SHORT REPORT

Combined mineralocorticoid and glucocorticoid deficiency is caused by a novel founder nicotinamide nucleotide transhydrogenase mutation that alters mitochondrial morphology and increases oxidative stress

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ABSTRACT

Background Familial glucocorticoid deficiency (FGD) reflects specific failure of adrenocortical glucocorticoid production in response to adrenocorticotropic hormone (ACTH). Most cases are caused by mutations encoding ACTH-receptor components (*MC2R*, *MRAP*) or the general steroidogenesis protein (StAR). Recently, nicotinamide nucleotide transhydrogenase (*NNT*) mutations were found to cause FGD through a postulated mechanism resulting from decreased detoxification of reactive oxygen species (ROS) in adrenocortical cells.

Methods and results In a consanguineous Palestinian family with combined mineralocorticoid and glucocorticoid deficiency, whole-exome sequencing revealed a novel homozygous *NNT*_c.598 G>A, p.G200S, mutation. Another affected, unrelated Palestinian child was also homozygous for NNT_p.G200S. Haplotype analysis showed this mutation is ancestral; carrier frequency in ethnically matched controls is 1/200. Assessment of patient fibroblasts for ROS production, ATP content and mitochondrial morphology showed that biallelic *NNT* mutations result in increased levels of ROS, lower ATP content and morphological mitochondrial defects.

Conclusions This report of a novel NNT mutation, p.G200S, expands the phenotype of *NNT* mutations to include mineralocorticoid deficiency. We provide the first patient-based evidence that *NNT* mutations can cause oxidative stress and both phenotypic and functional mitochondrial defects. These results directly demonstrate the importance of *NNT* to mitochondrial function in the setting of adrenocortical insufficiency.

INTRODUCTION

Familial glucocorticoid deficiency (FGD) (OMIM #202200) is a genetically heterogeneous autosomal-recessive disorder defined by lack of adrenocortical glucocorticoid production in response to adrenocorticotropic hormone (ACTH). Mineralocorticoid and androgen levels are normal. Patients present in infancy with hypoglycaemia or severe hypotension associated with low cortisol and significantly elevated ACTH levels. Most cases are caused by mutations in the *MC2R* and *MRAP* genes, encoding components of the adrenocortical ACTH receptor complex, and in the steroidogenic acute regulatory protein (*StAR*) gene, encoding a critical steroidogenesis protein. The specific effect of some *StAR* mutations on glucocorticoid production alone is notable, even though *StAR* is present in all adrenocortical layers and required for steroidogenesis of all three types of steroids. The most recently identified genetic basis of FGD are biallelic mutations in the ubiquitously expressed nicotinamide nucleotide transhydrogenase (*NNT*) gene and are thought to account for 5–10% of cases.[1 2]

NNT encodes an integral protein of the inner mitochondrial membrane (IMM) that translocates protons across the IMM from the cytosol to the mitochondrial matrix, generating nicotinamide adenine dinucleotide phosphate (NADPH).[3] NADPH is used both in biosynthesis, for example, as an essential cofactor of the P450 enzymes that catalyse steroidogenesis reactions in the mitochondria (cholesterol side chain cleavage and 11β-hydroxylation), and in detoxification of free reactive oxygen species (ROS), where it is required for glutathione peroxidase and glutathione reductase activity.

The recently identified *NNT* variants[4] span the entire gene and include abolishment of the initiating methionine, splice, missense and nonsense mutations. This mutational spectrum suggests that disease-associated mutations result in loss of NNT function. *Caenorhabditis elegans* lacking functional *NNT* display increased sensitivity to oxidative stress,[5] and knockdown of *NNT* in human adrenocortical H295R cell lines increases mitochondrial ROS levels.[1] Thus, although no direct studies of specific *NNT* mutations have been performed, they are hypothesised to cause FGD by impairing ROS detoxification in adrenocortical cells.[4]

Here we report a novel *NNT* mutation observed in four cases from two unrelated consanguineous Palestinian families, underlying not only glucocorticoid but also mineralocorticoid deficiency, differing from the FGD phenotype previously reported in other *NNT* mutations. Furthermore, for the first time, we describe the results of functional assessment of patient fibroblasts harbouring

biallelic *NNT* mutations and their effect on ROS levels, ATP content and mitochondrial morphology.

