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ORIGINAL RESEARCH

Expanding the Phenotypic Spectrum of Nicotinamide Nucleotide Transhydrogenase (*NNT*) Mutations and using Whole Exome Sequencing to Discover Potential Disease Modifiers

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Abstract: Mutations in the *NNT* gene (nicotinamide nucleotide transhydrogenase), which is involved in NADPH generation in mitochondria, have recently been described in familial glucocorticoid deficiency. We report two patients, one with isolated glucocorticoid deficiency and the other with a combined glucocorticoid and mineralocorticoid deficiency. Through whole exome sequencing, both cases were found to carry two different *NNT* mutations, confirming previous results for these patients. Each patient also carries multiple heterozygous protein-altering mutations in other genes involved in steroid hormone biogenesis and regulation. The patient with a combined glucocorticoid and mineralocorticoid deficiency is a compound heterozygote for common missense variants in the *ME3* gene (mitochondrial malic enzyme 3), the product of which also generates NADPH in mitochondria. Mutations in *NNT* are the likely proximal cause of the glucocorticoid deficiency in both patients, but genetic background effects may be important in modulating the specific phenotype of adrenal insufficiency.

Keywords: glucocorticoid and mineralocorticoid deficiency, *NNT*, exome sequencing

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Introduction

Familial glucocorticoid deficiency (FGD) is an autosomal recessive disorder defined by adrenocorticotropin (ACTH) resistant isolated cortisol deficiency (MIM [202200]). It can be caused by mutations in the genes *MC2R* (MIM [607397]), *MRAP* (MIM [699196]) and rarely steroid acute regulatory protein (*STAR*, MIM [600617]).[1] Recently, mutations in the genes encoding minichromosome maintenance-deficient 4 (*MCM4*, MIM [602638]) and nicotinamide nucleotide transhydrogenase (*NNT*, MIM [607878]) have also been reported as causal.[2–5] *NNT* is involved in NADPH biosynthesis in mitochondria, and is important to maintain the appropriate oxidoreductive state.[6–9] Adrenal insufficiency, which lead to both glucocorticoid and mineralocorticoid deficiency, may also be caused by mutations in *STAR*, or in other genes such as *NR5A1* [MIM 184757] or *NR0B1* [MIM 300473]. Mutations in the latter, however, lead to more severe phenotypes which include defects in sexual development.

Two ACTH-resistant patients with different clinical presentations in one important aspect (isolated glucocorticoid deficiency versus combined gluco- plus mineralocorticoid deficiency) were analyzed by whole exome sequencing after provisional exclusion of causal mutations in *MC2R* and *MRAP*. This lead to the identification of compound heterozygous mutations in *NNT* in both patients. These results confirmed the same mutations, previously reported in these patients.[5] The different presentations of the two patients were not obviously explained by the *NNT* variants; therefore we looked for additional genetic variants in genes involved in steroid hormone biosynthesis or regulation that might be potential genetic modifiers. Multiple interesting protein-coding variants were identified, although the functional consequences of these will be challenging to validate.

Materials and Methods

Ethics statement

This study was approved by the Ethics Committee of the CHU Sainte-Justine. The parents of the patients gave written informed consent for the genetic analyses.

Laboratory analyses and whole exome sequencing

Genomic DNA from patients and unaffected family members was obtained from venous blood using standard methods. Exome analysis was performed using the Agilent 50Mb SureSelect library of biotinylated RNA oligonucleotides, followed by paired-end 100 nt sequencing on Illumina HiSeq2000. Hybrid capture and next-generation sequencing were performed at the McGill University and Genome Quebec Centre for Innovation. Data were analyzed using NextGene (SoftGenetics, Inc.) as well as separately with a custom pipeline using BWA, Picard, Samtools and Annovar to align reads to the human consensus genome sequence, and identify and annotate variants.[10] The two exome samples generated 10 or 14 gigabases (Gb) of high quality aligned bases, with median 60 or 112-fold coverage respectively of targeted exons after filtering for PCR-generated duplicate reads. More than 90% of CCDS annotated coding region bases were covered by at least 10 independent reads. For primary mutation analysis, variants were filtered using data from the 1000 Genomes project and the NHLBI exome sequencing project. Common variants and potential false positives due to technical artifacts were removed by comparison with a dataset of variants from exomes of other unrelated projects analyzed with the same informatics pipeline. PCR-based Sanger capillary fluorescent sequencing was performed using standard methods, and data were analyzed using MutationSurveyor (SoftGenetics, Inc.).

