of the B4 surface that could promote a new protein-protein interaction surface.

[0117] In order to test the possibility of stable dimerization based on the B4 surface, two additional molecular dynamics simulations were performed on the wild-type and on the P341L B4-mutant based dimers as modeled by the program GRAMM-X. The P341L mutant was chosen because it showed higher overall r.m.s.d. with respect to wild-type GATM during the previous simulations (shown in Table 6, above) and, therefore, was the most variable during the simulation time.

[0118] Along the simulation, the wild-type dimer analyzed by the program PISA (Protein Interfaces, Surfaces, and Assemblies service at the European Biology Institute) adopted different configurations compatible with stable and unstable dimeric assemblies. Interestingly, the P341L dimer promptly evolved toward stability (after 10 nanoseconds) that was maintained along the remaining simulation time. Table 7, below, depicts the results for the propensity to form stable dimeric assemblies based on the B4 surface for wild-type (WT) GATM and for the P341L mutant. A “no” in the stable column indicates that the molecule

will not form stable dimeric assemblies. A “maybe” indicates that the molecule may form stable dimeric assemblies. A “yes” indicates that the molecule will form stable dimeric assemblies.

TABLE 7

Propensity to form dimeric assemblies based on the B4 surface for WT GATM or P341L mutant

time [ns]	WT				P341L			
	interface	DG	P	Stable	interface	DG	P	Stable
0	954	-4.6	0.38	no	994	-6.2	0.29	no
5	842	-7.3	0.18	no	833	-6.0	0.21	no
10	832	-5.4	0.24	no	870	-9.0	0.10	maybe
15	799	-9.2	0.09	no	939	-12.5	0.03	yes
20	765	-9.8	0.07	maybe	978	-13.2	0.03	yes
25	890	-7.9	0.15	no	991	-9.1	0.12	maybe
30	832	-7.8	0.17	no	950	-9.5	0.09	yes
35	890	-8.5	0.13	maybe	927	-10.2	0.07	maybe
40	984	-8.6	0.6	maybe	982	-11.3	0.05	yes

[0119] Such simulation results show, on one hand, how the B4 interface is prone to protein-protein interaction also in the wild-type protein and, on the other hand, how it is sufficient for only a point mutation (P341L) to shift the B4 based monomer-dimer equilibrium towards another stable quaternary assembly. This analysis, as the one shown before, supports the hypothesis that the mutation-induced structural rearrangements of the B4 surface promote a new protein-protein interaction surface that can lead to linear aggregation of GATM.

EXAMPLE 8

[0120] This example indicates that oral creatine may be a treatment for renal Fanconi syndrome and kidney failure patients with *GATM* mutations. Treatment with oral creatine might lead to a slower progression of chronic kidney disease as less mutant GATM protein is produced.

[0121] Since mutant GATM aggregates appeared to constitute the pathogenic mechanism, a way to reduce GATM production was investigated. Wild-type mice (n=4)

were supplemented with 1% creatine in their drinking water for one week, whereas a control group (n=4) received normal water. Afterwards, mRNA and protein expressions of Gatm in kidneys were determined using real-time PCR and Western blot. As shown in Fig. 10A, treatment of mice with creatine led to a 27% ($p=0.0125$) reduction of GATM mRNA expression. Fig. 10B shows that protein expression was diminished by 58.5% ($p=0.029$) in mice treated with creatine.

[0122] All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[0123] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning “including, but not limited to,”) unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0124] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIM(S):

1. One or both of creatine and a creatine substitute for use in the treatment of a renal disorder in a patient, wherein the patient has at least one dominant heterozygous missense mutation in a glycine amidinotransferase (GATM) protein associated with the renal disorder.
2. One or both of creatine and a creatine substitute for use in the stabilization of renal function in a patient suffering from a renal disorder, wherein the patient has at least one dominant heterozygous missense mutation in a GATM protein associated with the renal disorder.
3. One or both of creatine and a creatine substitute for the use of claim 1 or 2, wherein the renal disorder is one or both of Fanconi syndrome and glomerular failure.
4. A method of diagnosing a renal disorder in a patient, the method comprising:
 - assaying a biological sample obtained from the patient to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein in the biological sample;
 - wherein the detection of the at least one dominant heterozygous missense mutation is indicative of the renal disorder in the patient.
5. One or both of creatine and a creatine substitute for use in stabilizing renal function in a patient, wherein the patient is diagnosed with a renal disorder by the method of claim 4.
6. One or both of creatine and a creatine substitute for use in the treatment of a renal disorder in a patient, wherein the patient is diagnosed with the renal disorder

by the method of claim 4.

7. The method of claim 4 or the one or both of creatine and a creatine substitute for the use of claim 5 or claim 6, wherein the renal disorder is one or both of Fanconi syndrome and glomerular failure.

8. A method for detecting a GATM mutation in a biological sample from a patient, the method comprising:

assaying the biological sample to detect at least one dominant heterozygous missense mutation in one or both of (i) at least one copy of glycine amidinotransferase (GATM) genetic sequence and (ii) GATM protein.

9. The method of any one of claims 4 and 7-8, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7, wherein assaying the biological sample to detect at least one dominant heterozygous missense mutation comprises carrying out one or more of polymerase chain reaction (PCR) amplification, reverse-transcriptase PCR analysis, single-strand conformation polymorphism analysis (SSCP), mismatch cleavage detection, heteroduplex analysis, Southern blot analysis, Western blot analysis, deoxyribonucleic acid sequencing, restriction fragment length polymorphism analysis, haplotype analysis, serotyping, and any combination thereof.

10. The method of any one of claims 4 and 7-9, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9, wherein the biological sample is a sample of blood, urine, kidney, or skin.

11. The method of any one of claims 4 and 7-10, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9-10, wherein assaying comprises obtaining the sequence of at least a portion of the GATM genetic sequence.

12. The method of any one of claims 4 and 7-11, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9-11, wherein assaying comprises obtaining the sequence of substantially all of the GATM genetic sequence.

13. The method of any one of claims 4 and 7-12, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9-12, further comprising comparing the genetic sequence of the patient to the sequence of the wild type GATM genetic sequence and identifying any differences between the genetic sequence of the patient and the wild type GATM genetic sequence.