METHODS

Further information can be found in the online supplementary methods.

Genetic analysis

Genomic DNA was extracted from peripheral blood mononuclear cells. Regions of homozygosity were mapped using the Affymetrix Gene Chip 250K/750K Nsp SNP array. Candidate genes in homozygous regions shared only by affected individuals were Sanger sequenced. Whole-exome, massively parallel sequencing (WES) was performed in individuals IV-1 and IV-2 from family A (figure 1A). Following enrichment using Illumina's TruSeq Exome Enrichment Kit, sequencing was performed on the Illumina HiSeq2500 platform at the Technion Genome Center, Israel. Sanger sequencing was used to validate the NNT_p.G200S mutation identified. Mutation frequency in 200 ethnically matched controls was assayed by *AciI* digestion of PCR products surrounding the NNT_p.G200S mutation. Haplotype analysis of a 4 Mb region flanking the *NNT* gene was performed using six short tandem repeat (STR) markers. Analysis of *NNT* splicing was performed on cDNA from patient and control fibroblasts by amplification of exons 3–6 surrounding the *NNT*_c.598 G>A mutation at the end of exon 4.

Cell culture

Patient and control fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM). Cell growth was measured using a colorimetric method based on the staining of basophilic cellular compounds with methylene blue (MB) at A_{620nm}.[6] Intracellular ROS production was measured using 2′,7′-dichlorodihydrofluorescein diacetate (DCF, Sigma-Aldrich).[7] Relative fluorescent units (RFU) were calculated by normalising the DCF fluorescence to the number of cells assayed by MB.[6] ATP content was measured by luciferin-luciferase using the ATPlite luminescence assay system (Perkin-Elmer, Waltham Massachusetts, USA). Relative luminescence units (RLU) were calculated by normalising to growth as measured by MB in parallel wells for each separate experiment. Mitochondrial morphology was assessed using MitoTracker Green (Life Technologies) staining.[8]

Statistics

Statistical significance was assessed by a two-tailed, unpaired Student's t test. $p \leq 0.05$ was considered significant.

CLINICAL PRESENTATIONS

Family A

Patient I (IV-6)

A 3-day-old female (birth weight 2.08 kg) born following a normal pregnancy to consanguineous parents presented with hyperpigmentation and neonatal Addisonian crisis including hyponatraemia, hyperkalaemia, inappropriate concomitant natriuria (47 mmol/L) and hypoglycaemia (figure 1A). She responded to a maintenance dose of hydrocortisone and required relatively high doses of mineralocorticoids, up to 200 μg of fludrocortisone, to maintain normal sodium levels in the first 2.5 years of life. Re-evaluation at age 2.5 years showed normal tear secretion, parathyroid hormone levels, thyroid function tests, thyroid antibodies, coeliac screen and Barium esophagography. These results deemed achalasia, alacrima, adrenal insufficiency (AAA) (MIM #231550) or autoimmune-polyendocrine (MIM #240300) syndromes as unlikely. During transient cessation of treatment with glucocorticoids and mineralocorticoids (at Na of 127 mmol/L), cortisol and aldosterone levels were low despite high levels of ACTH and plasma renin activity (PRA) levels (figure 1C). Bone age was 2.5 years, and karyotype was 46, XX.

Patient II (IV-2, cousin of patient I)

A 27-day-old male (birth weight 3.29 kg) born to consanguineous parents presented with Addisonian crisis. His sodium and potassium levels were 118 and 6 mmol/L respectively with concomitanat hypernatriuria of 50 mmol/L. His physical examination was normal, with male genitalia. Clinical features are summarised in figure 1C. Individual IV-1 and two other family members in generation III presented in Amman, Jordan, with similar clinical phenotypes of neonatal Addisonian crisis, including hyponatraemia (<125 mmol/L), hyperkalaemia (>6 mmol/L), concomitant natriuria (>40 mmol/L), low cortisol (<5 μg/dL) and requirement of both mineralocorticoid (fludrocortisone 0.1–0.2 mg daily) and a maintenance dose of glucocorticoid (5–10 mg hydrocortisone) for a good clinical response (personal communication).