Bioinformatics

Potential effects of amino acid substitutions were analyzed using the mutation prediction tools Polyphen2,[11] SIFT,[12] SNPS&GO,[13] and Provean.[14] The *NNT* gene encodes two annotated mRNAs according to RefSeq, with different first exons but identical coding potential. Mutations in *NNT* were numbered consistently with the previous report.[5]

Results

Clinical ascertainment

Over the course of two years, one infant was referred to our service with severe hypoglycemia and another with salt wasting, hyponatremia, and hyperkalemia. Both had elevated serum ACTH, consistent with primary adrenal insufficiency. After stepwise exclusion of the various causes of primary adrenal insufficiency according to our published algorithm,[15] we considered ACTH resistance. However, PCR-based Sanger

sequencing of annotated protein coding exons was negative for potential causal mutations in *MC2R* and *MRAP*, genes known to cause ACTH resistance when mutated (data not shown).[16] Later, in the course of the present study, these two patients were sequenced in the *NNT* gene by another team, leading to the identification of mutations in *NNT* in both children.[5]

Case 1

A 14 month-old girl, the second child born to non-consanguineous French-Canadian parents, without any relevant personal or family history, was admitted for severe fasting hypoglycemia (0.7 mmol/L) and metabolic acidosis (pH 7.15; bicarbonates 14.6 mmol/L) but normal serum electrolytes (sodium 139 mmol/L, potassium 5.1 mmol/L) occurring during a febrile episode (40 °C). On admission, weight was 9.4 kg (50th percentile), length 80 cm (85th percentile). Growth had been normal before this episode. Examination noted deeply hyperpigmented skin but was otherwise unremarkable. The external genitalia were normal. Resuscitation including glucose and hydrocortisone led to a fast recovery.

Twenty-four hours after hydrocortisone withdrawal, serum cortisol at 08h00 was undetectable (<11 nmol/L) and did not rise after stimulation with 1 µg of ACTH intravenously. Serum ACTH at 08h00 on the same day was 713 pmol/L (N: 0.33–15.6 pmol/L). Abdominal ultrasound showed that both adrenal glands were calcified. Antibodies to CYP21 were undetectable and there was no evidence in favor of a defect in *STAR* or in any enzyme involved in glucocorticoid synthesis, adrenoleukodystrophy, or triple-A syndrome.

The patient was diagnosed as having ACTH-resistant isolated glucocorticoid deficiency and has been treated with hydrocortisone (10–13 mg/m^2/d) for the past 6 years. She has normal serum electrolytes and renin, and is growing and developing normally. Currently the patient is 8 years old and has a 14-year old sister, who is healthy with no clinical evidence of adrenal dysfunction.

Case 2

A 13-month-old boy, a first child born to non-consanguineous French Canadian parents after a normal pregnancy, was admitted for a retro-pharyngeal abscess with thrombosis of the internal jugular vein and compression of the internal carotid artery leading to infarction of the left parietal lobe. On admission, weight was 10.4 kg (25th percentile), length 78 cm (50th percentile). Growth had been normal before this episode. During hospitalization, he was treated with intravenous antibiotics and was tube-fed but he nevertheless continued to lose weight, with vomiting and hypotension. Serum sodium and potassium were 126 mmol/L and 8.2 mmol/L, respectively. Simultaneously, urinary sodium was inappropriately high (63 mmol/L) and serum cortisol inappropriately low (30 nmol/L). High serum ACTH (466 pmol/L) and renin (202 ng/L) were consistent with combined gluco- and mineralocorticoid deficiency. The patient promptly recovered under hydrocortisone and fludrocortisone replacement therapy. Ultrasound scan showed normal adrenal glands. Serum 17-OH-Progesterone was normal and anti-CYP21 antibodies were undetectable. There was no clinical argument for triple A syndrome and VLCFA levels were normal, ruling out adrenoleukodystrophy. The *DAX1* and *SF1* genes, in addition to *MC2R* and *MRAP*, were normal by traditional coding exon sequencing. The patient has been maintained on combined hydrocortisone and fludrocortisones. Currently, the patient is 9-years old and has one 2-year old sister, who is healthy with no clinical evidence of adrenal dysfunction.

Exome sequencing and mutation validation

At the time the study was initiated, no etiology had been established for the two patients' adrenal insufficiency, and we therefore commenced exome sequencing to look for causal mutations in potentially novel genes. A recessive mode of inheritance was suggested by the normal status of all four parents, and a founder effect was considered based on the French-Canadian ethnicity of both families. However, considering variants in or near coding regions, no annotated protein-coding gene was found to carry a private shared homozygous pathogenic variant in both patients (simultaneously rare, with minor allele frequency < 0.01 in the 1000 Genomes database, and not seen in other exomes of unrelated samples; potentially protein-altering by nonsynonymous codon change, insertion/deletion with our without frameshift, gain or loss of stop codon, intronic or exonic consensus splice

site mutation, or more extended splice site mutation). In patient 1, 7 genes contained either a homozygous or two heterozygous private non-synonymous variants. In patient 2, 23 genes contained either a homozygous or two heterozygous private non-synonymous variants. The only gene from these two lists shared between both patients was *NNT*, encoding nicotinamide nucleotide transhydrogenase. Patient 1 had two different heterozygous truncating frameshift alleles in *NNT* (c.63delG, p.S22Pfs*6; c.1864-1G > T, p.I622Dfs*1) (Fig. 1A and Supplementary Fig. S1); patient 2 had one heterozygous truncating frameshift allele in *NNT* (c.1107_1110delTCAC, p.H370*) and one heterozygous missense in *NNT* (c.3027T > G, p.N1009K) (Fig. 1B and Supplementary Fig. S1). Patient 1 was also heterozygous for two missense variants in *NNT* present in dbSNP (Supplementary Table 2).