14. The method of any one of claims 4 and 7-13, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9-13, wherein assaying the biological sample comprises contacting the sample with an antibody that specifically binds to GATM protein having the at least one mutation and does not bind to wild type GATM protein, thereby forming a complex, and detecting the complex.

15. The method of any one of claims 4 and 7-14, or the one or both of creatine and a creatine substitute for the use of any one of claims 5-7 and 9-14, wherein assaying comprises contacting the sample with an antibody that specifically binds to wild type GATM protein and does not bind to GATM protein having the at least one mutation, thereby forming a complex, and detecting the complex.

16. The method of any one of claims 4 and 7-15, or the one or both of creatine and a creatine substitute for the use of any one of claims 1-3, 5-7, and 9-15, wherein the at least one dominant heterozygous missense mutation is located within the non-catalytic domain of GATM.

17. The method of any one of claims 4 and 7-16, or the one or both of creatine and a creatine substitute for the use of any one of claims 1-3, 5-7, and 9-16, wherein the at least one dominant heterozygous missense mutation in GATM protein is at least one mutation selected from the group consisting of T336A, T336I, and P341L.

18. The method of any one of claims 4 and 7-17, or the one or both of creatine and a creatine substitute for the use of any one of claims 1-3, 5-7, and 9-17, wherein the at least one dominant heterozygous missense mutation in GATM genetic sequence is at least one mutation selected from the group consisting of (i) c.1006A>G, (ii) c.1007C>T, and (iii) c.1022C>T.

19. The method of any one of claims 4 and 7-18, or the one or both of creatine and a creatine substitute for the use of any one of claims 1-3, 5-7, and 9-18, wherein the method or use does not comprise kidney dialysis.