Family-B

Patient III (II-2)

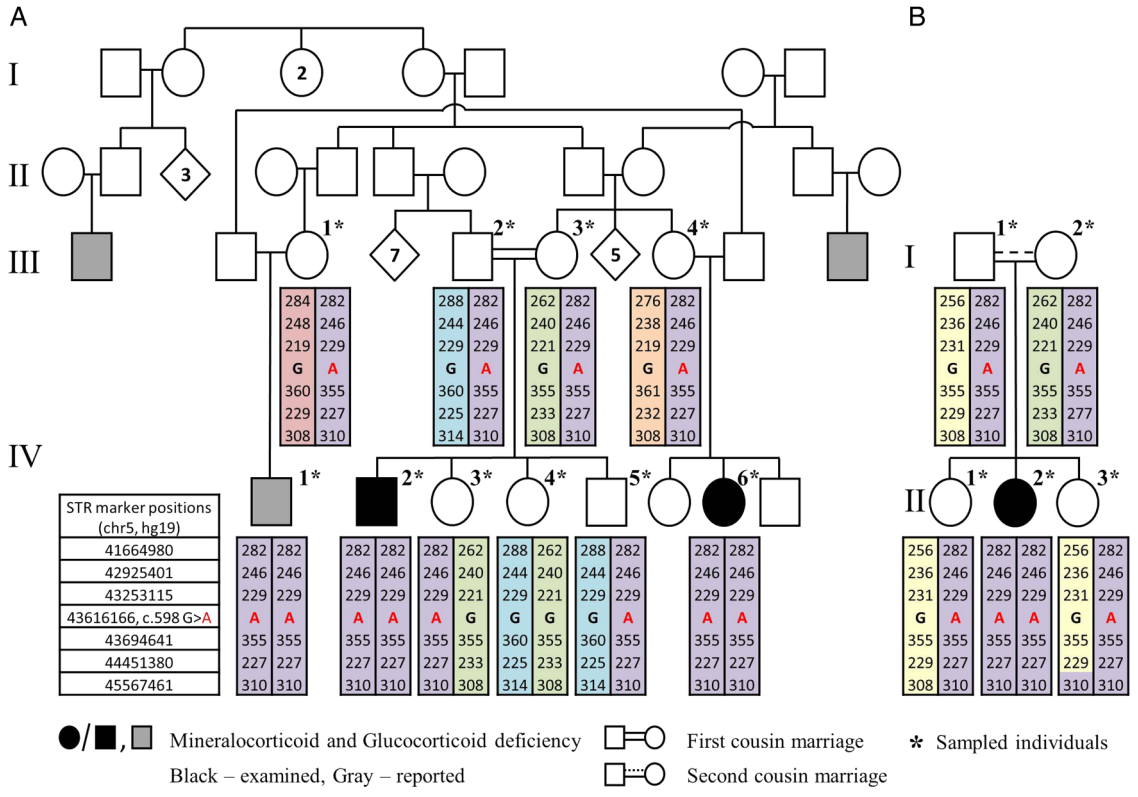
A 9.5-year-old female was followed since age 11 m, when she presented with Addison disease including hyponatraemia, hyperkalaemia, hypotension and skin hyperpigmentation (figure 1B). Poor compliance with hydrocortisone and mineralocorticoid replacement therapy led to frequent hospitalisations mainly due to severe hyponatraemia. Her karyotype was 46, XX. Further clinical features are summarised in figure 1C. Her parents are second cousins but her family history was negative for similar disorders.