To verify the mutations found in *NNT*, and to document inheritance phase, we performed PCR-based Sanger sequencing on DNA from the patients and their parents. In the case of patient 1, the father was heterozygous for mutation c.63delG, p.S22Pfs*6, the mother was heterozygous for mutation c.1864-1G > T, p.I622Dfs*1 and the patient was thus a compound heterozygote (Fig. 1A and data not shown). The unaffected sister showed no *NNT* mutations. In the case of patient 2, the father was heterozygous for mutation c.T3027T > G, p.N1009K, but neither parent carried the mutation c.1107_1110delTCAC, p.H370* (Fig. 1B and data not shown). Therefore this second mutation in the patient presumably arose *de novo* in a parental germ line, but there is no way to distinguish in which parent. The data are nonetheless consistent with the patient being a compound heterozygote. The unaffected sister showed no *NNT* mutations.

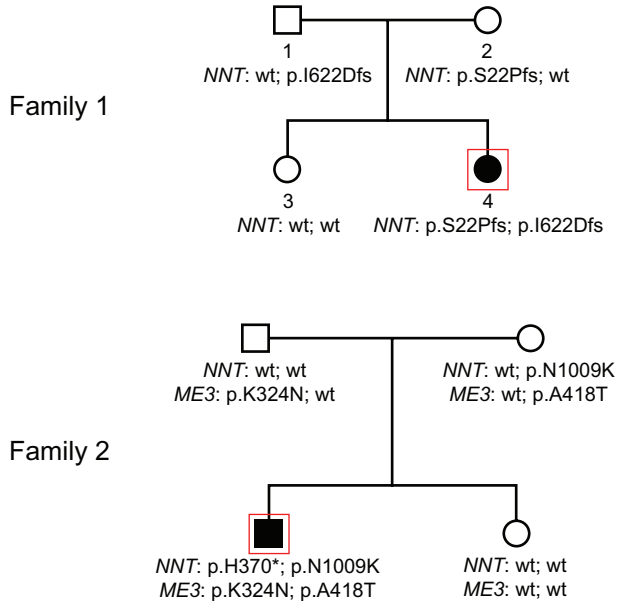

Figure 1. Pedigrees of the two families.
Notes: *NNT* biallelic mutations are found exclusively in the affected index cases (filled symbols). *ME3* biallelic variants are also found exclusively in the affected index case of family 2. All unaffected cases have either only monoallelic *NNT* or *ME3* mutations/variants or two normal wild-type alleles.

The mutation p.N1009K is predicted as probably damaging by Polyphen2 both HumDiv (score of 1.000, sensitivity: 0.00; specificity: 1.00) and HumVar (score of 0.989, sensitivity: 0.52; specificity: 0.95), not tolerated by SIFT (threshold 0.05), as deleterious by Provean (score −5.581), and as disease related by SNPs&GO.

In the course of our work, we were informed by Dr. L.A. Metherell that mutations in the gene *NNT* had been found in previously obtained DNA from our two patients (our family 1 is equivalent to their kindred 12, our family 2 is equivalent to their kindred 9) through candidate gene sequencing based on other results from their cohort.[5] The four *NNT* mutations reported in that study in our patients are identical to the ones discovered by our whole exome sequence analysis. Importantly, in that study only glucocorticoid deficiency was mentioned as a clinical feature of mutations in *NNT*; the mineralocorticoid deficiency of our patient 2 was not reported, nor was a full case report of either patient presented. Moreover, segregation of the variants in *trans* which we have determined, was not assessed in the previous study due to lack of parental or sibling samples.

Additional genetic variants

The significant difference in clinical presentation in the two patients is not obviously explained by the mutations found in *NNT*; patient 1, who is clinically less severe, has the more severe mutations formally. Therefore, taking advantage of the whole exome variant data on both patients, we looked for additional genetic variants in genes involved in steroid hormone biosynthesis or regulation. We generated a list of 27 such genes (including *NNT*, Table 1 in Supplementary Data) and filtered all exome variants in the two patients for variants potentially altering protein-coding potential. In looking for potential genetic modifiers, we considered known variants

(ie, not private), since such variants might conceivably influence the phenotype in our patients carrying pathogenic mutations in *NNT*, even if having no phenotypic consequences on their own. Some of the variants called in the exome data were verified by Sanger sequencing (Supplement Table 2).

Not surprisingly, both patient exomes contain additional potential variants in relevant pathway genes (Supplementary Fig. S2 and Supplementary Table 2). In particular, patient 2 has two heterozygous variants in the gene *ME3* (c.G972C, p.K324N equivalent to dbSNP rs1042780, c.G1252A, p.A418T equivalent to dbSNP rs3182235, Supplementary Fig. S2); each parent is singly heterozygous, thus the patient is a compound heterozygote (data not shown). *ME3* variants are of particular interest as *ME3* encodes a subunit of malic enzyme that also generates mitochondrial NADPH. The minor allele frequencies of p.K324N and p.A418T are 0.4 and 0.02 respectively based on 1000 Genomes project data. The p.K324N SNP is predicted as benign by Polyphen2 HumDiv (score of 0.015, sensitivity: 0.96; specificity: 0.79) and HumVar (score of 0.056 sensitivity: 0.93; specificity: 0.64), as tolerated by SIFT (threshold 0.05), as neutral by Provean, and could not be interpreted by SNPs&GO. The lysine at position 324 is well conserved in mammals, chicken and zebrafish, but not in *Xenopus*. The p.A418T SNP is predicted as possibly damaging by Polyphen2 HumDiv (score of 0.610, sensitivity: 0.87; specificity: 0.91) and benign by HumVar (score of 0.148, sensitivity: 0.89; specificity: 0.71), as damaging by SIFT, as neutral by Provean, and as neutral by SNPs&GO. The alanine at position 418 is well-conserved among mammals and chicken, but not in *Xenopus* or the pigeon (Supplementary Fig. S3); presumably the incomplete conservation caused the different prediction tools to yield different interpretations of the variant.