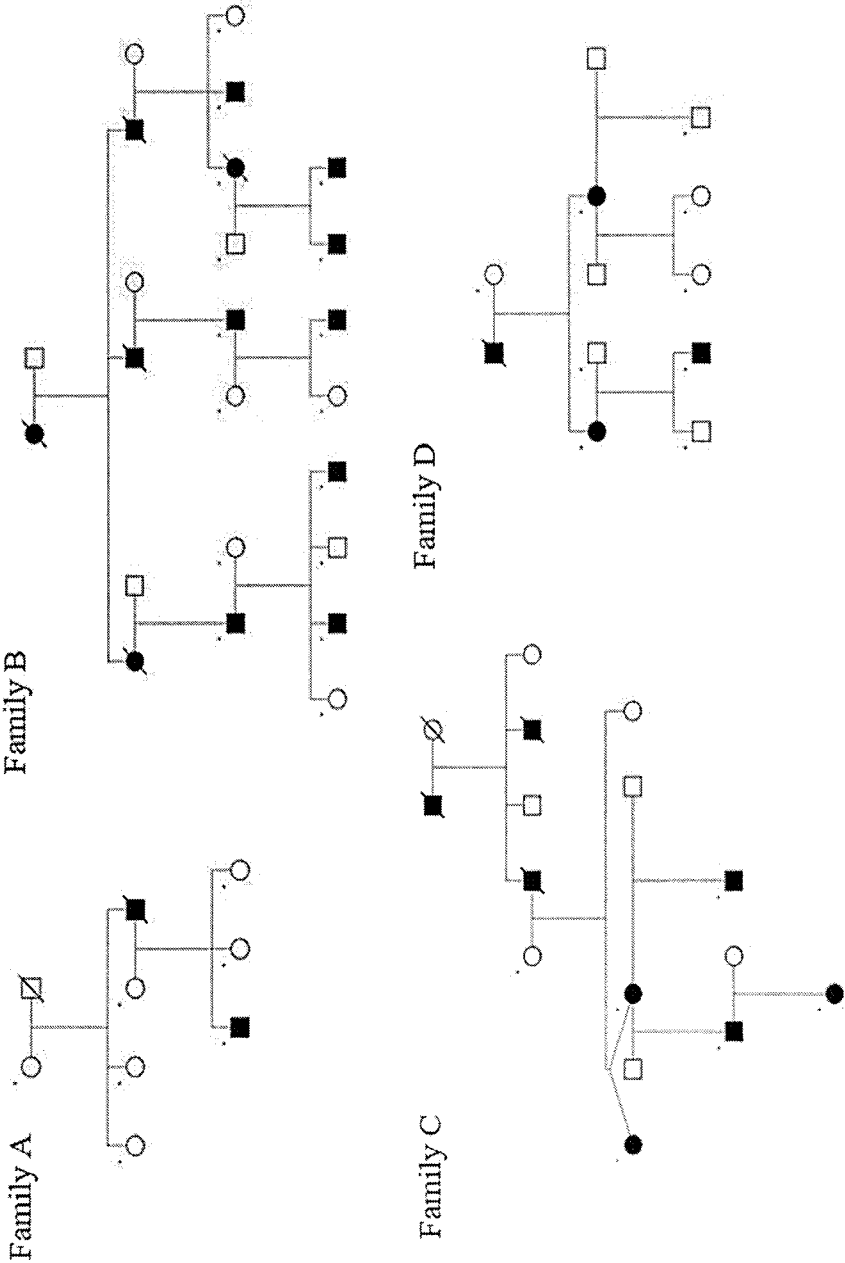


Figure 1

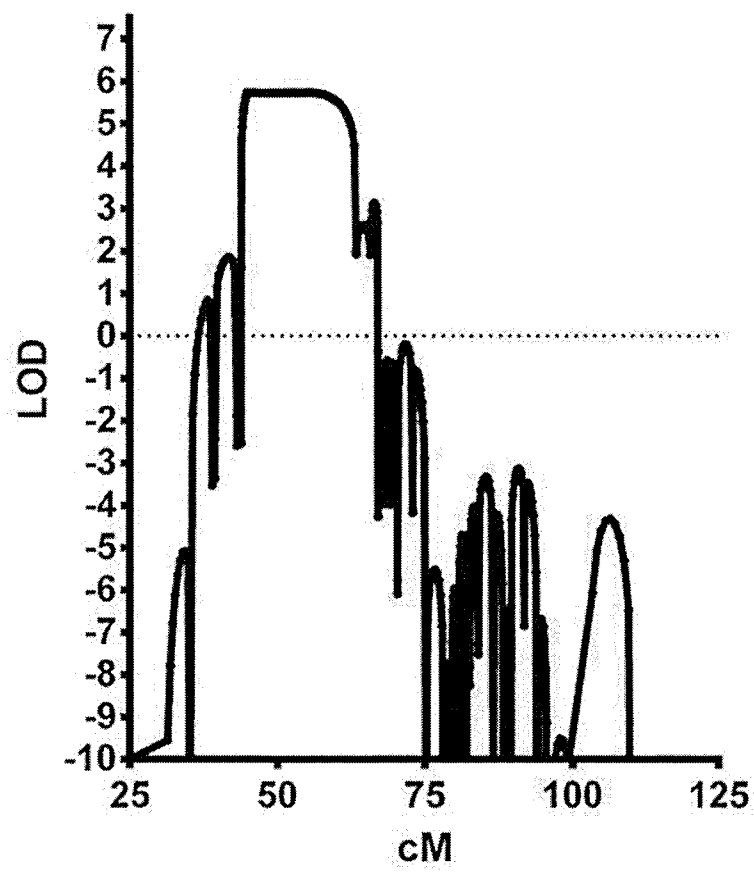
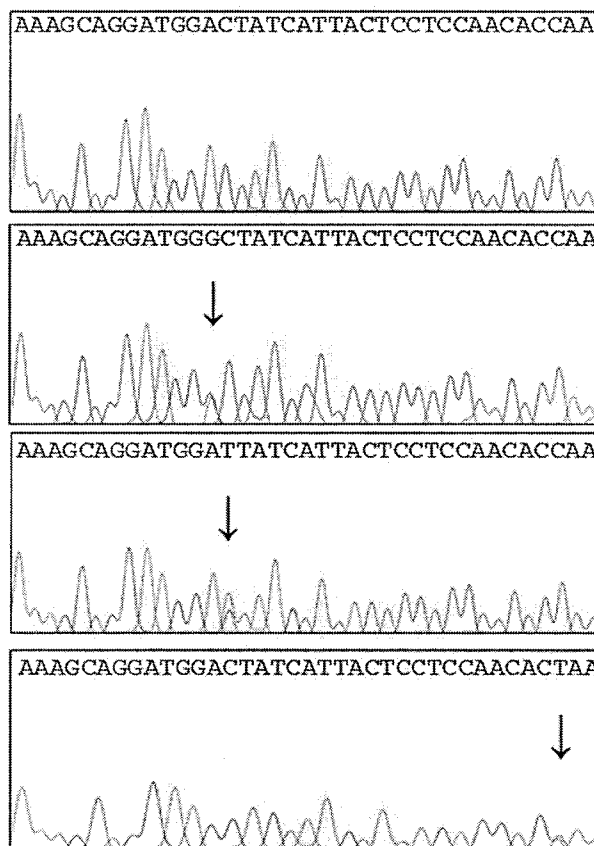


Figure 2

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WT 2
SEQ ID NO: 17

c.1006A>G
SEQ ID NO: 18

c.1007C>T
SEQ ID NO: 19

c.1022C>T
SEQ ID NO: 20

Figure 3

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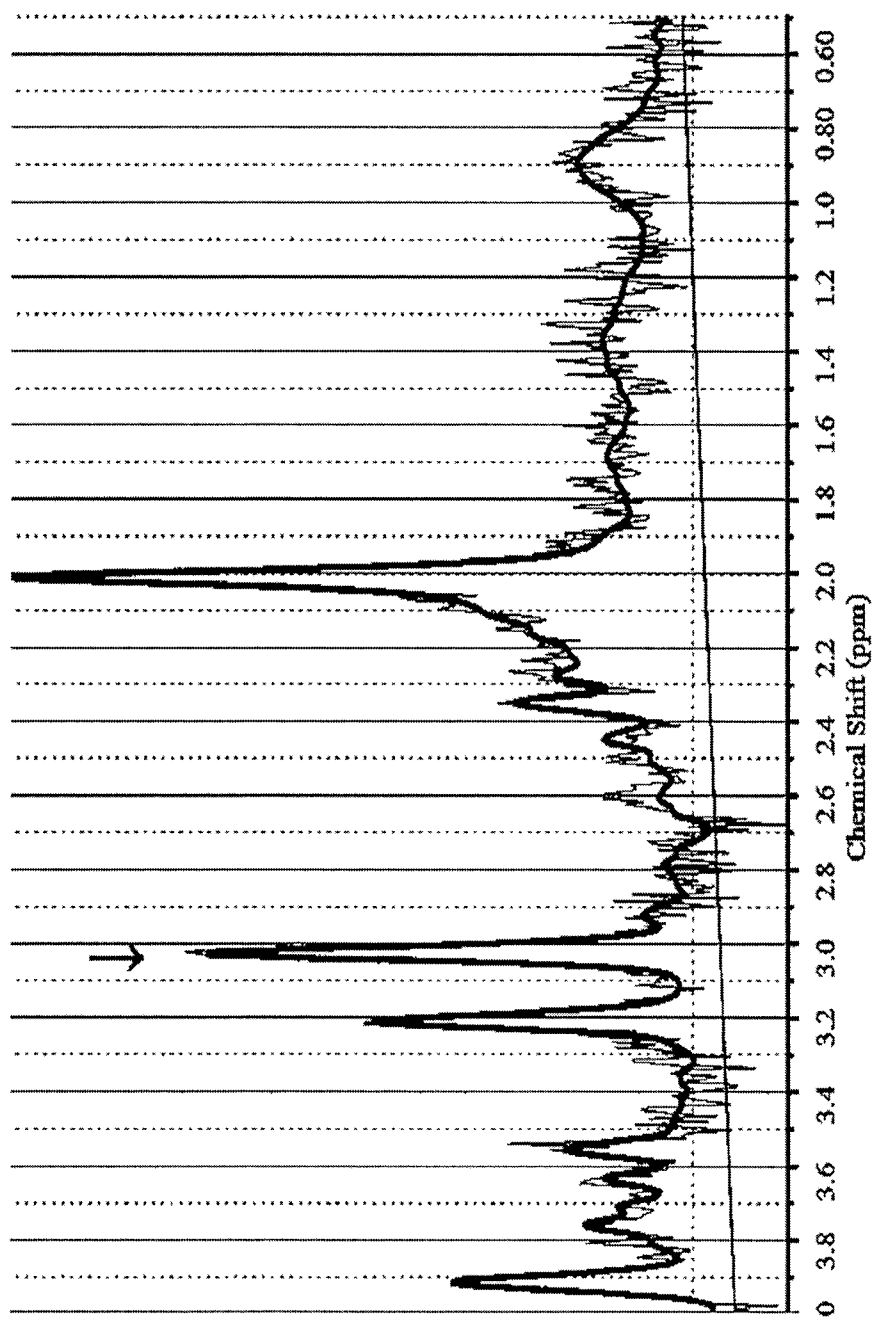