RESULTS

In family A (figure 1A), we identified a single, large 22.3 Mb region fulfilling homozygosity criteria on chromosome 5 (chr5:31656768-54018232, hg19). This region contained over 80 genes (UCSC Genome Browser), including several candidate genes that were Sanger sequenced: *RXFP3*, encoding the relaxin 3 receptor 1 that is highly expressed in the adrenal gland, and *CYP11B2*, although it is classically associated with corticosterone methyloxidase deficiency, and isolated hypoaldosteronism, without glucocorticoid deficiency (as observed in our patients). The *StAR* and *NR5A1* genes were also Sanger sequenced, but no disease-causing mutations were found. Homozygosity mapping in family B identified a total of 144 Mb of homozygous regions.

WES of two affected cousins, IV-1 and IV-2 from family A (figure 1A), revealed a novel G>A missense mutation in the *NNT* gene (position chr5:43616166, *NNT*_c.598 G>A, p.G200S, hg19, NM_012343) (figure 2A). This mutation, replacing the highly conserved glycine residue at position 200 of the protein (figure 2B) with serine, was homozygous in all affected individuals in family A and segregated as expected (figures 1A and 2A). Surprisingly, the affected individual in family B was also homozygous for the *NNT*_c.598 G>A mutation, with consistent segregation in this family as well (figure 1B). Haplotype analysis showed that the mutation was ancestral: a shared haplotype harboured the mutation in both unrelated families (figure 1A and B). Mutation testing in 200 healthy ethnically matched controls revealed only one heterozygote carrier (allele frequency of 0.0025) and no homozygotes.

NNT is expressed in skin; we therefore examined the functional implications of this novel mutation, in fibroblasts obtained by skin biopsy from individual IV-2 in family A (figure 1A).



C

Clinical characteristics of the patients affected with combined Glucocorticoid and Mineralocorticoid deficiency

Case		Age of presentation	Cortisol [nmol/l]	17OHP [nmol/l]	DHEAS [micromol/l]	ACTH [pmol/l]	PRA* [ng/ml/hr]	Aldosterone * [pmol/l] **
Family A	IV-6	3 days	0.55	<0.3	<0.27	>278	4.54	238
	IV-2	27 days	< 0.69	13.3	< 0.41	> 278	>15	114
Family B	II-2	11 months	< 27.6	2.49	< 0.41	1591	8.7	170

* During hyponatremia, Supine .for 15 minutes.

Normal ranges:

Cortisol:	130-690 nmol/l
17OHP:	1-6 months – 1-14.1 nmol/l, >6months – 0.6-4.25nmol/l
DHEAS:	0.14-1.35 micromol/l
ACTH:	1.9-10.2 pmol/l
Aldosterone:	Supine 194-5000 pmol/l **expected over 700 during hyponatremia
Plasma Renin Activity (PRA):	Supine 0.2-2.8 ng/ml/hr

Figure 1 Families with combined mineralocorticoid and glucocorticoid deficiency. The nicotinamide nucleotide transhydrogenase (*NNT*) c.598 G>A, G200S, mutation segregation, haplotype analysis and unique clinical characteristics of the patients affected with combined glucocorticoid and mineralocorticoid deficiency. (A and B) Pedigrees of families A and B. The genomic positions of tested short tandem repeat (STR) markers flanking the *NNT* gene are indicated in the table, bottom left. STR and *NNT* c.598 G>A results are shown in the table beneath each individual. Numbers indicate amplicon size for each STR marker. The *NNT* c.598 G>A wildtype nucleotide G and the variant nucleotide A (red) are indicated. Segregation is consistent with autosomal-recessive inheritance, and only affected individuals are homozygous for the *NNT* c.598 G>A variant (family A: IV-1, IV-2, IV-6; family B: II-2). The mutant ancestral haplotype, shared in both unrelated families, is marked in purple. (C) Clinical characteristics of the patients affected with combined glucocorticoid and mineralocorticoid deficiency. 17OHP, 17-hydroxyprogesterone; ACTH, adrenocorticotropic hormone; DHEAS, dehydroepiandrosterone sulfate.

We first assayed cDNA obtained from mutant and control fibroblasts to determine whether *NNT* splicing is affected by the c.598_G>A mutation that is located 2 bp upstream of the splice junction at the end of exon 4. No alternative splicing was observed in the mutant fibroblasts (see online supplementary figure S1).