Discussion

In the only paper to date describing patients with *NNT* mutations (which included our two patients), the phenotype was reported as isolated glucocorticoid deficiency, yet one of our patients had clear evidence of combined gluco- and mineralocorticoid deficiency. Thus our findings expand the genotype-phenotype correlation for *NNT*, which should be considered as a candidate gene in patients with otherwise unexplained primary adrenal insufficiency. We have also documented that the mutations in *NNT* are in *trans*, a point which was left open in the previous report on these patients.[5] The differential diagnosis between isolated glucocorticoid deficiency and combined gluco- and mineralocorticoid deficiencies is not always easy, especially at the time of presentation of an acutely and severely ill infant. This makes it challenging to determine the optimum hormonal treatment regime, since treatment with cortisol replacement alone leaves the patient at potential risk for a severe reaction if isolated glucocorticoid deficiency progresses spontaneously to more severe adrenal insufficiency. In the present study however, the presentation of the two patients was clearly different from an early age.

We sought to explain this phenotypic difference by looking for additional genomic variants in our exome sequence data. Among several additional protein-altering variants found in relevant genes, compound heterozygous *ME3* variants were noted in patient 2. These were of particular interest as malic enzyme 3 also generates mitochondrial NADPH, thus even modest functional variation in this gene might have significant effects in patients sensitized by severe mutations in *NNT*. Further work will be required to test whether these variants affect *ME3* activity.

NADPH metabolism is complex, and proceeds via multiple cytosolic and mitochondrial pathways. In mitochondria, NADPH can be generated through three different reactions: NADP-linked malic enzyme, isocitrate dehydrogenase, and nicotinamide nucleotide transhydrogenase (*NNT*).[17] These various pathways to NADPH are differentially relevant in various tissue or cell types. NADPH itself functions as a critical cofactor of many enzymatic conversions. Meimaridou et al, in their discussion of the role of *NNT* in glucocorticoid deficiency, emphasize its function in the regeneration of reduced glutathione.[5] Mice carrying a severe spontaneous allele of *NNT* (deleted for five coding exons in frame), as well as those with new ENU-derived alleles, exhibit increased toxicity due to oxidative stress together with insulin resistance.[18,19] However, NADPH is a required cofactor for many steroid hormone biosynthetic reactions. Given the multiple potential sources of mitochondrial NADPH, it is difficult to assess how the rates of these various biosynthetic steps might be affected

by changes in *NNT* gene function. But a primary role of *NNT* through production of metabolites in hormone biosynthesis may be as important as a role in regeneration of glutathione for protection against cytotoxic oxidative products.

In conclusion, our exome sequencing approach identified *NNT* mutations, which were simultaneously found by a candidate gene approach, in two infants with very low cortisol and high ACTH. It underlines the power of exome sequencing in pediatric endocrinology, as also illustrated by our recent discovery of mutations in *POMC* leading to bio-inactive ACTH.[20] In the present work, exome sequencing also led to the identification of variants in *ME3*, a potential disease modifier, in our patient with *NNT* mutations and additional mineralocorticoid deficiency.

Acknowledgements

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Author Contributions

CH participated in clinical review of patients and in molecular validation studies of mutations and in manuscript preparation. JD supervised clinical review and sampling of patients and in primary manuscript preparation. J-MV participated in bioinformatic analysis of mutations. LP participated in molecular validation studies. NA participated in bioinformatic analysis of exome sequence data. JS participated in bioinformatic analysis of exome sequence data. CLD participated in clinical review of patients and in manuscript preparation. GVV participated in clinical review of patients and in manuscript preparation. JM supervised bioinformatic analysis of exome sequence data. MES participated in all aspects of the project and in manuscript preparation.

Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosure and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation (pending) of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Supplementary Data