Figure 4A

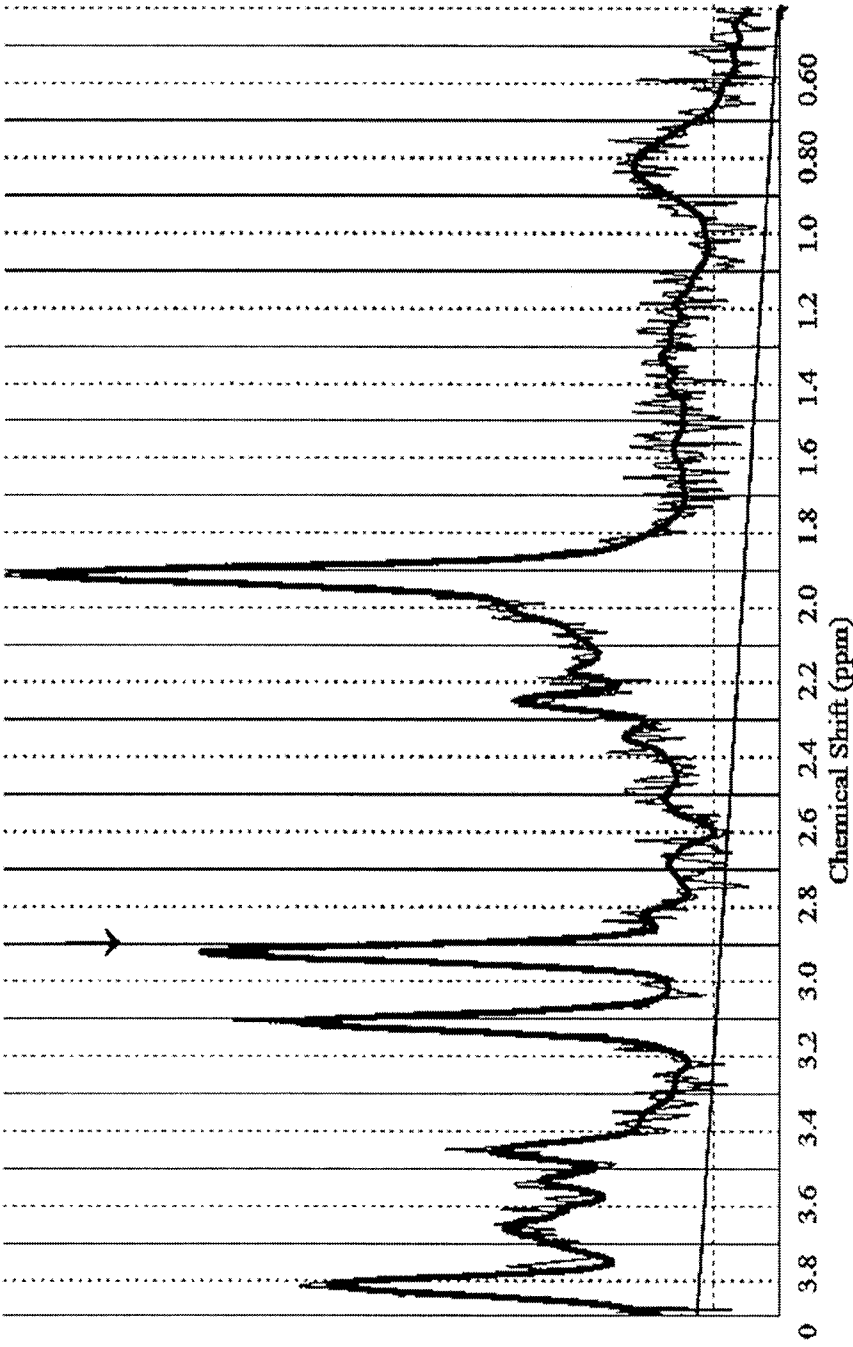


Figure 4B

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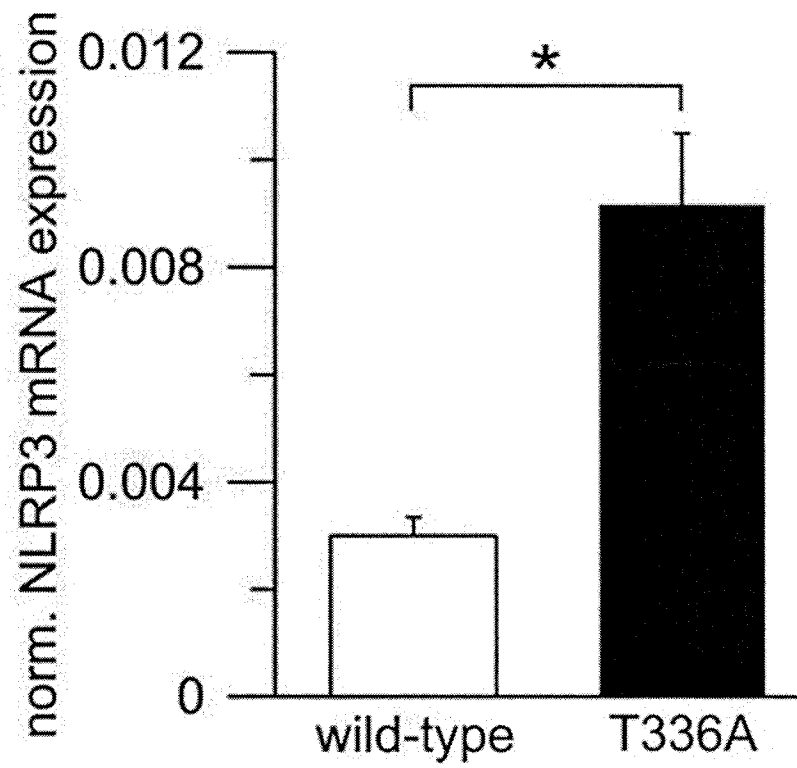
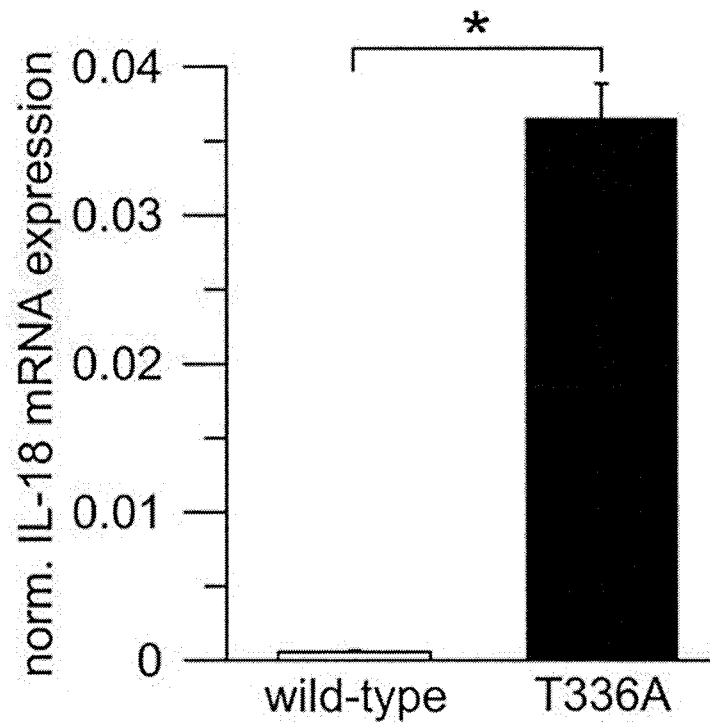