We then assessed ROS production, ATP content and mitochondrial morphology. ROS levels, measured by the DCF, were 40% higher in NNT_p.G200S homozygous fibroblasts compared with control fibroblasts ($p<0.01$) (figure 2D). We also found that ATP content was significantly reduced in NNT_p.G200S homozygous cells, 25% less than in a healthy control

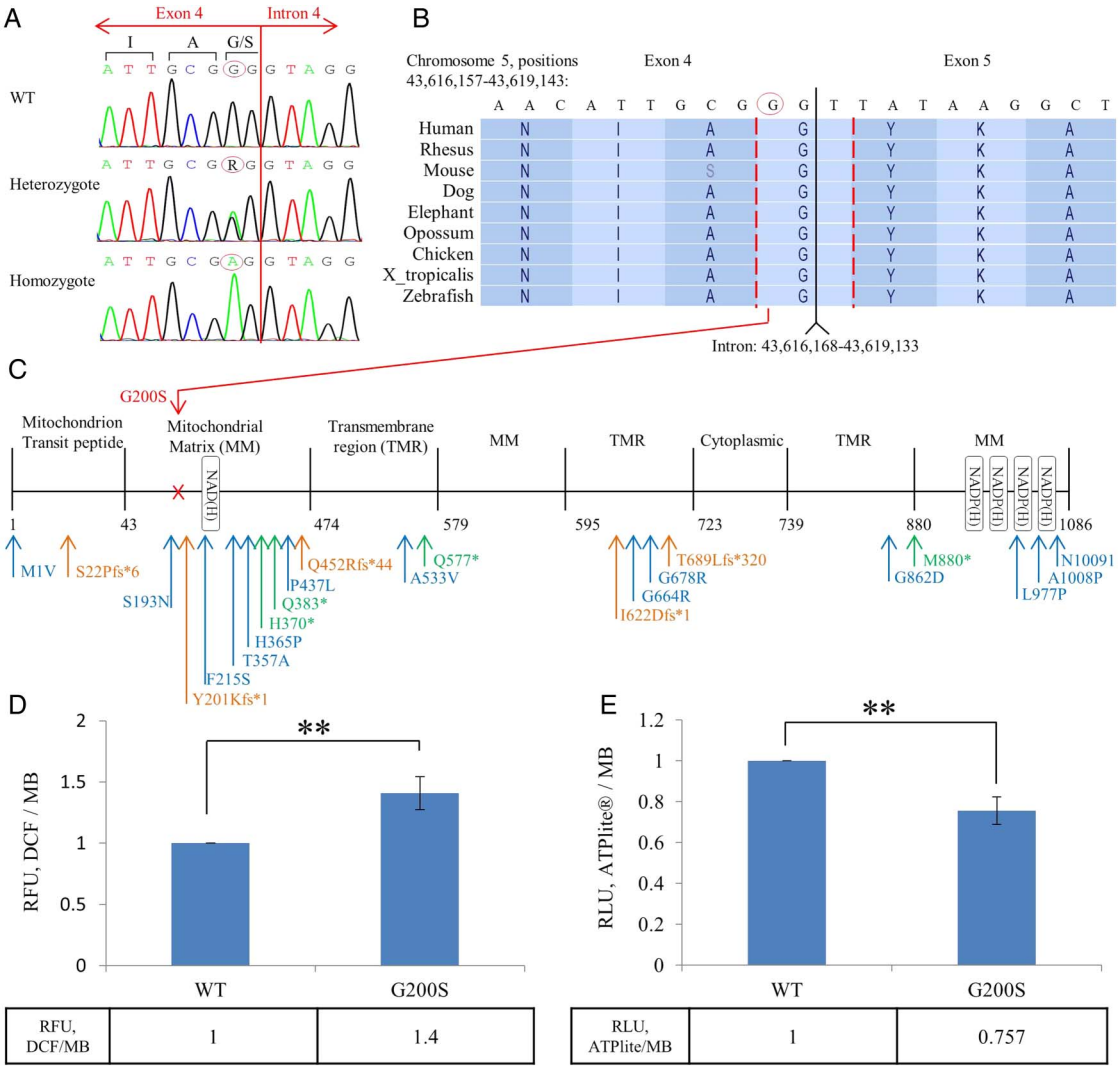
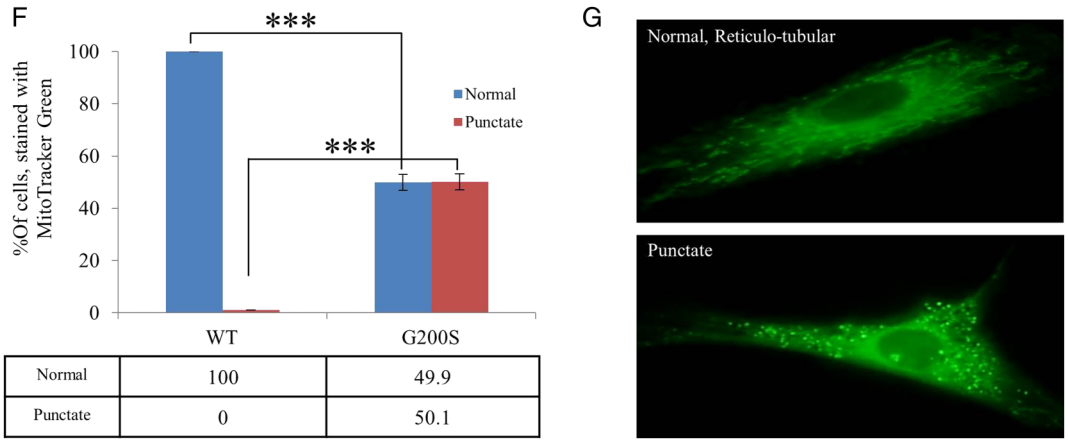