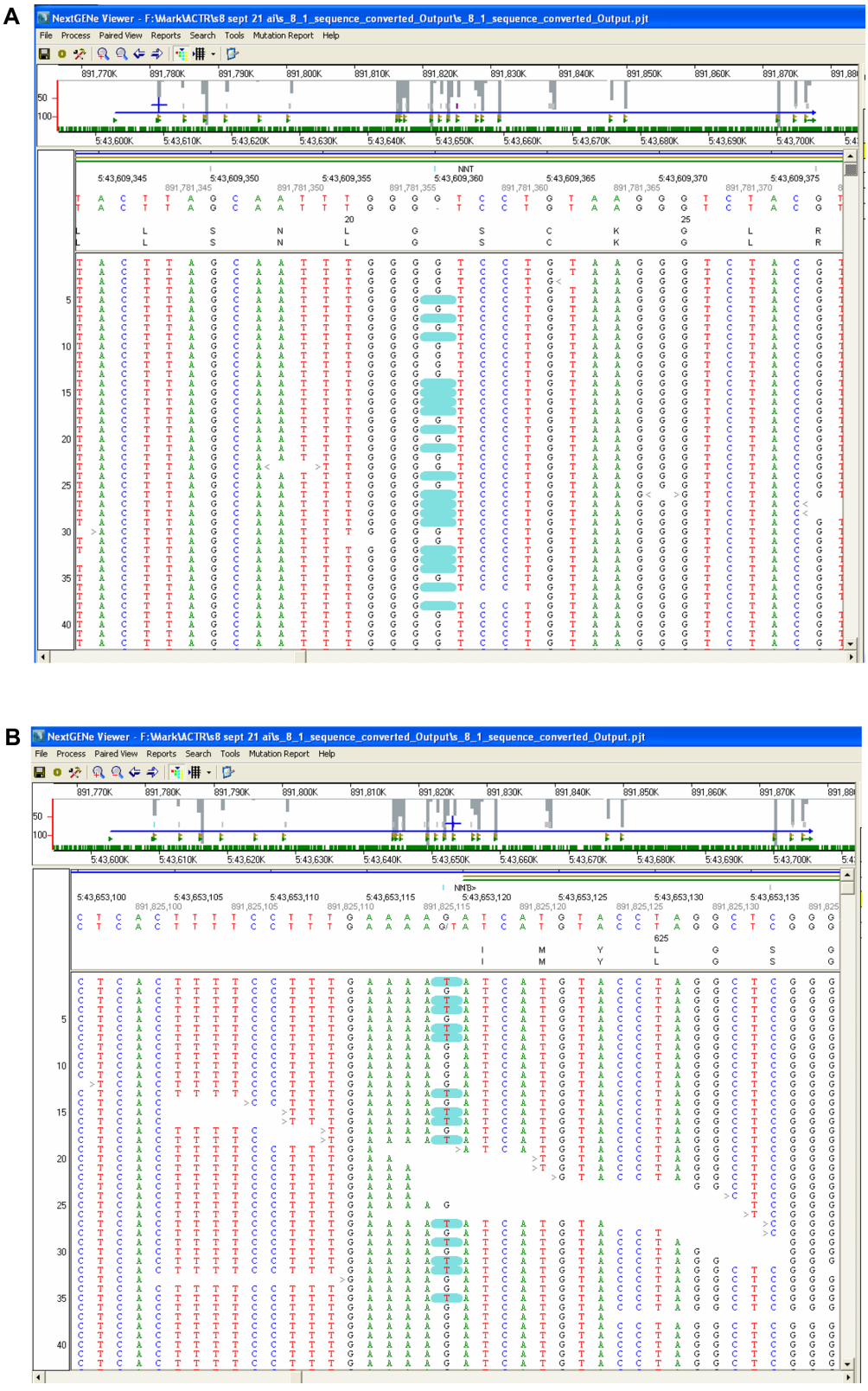


Figure S1. (*Continued*)