Figure 5A

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**Figure 5B**

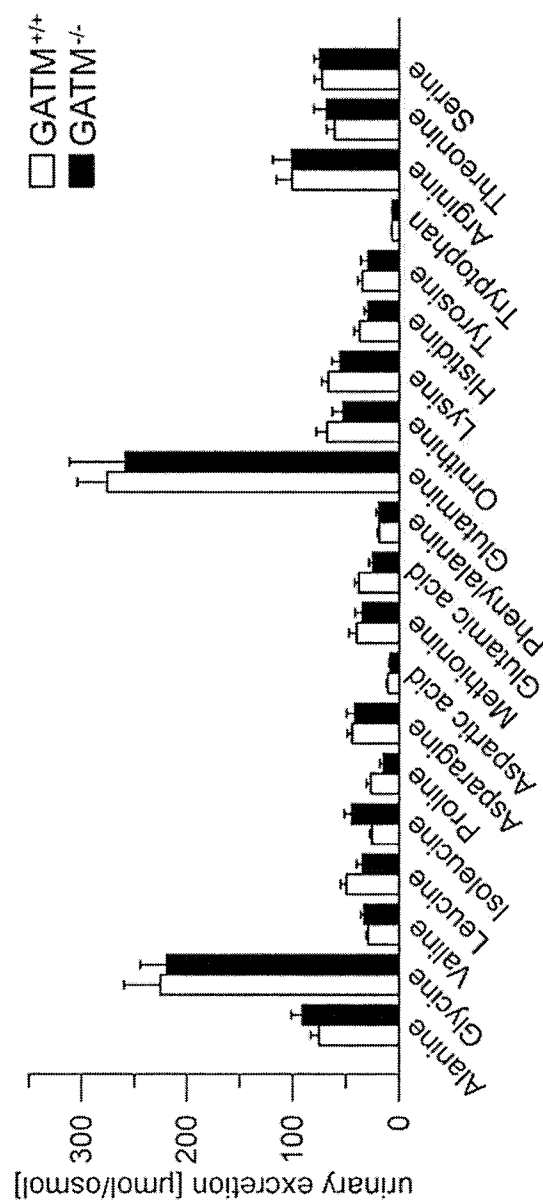


Figure 6A

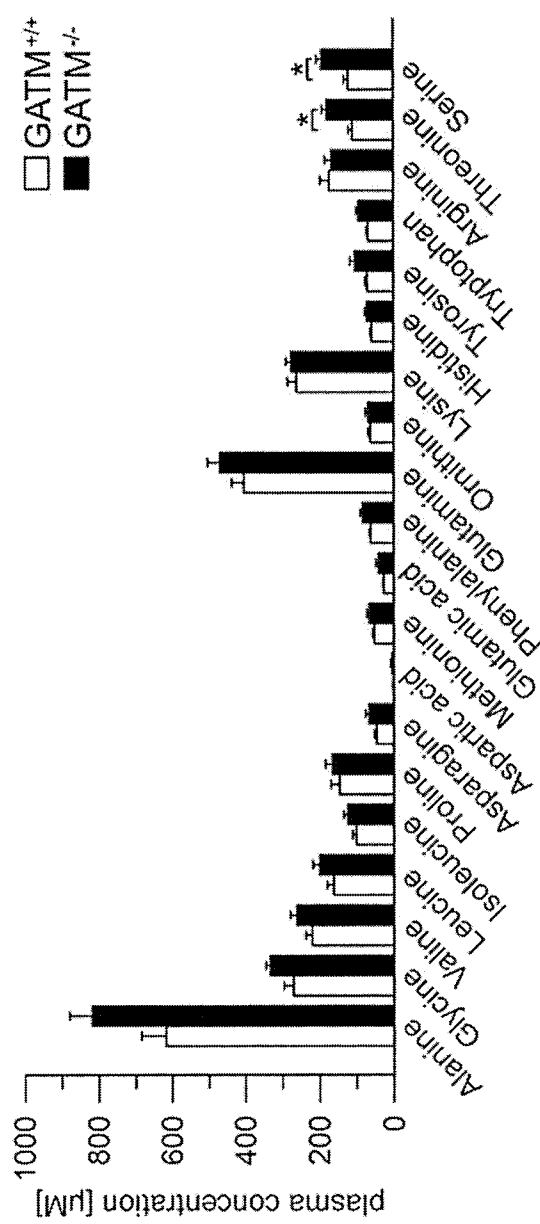


Figure 6B

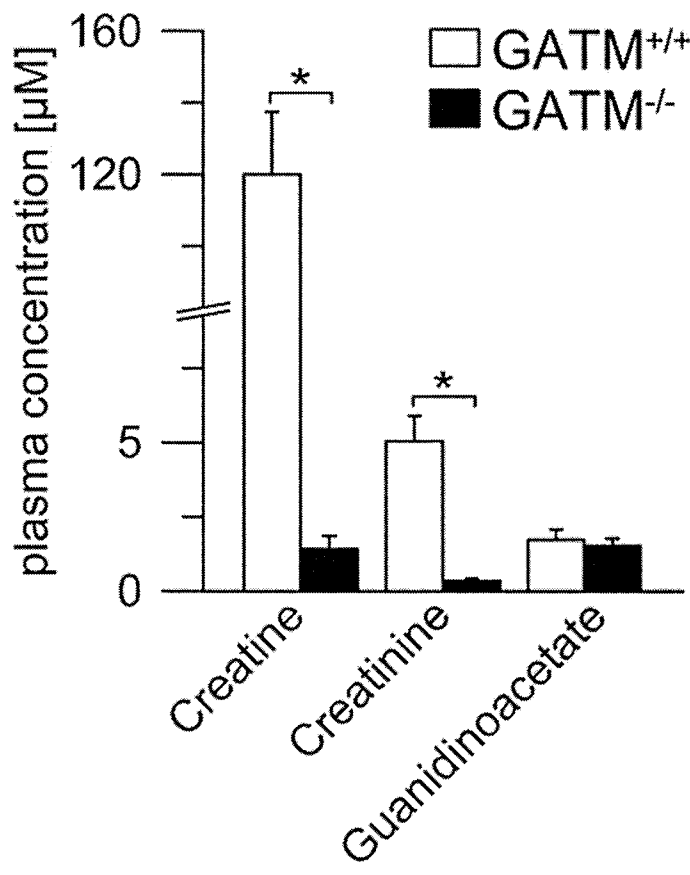