Figure 2 The nicotinamide nucleotide transhydrogenase (*NNT*) c.598 G>A, G200S, mutation sequencing, conservation and protein illustration. NNT G200S homozygous fibroblasts: reactive oxygen species (ROS) production, ATP content and mitochondrial morphology. (A) Sanger sequencing of *NNT* c.598 G>A in genomic DNA of wildtype (WT), heterozygote and homozygote individuals. Exon/intron junction (black vertical line), nucleotides and amino acid residues are indicated above, c.598 variant position circled in red. (B) Evolutionary conservation of NNT residue G200 and flanking residues (mutation position is circled in red). The exon 4/5 junction is indicated by a black vertical line. (C) Schematic representation of the NNT protein, indicating its domains, and the residues included in each domain. The G200S mutation is labelled above the diagram in red (and indicated by an x). Previously described mutations are marked below the diagram; missense mutations in blue, nonsense mutations in green and frameshift mutations in orange. *NADP(H)*, NADP(H) known nucleotide phosphate-binding region; *NAD(H)*, predicted NAD(H) nucleotide phosphate-binding region. (D) ROS production, quantified with 2′,7′-dichlorodihydrofluorescein diacetate (DCF, Sigma-Aldrich) and normalised to methylene blue (MB), as described in the 'Methods' section. Fibroblasts obtained from a skin biopsy (2000 cells/0.32 cm^2) from individual IV-2, homozygote for the G200S mutation in the *NNT* gene, show a 40% increase in ROS production. (E) ATP content in fibroblasts, measured with ATPlite (Perkin Elmer) and normalised to MB, as described in the 'Methods' section. Fibroblasts obtained from a skin biopsy (3000 cells/0.32 cm^2) from individual IV-2, homozygote for the G200S mutation in the *NNT* gene, show a 25% decrease in ATP content. Results in both (D) and (E) represent four experimental repeats conducted in triplicate (**p<0.01). (F and G) Mitochondrial morphology in fibroblasts stained with MitoTracker Green (Life Technologies), and quantified as normal (reticulotubular) or punctate. Fifty per cent of mitochondria in fibroblasts obtained from a skin biopsy from individual IV-2, homozygote for the G200S mutation in the *NNT* gene, were found to be punctate, control fibroblasts showed 100% normal filamentous morphology (***p<0.001, n=375 counted cells in each cell type, in a total of six separate stainings). RFU, relative fluorescence units; RLU, relative luminescence units.