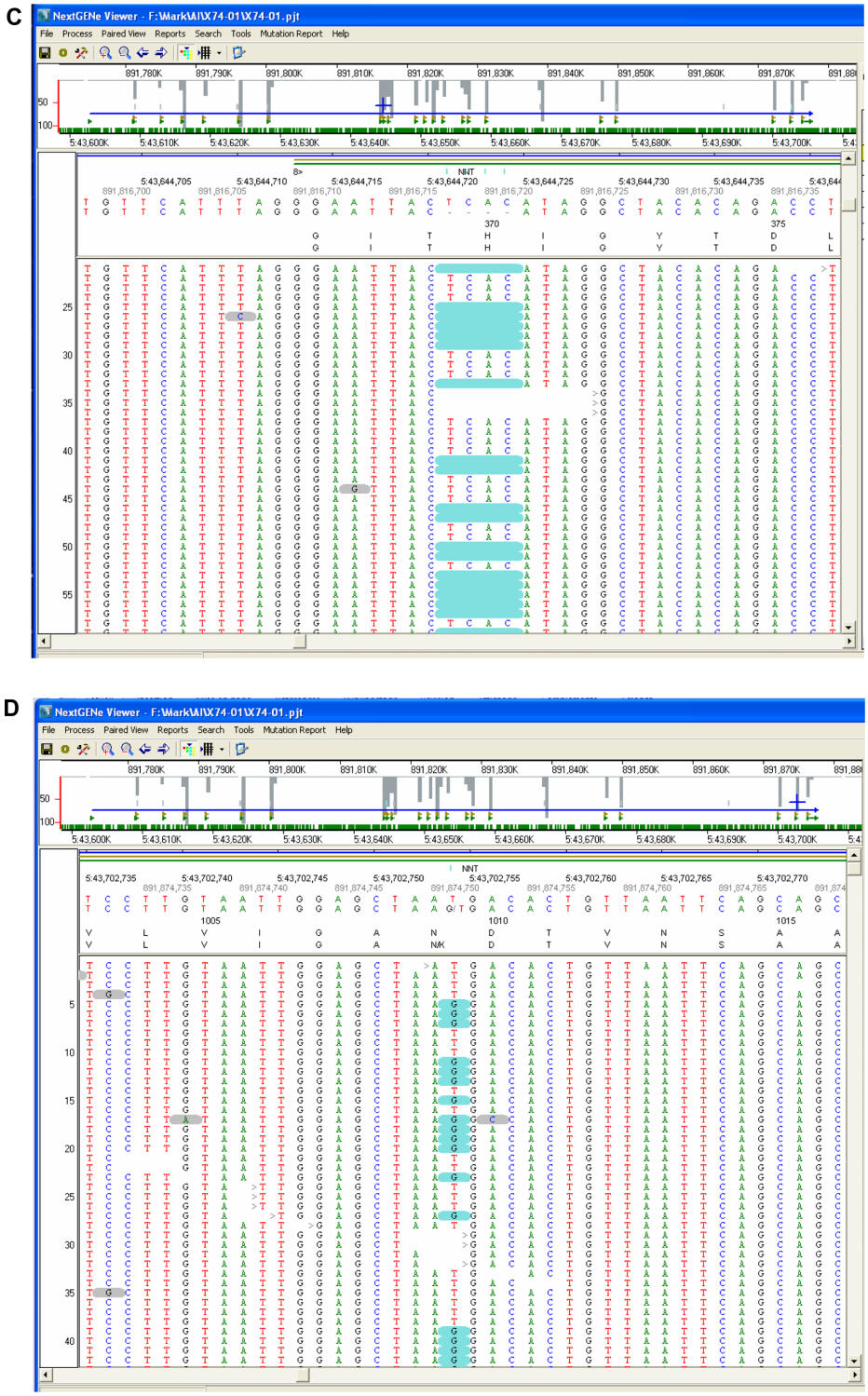


Figure S1. Mutations in *NNT* in probands of families 1 and 2. (**A**) Family 1, c.63delG, p.S22Pfs*6, heterozygous frameshift deletion, visualized with NextGene. Upper box in each panel shows the annotated *NNT* gene, 5′-3′ left to right as gene is on forward chromosomal strand (light blue arrow). Exons are shown as green boxes, coding exons as gold boxes. Read coverage is shown on the left, and is concentrated over target exons. Reported variants (in dbSNP as implemented in the custom software database) shown as red ticks, novel SNPs as blue ticks. Blue cross indicates location of sequence alignment in lower window. Numbering across top of upper window is in bp across entire genome starting with left end of chromosome 1 as per genome assembly hg19. Lower window shows a closeup of the gene region containing the heterozygous mutation in the sample. Upper sequence track is the genome consensus, lower track is the sample showing the nucleotide change, followed by the same two tracks with predicted protein coding effect and amino acid numbering. In some cases window shows only a subset of reads. Novel variant bases are highlighted in blue, variant bases present in dbSNP are in purple (none in these screenshots), candidate variants removed by the software are in grey. (**B**) Family 1, c.1864-1G > T, p.I622Dfs*1, splice acceptor site AG-to-AT heterozygous mutation, visualized with NextGene. (**C**) Family 2, c.1107_1110delTCAC, p.H370* heterozygous frameshift deletion, visualized with NextGene. (**D**) Family 2, c.3027T > G, p.N1009K heterozygous missense mutation, visualized with NextGene.