Figure 6C

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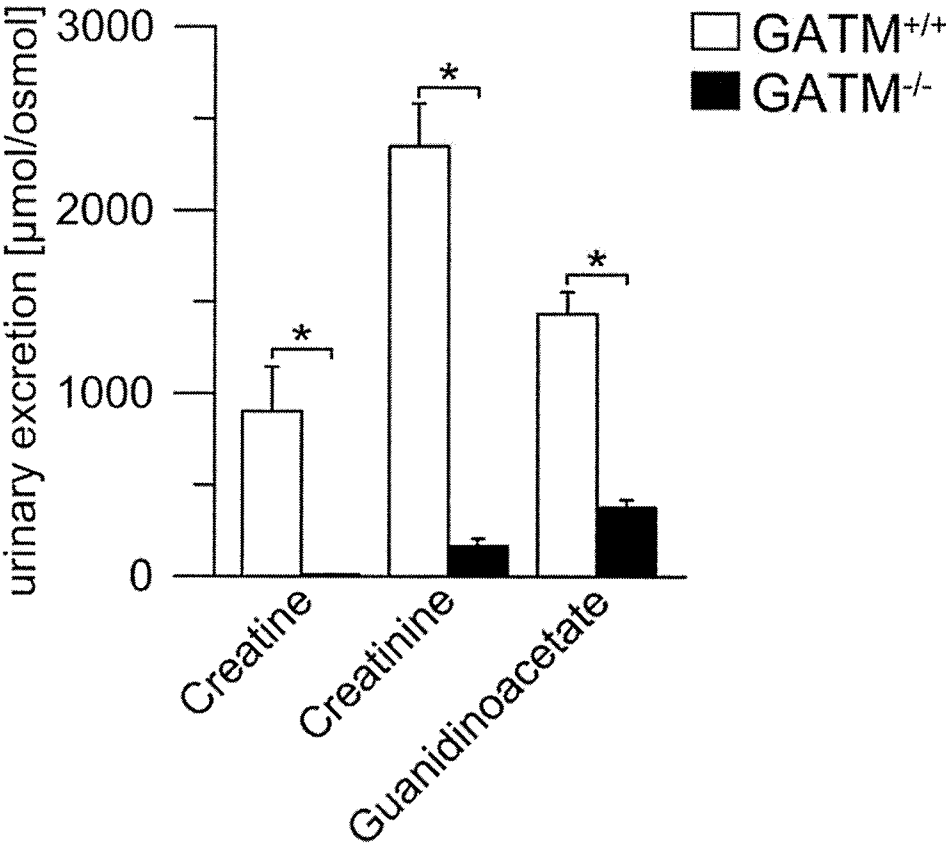


Figure 6D

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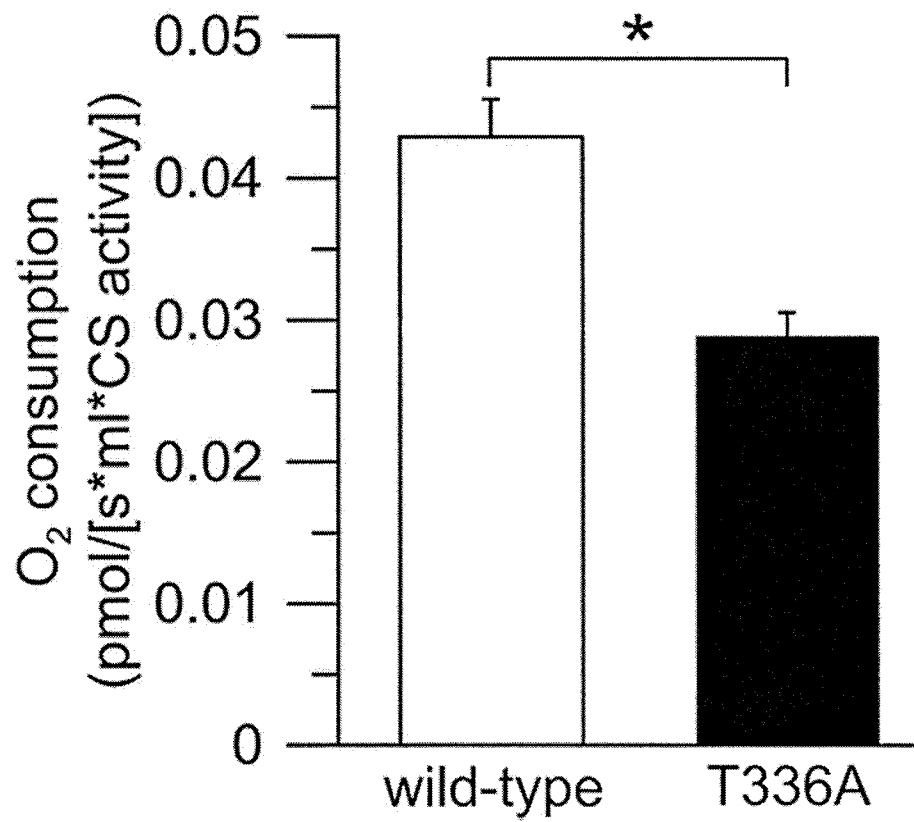