(p<0.01) (figure 2E). Finally, analysis of mitochondrial morphology revealed that in 50% of the patient's cells the mitochondria had a pathological punctate appearance, in contrast to the normal elongated reticulotubular appearance of mitochondria in 100% of control cells (n=375, p<0.001) (figure 2F and G).

DISCUSSION

Recessive *NNT* mutations have been recently shown to cause FGD.[1 2] We report a novel *NNT* mutation, c.598 G>A, p.G200S, that expands the *NNT*-associated phenotype to include neonatal mineralocorticoid deficiency. Patients homozygous for this mutation presented with classical, complete life-threatening neonatal Addisonian crisis, including severe hyponatraemia, hyperkalaemia, hypoaldosteronemia and high PRA, which are consistent with a role for NNT in synthesising both steroid classes. NNT is an IMM proton pump that regenerates NADPH from NADP+ using energy provided by the mitochondrial proton gradient.[3] The NADPH produced is an essential cofactor of the P450 enzymes, which catalyse two key steroidogenesis reactions occurring in the mitochondria:[9 10] the initial step of cholesterol side chain cleavage



	WT	G200S
Normal	100	49.9
Punctate	0	50.1

Figure 2 Continued.

and the almost final 11β-hydroxylation step leading to production of cortisol in the zona fasciculata and corticosterone (processed into aldosterone) in the zona glomerulosa. Thus, it should not come as a surprise that the NNT_p.G200S mutation leads to both mineralocorticoid and glucocorticoid deficiency, except that previously reported *NNT* mutations caused only glucocorticoid deficiency.[1 2 11 12] Notably, one previously reported infant with an *NNT* mutation did have borderline low serum sodium, markedly high PRA and low aldosterone with no serious clinical significance while on glucocorticoid replacement therapy.[2] The mutation in this infant, NNT_p.F215S, is very close to the NNT_p.G200S we describe as causing both mineralocorticoid and glucocorticoid deficiency. This suggests that previously reported patients with *NNT* mutations may have had some subclinical mineralocorticoid deficiency together with their clinically evident glucocorticoid deficiency. Nevertheless, further research and possibly more detailed clinical studies during early infancy may explain why other *NNT* mutations apparently do not severely impair mineralocorticoid synthesis.

The NNT protein is a homodimer that couples the NADPH redox reaction to proton translocation,[13] and thus maintains the high GSH:GSSG ratio required for ROS detoxification.[14] Each NNT subunit includes two matrix spanning regions, each harbouring four C-terminal NADP(H) nucleotide phosphate-binding (catalytic) sites and one N-terminal NAD(H) nucleotide phosphate-binding domain. NADH serves as the electron donor, and interestingly, approximately 40% of previously described mutations and the novel NNT_p.G200S mutation we report are located near the predicted NAD(H)-binding domain (figure 2C, amino acids 229–259). Mutations near this domain may diminish NNT's ability to regenerate NADPH, and thus increase oxidative stress. To examine this possibility, we performed the first studies in human fibroblasts harbouring disease-associated mutations. In NNT_p.G200S homozygous fibroblasts, we found a 40% increase in ROS levels (figure 2D), similar to *NNT* knockdown, which has been shown to increase mitochondrial ROS levels by 15% in human adrenocortical H295R cell lines.[1] We also observed a 25% decrease in ATP content (figure 2E), which is consistent with reported loss of ATP production in adrenal glands from C57BL6/J mice with a spontaneous *NNT* mutation.[15] In addition to these defects in mitochondrial function, we also discerned altered mitochondrial morphology, with a marked punctate appearance of patient mitochondria (figure 2F and G). This punctate appearance, in contrast to the normal reticulotubular morphology, has been previously shown to be caused by various apoptotic stimuli.[16] Taken together, these results provide the first direct evidence that *NNT* mutations associated with defects in steroid synthesis increase cellular oxidative stress and impair mitochondrial function and morphology. As noted above, NADPH regeneration by NNT is also necessary as a cofactor for steroidogenesis enzymes, for example, *CYP11B1*. However, we did not observe accumulation of 17-OH-progesterone in the NNT_pG200S homozygous patients. Thus, current evidence suggests that defects in ROS detoxification underlay the adrenal insufficiency observed in patients with *NNT* mutations.