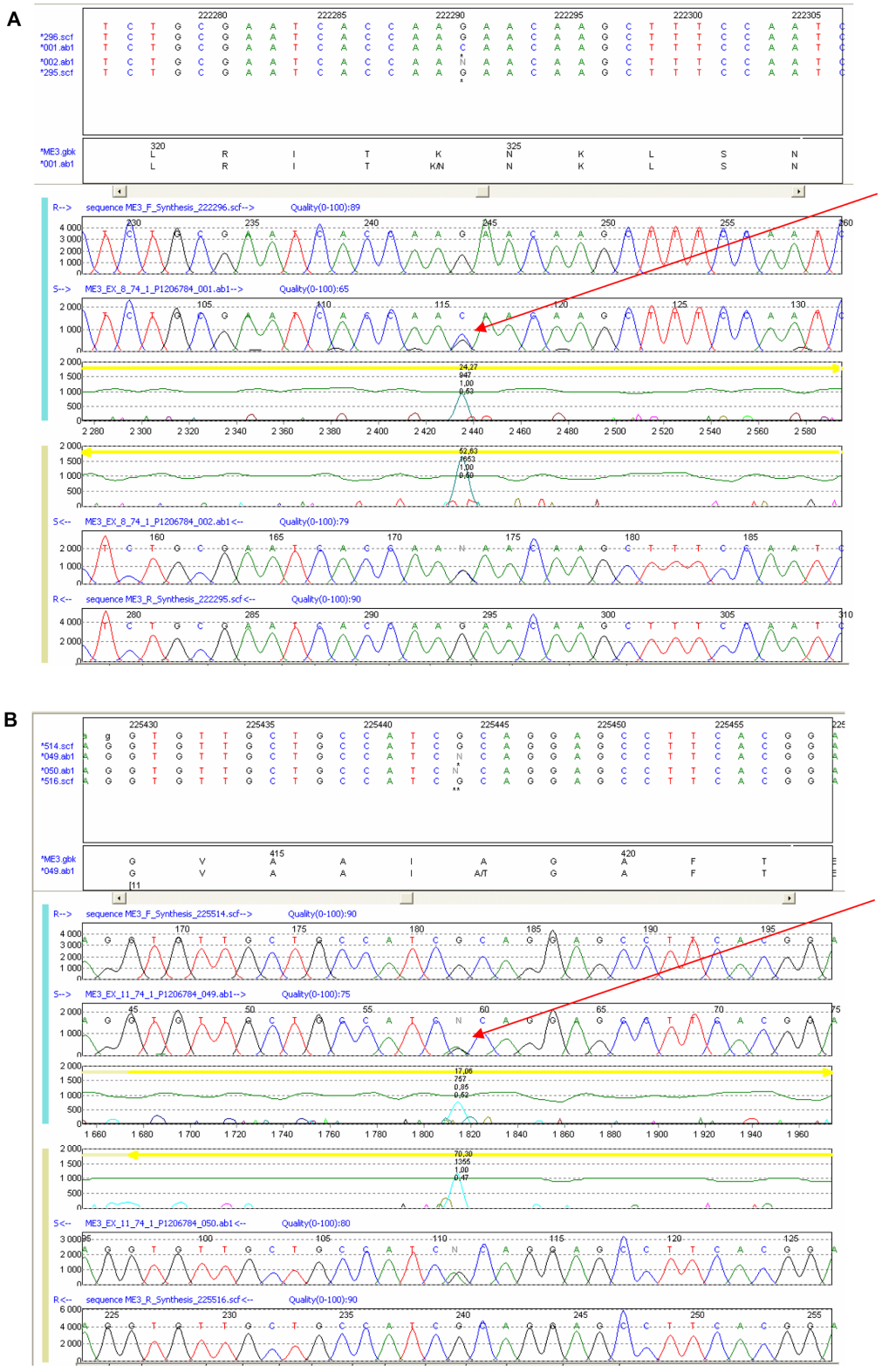
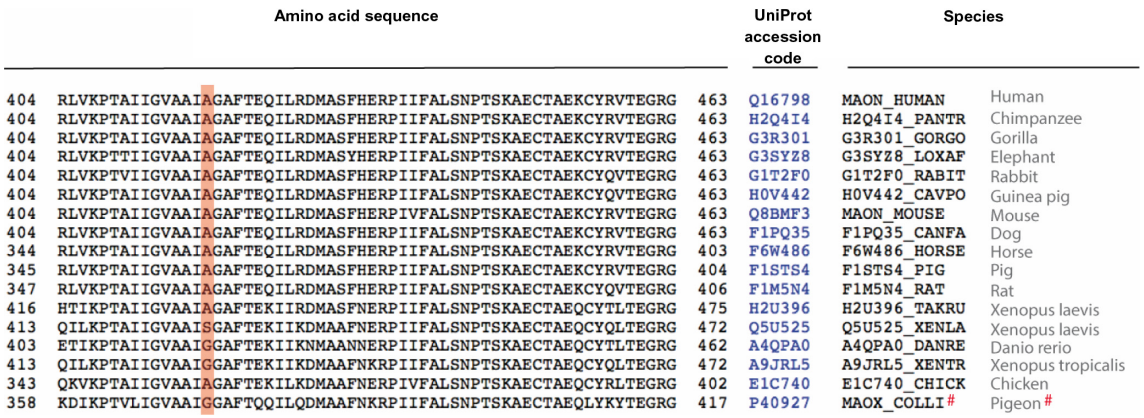


Figure S2. Mutations in *ME3* in proband of family 2. (**A**) p.K324N heterozygous mutation visualized with MutationSurveyor (Soft Genetics, Inc.), using NM_001014811 as the reference sequence. Upper window of panel shows nucleotide sequences in upper panel, amino acid sequences in lower panel with wild type amino acid sequence above, mutant amino acid sequence below. Lower window of panel has six lanes: uppermost (first) and lowermost (sixth) track are virtual forward and reverse chromatogram traces respectively, created by the software from the consensus wild type sequence, second and fifth tracks are forward and reverse chromatograms respectively from patient sample. The two centre tracks (third and fourth) are mutation detection calls by MutationSurveyor for forward and reverse sequences, peaks above green curve reach significance according to the software calling algorithm. In this software genes are automatically shown 5′-3′ left to right regardless of chromosomal orientation in the genome assembly. Red arrow shows heterozygous mutation in forward chromatogram. (**B**) p.A418T heterozygous mutation as visualized in MutationSurveyor.