Figure 7

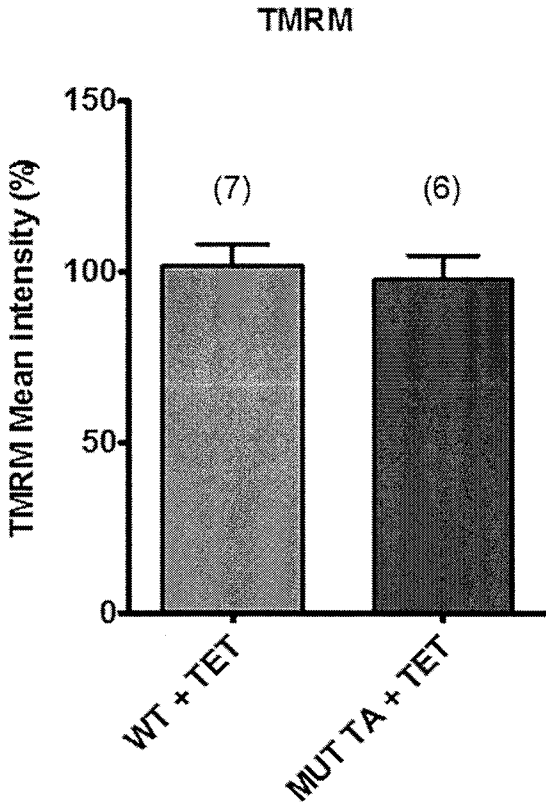


Figure 8A

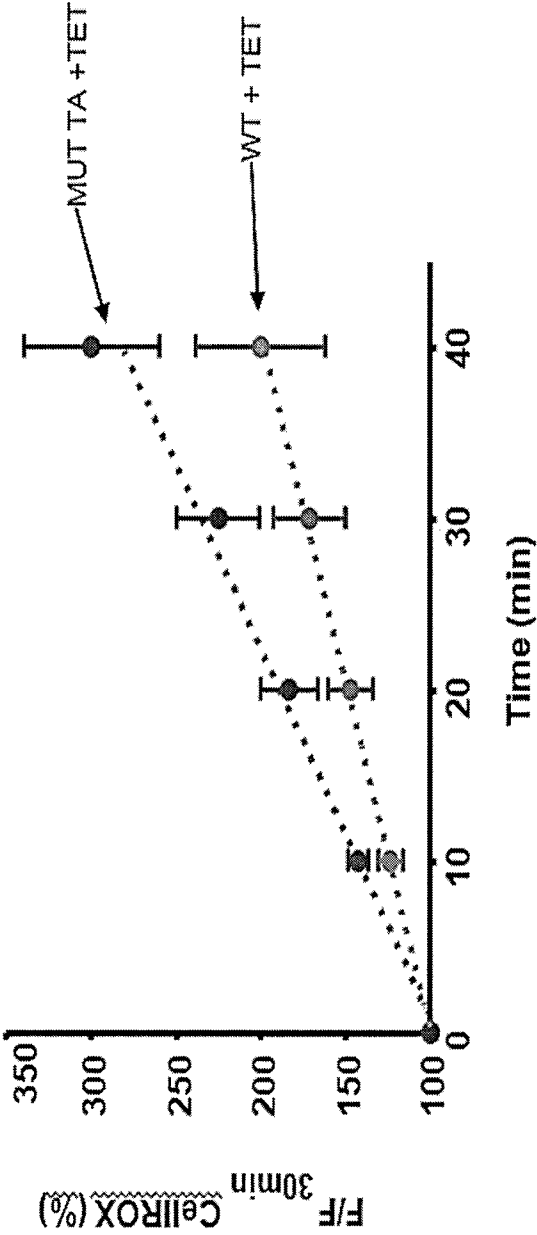


Figure 8B

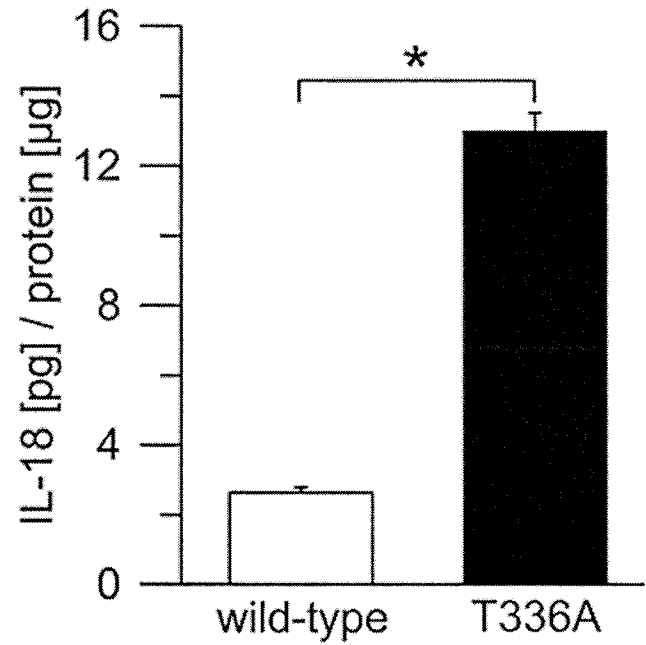


Figure 9A

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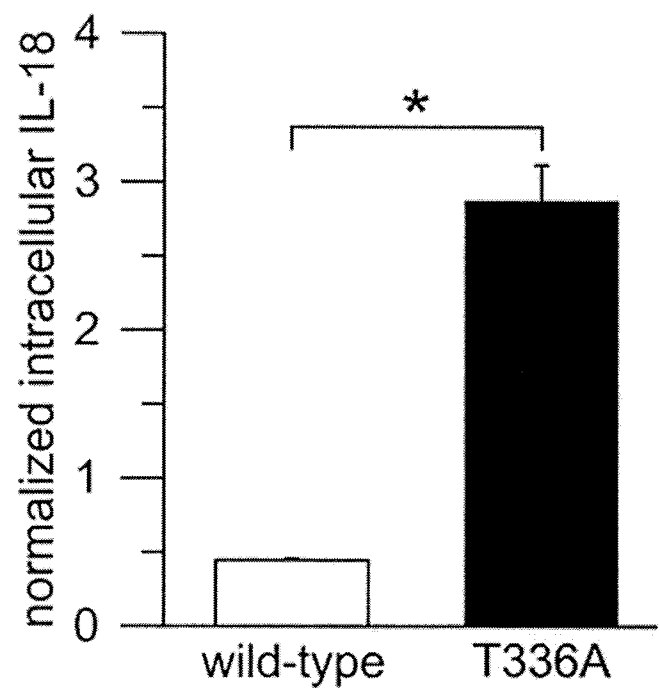


Figure 9B

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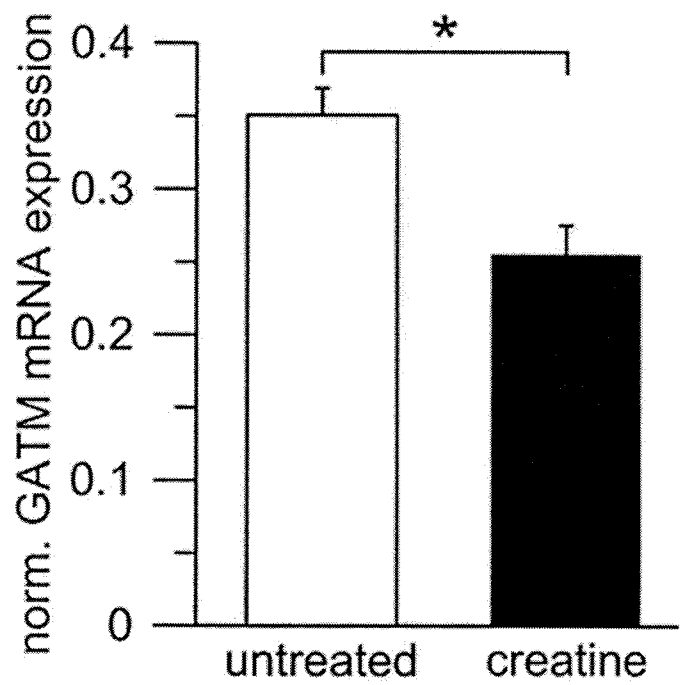


Figure 10A

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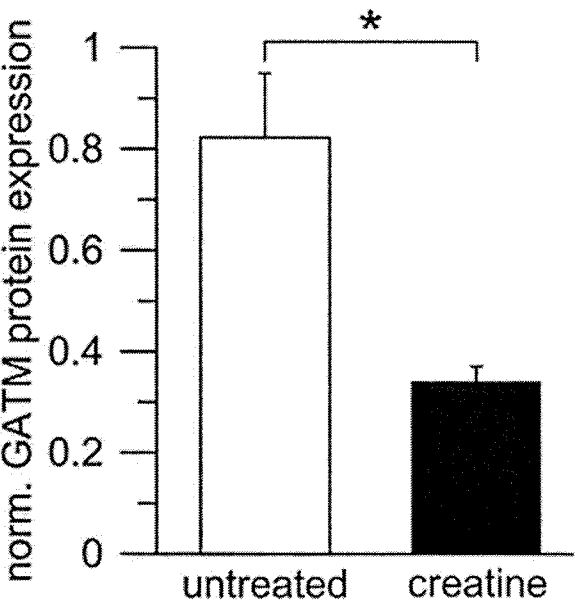


Figure 10B

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/035423

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/035423

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K31/198 A61P13/12
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ENRIKO D. KLOOTWIJK ET AL: "Renal Fanconi syndrome: taking a proximal look at the nephron", NEPHROLOGY DIALYSIS TRANSPLANTATION., vol. 30, no. 9, 9 December 2014 (2014-12-09), pages 1456-1460, XP055496108, GB ISSN: 0931-0509, DOI: 10.1093/ndt/gfu377 table 1 ----- -/-	1-19



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

3 August 2018

Date of mailing of the international search report

24/08/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Borst, Markus

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2018/035423