Whereas the genes previously implicated in FGD are specific to ACTH signal transduction (*MC2R*, *MRAP*) or steroidogenesis (*StAR*), *NNT* has a much more general role in cellular metabolism. In this respect, *NNT* echoes *MCM4*, which is part of a highly conserved complex responsible for DNA replication and genome stability, and in which a mutation has been reported to cause a relatively mild syndrome that includes childhood-onset glucocorticoid deficiency.[11 12] Despite its ubiquitous role, the phenotype associated with *NNT* mutations has so far been limited to adrenocortical dysfunction. This tissue-specificity may be explained by the unique functions and effects of ROS in adrenocortical mitochondria. Steroidogenesis is accompanied by high ROS production because both cholesterol side-chain cleavage and 11-β hydroxylation are relatively 'leaky'. Respectively, approximately 15% and 40% of electron flow in these reactions results in ROS formation,[17] compared with <10% in the tightly coupled electron transfer chain (ETC) of ATP production.[18] Steroidogenesis-induced ROS have been shown to play a regulatory role in local feedback inhibition of steroid synthesis[19] by inactivating peroxiredoxin-3, which normally detoxifies mitochondrial H_2O_2. Increased steroid synthesis thus results in increased H_2O_2. Excess H_2O_2 diffuses to the cytosol and activates p38-MAPK signalling, which inhibits StAR synthesis and suppresses steroidogenesis.[20] In addition to suppressing steroidogenesis, ROS can also oxidise OXPHOS proteins, reducing their activity.[21] This uncoupling of the ETC further increases ROS generation, creating a vicious cycle reducing both steroidogenesis and energy production. Adrenocortical cells and steroidogenesis, in particular, are thus uniquely vulnerable to impaired ROS homeostasis. Indeed, increased oxidative stress has also been observed in cellular models and fibroblasts of patients with AAA syndrome,[19] and in X-linked adrenoleukodystrophy, both in patients and in the Abcd1 knockout mouse model.[22 23]

In conclusion, the novel founder NNT_p.G200S mutation expands the NNT mutation phenotype to include severe combined mineralocorticoid and glucocorticoid insufficiency, distinct

from classical FGD. Using patient fibroblasts homozygous for this mutation, we provide the first direct evidence that disease-associated *NNT* mutations impair detoxification of ROS, reduce cellular ATP content and alter mitochondrial morphology, probably reflecting similar phenomenon in the adrenal cortex. Our results provide further support for the emerging theme of oxidative stress as a mechanism of adrenal insufficiency.[19] Given these findings and the ubiquitous nature of the NNT protein, patients should be closely monitored to detect extra-adrenal manifestations that may develop beyond childhood.

Contributors EL-L and DZ contributed equally to this study. AW-S performed all the laboratory research work in the study, analysed the data and wrote the manuscript. AA-L characterised the clinical features of the patients, obtained consent and samples from the families in the study. FZ and LC performed the bioinformatics analysis of the exome data. AK-L participated in the functional assessed in patient fibroblasts. LK and MK assayed 200 healthy ethnically matched controls for the mutation in the study. SZ performed the homozygosity arrays for homozygous mapping. PR and EL-L participated in the laboratory research, analysing the data and editing the manuscript. DZ characterised the clinical features of the patients, obtained consent and sampled from the families in the study. In addition, he participated in the analysis of the data and in editing the manuscript.

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Competing interests None declared.

Patient consent Obtained.

Ethics approval The IRB and the National Helsinki Committee for Genetic Studies.

Provenance and peer review Not commissioned; externally peer reviewed.

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