	Amino acid sequence		UniProt accession code	Species	
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404	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYRVTEGRG	463	H2Q4I4	H2Q4I4_PANTR	Chimpanzee
404	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYRVTEGRG	463	G3R301	G3R301_GORGO	Gorilla
404	RLVKPTTIIGVAAIAGAFTEQILRDMASYHERPIIFALSNPTSKAECTAEKCYRVTEGRG	463	G3SYZ8	G3SYZ8_LOXAF	Elephant
404	RLVKPTVIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYQVTEGRG	463	G1T2F0	G1T2F0_RABIT	Rabbit
404	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYQVTEGRG	463	H0V442	H0V442_CAVPO	Guinea pig
404	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIVFALSNPTSKAECTAEKCYRVTEGRG	463	Q8BMF3	MAON_MOUSE	Mouse
404	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYRVTEGRG	463	F1PQ35	F1PQ35_CANFA	Dog
344	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYRVTEGRG	403	F6W486	F6W486_HORSE	Horse
345	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYRVTEGRG	404	F1STS4	F1STS4_PIG	Pig
347	RLVKPTAIIGVAAIAGAFTEQILRDMASFHERPIIFALSNPTSKAECTAEKCYQVTEGRG	406	F1M5N4	F1M5N4_RAT	Rat
416	HTIKPTAIIGVAAIAGAFTEKIIRDMASFNERPIIFALSNPTSKAECTAEQCYTLTEGRG	475	H2U396	H2U396_TAKRU	Xenopus laevis
413	QILKPTAIIGVAAISGAFTEKIIKDMAAFNERPIIFALSNPTSKAECTAEQCYQLTEGRG	472	Q5U525	Q5U525_XENLA	Xenopus laevis
403	ETIKPTAIIGVAAIGGAFTEKIIKNMAANNERPIIFALSNPTSKAECTAEQCYTLTEGRG	462	A4QPA0	A4QPA0_DANRE	Danio rerio
413	QILKPTAIIGVAAIGGAFTEKIIKDMAAFNKRPIIFALSNPTSKAECTAEQCYQLTEGRG	472	A9JRL5	A9JRL5_XENTR	Xenopus tropicalis
343	QKVKPTAIIGVAAIAGAFTEKILKDMAAFNERPIVFALSNPTSKAECTAEQCYRLTEGRG	402	E1C740	E1C740_CHICK	Chicken
358	KDIKPTVLIGVAAIGGAFTQQILQDMAAFNKRPIIFALSNPTSKAECTAEQLYKYTEGRG	417	P40927	MAOX_COLLI#	Pigeon#

Figure S3. *ME3* interspecies protein alignment.
Notes: #Corresponding to the NADP-dependent malic enzyme (ME1) protein which was used as template for the homology modelling. Alignment *ME3* proteins from different species (noted in last column) performed on UniProt website. For comparison, the alignment of ME1 protein used for homology modeling is shown on the last row. The column corresponding to the alanine 418 in human *ME3* (first row) is highlighted in *red*.

Table 1. Steroid biosynthetic, NADP and related genes of interest.

Enzyme/activity	Gene
Cholesterol side chain cleavage	CYP11A1
17-α hydroxylase	CYP17A1
21 α-hydroxylase	CYP21A2
3 β-hydroxysteroid dehydrogenase	HSD3B2
11 β-hydroxylase	CYP11B1
Aldosterone synthase	CYP11B2
Ferredoxin	FDX1
Ferredoxin reductase	FDRX
P450 oxidoreductase	POR
Nicotinamide nucleotide transhydrogenase	*NNT*
NADP linked mitochondrial malic enzyme	*ME3*
NADP linked isocitrate dehydrogenase	IDH1, IDH2
NAD kinase	NADK
Glucose 6-P dehydrogenase	G6PD
6-P gluconate dehydrogenase	PGD
Corticotropin releasing hormone	CRH
CRH binding protein	CRHBP
Corticotropin releasing hormone receptor	CRHR1, CRHR2
Proopiomelanocortin	*POMC*
Gitelman syndrome	SLCI2A3
Bartter syndrome without deafness	SLC12A1, KCNJ1
Renin	REN
Angiotensin	AGT
Angiotensin converting enzyme	ACE

Table 2. Protein coding variants in pathway genes in probands of families 1 and 2.

Proband	Gene	Variant	Zygosity	Verified by Sanger seq'g.	dbSNP rs#	Allele frequency (dbSNP v.135)
1	ACE	p.S48N (alternative N-terminus)	Het	n.d.	Novel	n.a.
1	AGT	p.M268T	Het	n.d.	699	0.32
1	AGT	p.R375W	Het	n.d.	Novel	n.a.
1	IDH1	p.V178I	Het	+	34218846	0.05
1	NADK	p.F436/ E437ins(CTC)	Het	+	Novel	n.a.
1	NADK	p.N262K	Het	n.d.	4751	0.2
1	NADK	p.R249W	Het	n.d.	75816936	0.02
1	*NNT*	p.K63R	Het	n.d.	35201656	0.036
1	*NNT*	p.L663F	Het	n.d.	41271083	0.035
2	AGT	p.M268T	Het	n.d.	699	0.32
2	AGT	p.T207M	Het	+	4762	0.1
2	IDH1	p.V178I	Het	+	34218846	0.05
2	***ME3***	**p.A418T**	**Het**	**+**	**3182235**	**0.02**
2	***ME3***	**p.K324N**	**Het**	**+**	**1042780**	**0.39**
2	NADK	p.N262K	Hom	n.d.	4751	0.2

Notes: Mutations in *ME3* are highlighted in bold. Allele frequencies are from dbSNP v. 135 which incorporates data from the 1000 Genomes project; these frequencies may thus reflect combined results from multiple ethnicities as sequenced in the 1000 Genomes.