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CRISTIAN PATTARO ET AL: "Genome-wide linkage analysis of serum creatinine in three isolated European populations", KIDNEY INTERNATIONAL, vol. 76, no. 3, 1 August 2009 (2009-08-01), pages 297-306, XP055496072, LONDON, GB ISSN: 0085-2538, DOI: 10.1038/ki.2009.135 paragraph bridging left and right hand column on page 301 -----	1-7,9-19
Y	GARDAR SVEINBJORNSSON ET AL: "Rare mutations associating with serum creatinine and chronic kidney disease", HUMAN MOLECULAR GENETICS, vol. 23, no. 25, 31 July 2014 (2014-07-31), pages 6935-6943, XP055496085, gb ISSN: 0964-6906, DOI: 10.1093/hmg/ddu399 abstract; table 3 -----	1-7,9-19
Y	STOCKLER-IPSIROGLU SYLVIA ET AL: "Arginine:glycine amidinotransferase (AGAT) deficiency: Clinical features and long term outcomes in 16 patients diagnosed worldwide", MOLECULAR GENETICS AND METABOLISM, vol. 116, no. 4, 1 December 2015 (2015-12-01), pages 252-259, XP029346061, ISSN: 1096-7192, DOI: 10.1016/J.YMGME.2015.10.003 abstract; table 1 -----	8-19
X,P	REICHOLD M ET AL: "Glycine Amidinotransferase (GATM), Renal Fanconi Syndrome, and Kidney Failure", JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY 20180701 AMERICAN SOCIETY OF NEPHROLOGY USA, vol. 29, no. 7, 1 July 2018 (2018-07-01), pages 1849-1858, XP002783475, ISSN: 1046-6673 abstract -----	1-19