A novel homozygous insertion and review of published mutations in the \textit{NNT} gene causing familial glucocorticoid deficiency (FGD)

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Abstract

Familial glucocorticoid deficiency (FGD) is an autosomal recessive disorder characterized by low levels of cortisol despite high adrenocorticotropic hormone (ACTH) levels, due to the reduced ability of the adrenal cortex to produce cortisol in response to stimulation by ACTH. FGD is a heterogeneous disorder for which causal mutations have been identified in MC2R, MRAP, MCM4 and TXNRD2. Also mutations in STAR and CYP11A1 can sometimes present with a phenotype resembling FGD. Recently, it has been indicated that FGD can also be caused by mutations in NNT (nicotinamide nucleotide transhydrogenase).

We identified a 6.67 Mb homozygous region harboring the NNT gene by SNP haplotyping in a 1-year old Dutch boy presenting with FGD, but without mutations in MC2R and MRAP. Exome-sequencing revealed a novel homozygous mutation (NM_012343.3: c.1259dupG) in NNT that was predicted to be disease-causing. The mutation is located in exon 9 and creates a frameshift leading to a premature stop-codon (p.His421Serfs*4) that is known to result in FGD. Both parents were shown to be heterozygous carriers. We reviewed the literature for all the reported NNT mutations and their clinical presentation. The median age of disease onset in 23 reported patients, including the present patient, was 12 months (range 3 days to 39 months). There was no difference in age of disease onset between truncating and non-truncating NNT mutations. Based on recent literature, we advise to monitor patients with FGD due to NNT mutations for possible combined mineralocorticoid insufficiency and extra-adrenal manifestations.
Inroduction

Familial glucocorticoid deficiency (FGD; MIM 202200) is a rare, autosomal recessive disorder characterized by undetectable or low levels of plasma cortisol despite high plasma adrenocorticotropin (ACTH) levels. The first symptoms generally occur during early infancy. FGD can present as an acute adrenal crisis precipitated by an intercurrent illness, or with recurrent hypoglycemia (often associated with seizures). Nonspecific additional symptoms might be: lethargy, failure to thrive, pallor and delayed developmental milestones. In addition, hyperpigmentation due to elevated ACTH levels is often seen. Strikingly, serum electrolytes are usually normal because aldosterone production is regulated mainly by the renin-angiotensin system. Plasma cortisol levels can range from undetectable to low-normal in the presence of high ACTH levels and do not respond to exogenous ACTH [1].

FGD is a genetically heterogeneous disorder resulting from known mutations in MC2R (Melanocortin 2 receptor; MIM 607397), MRAP (MC2R accessory protein; MIM 609196), and MCM4 (Minichromosome maintenance 4; MIM 602638) [2,3]. Also mutations in STAR (Steroidogenic acute regulatory protein; MIM 600617) and CYP11A1 (Cytochrome P450, Family 11, Subfamily A, MIM 613743; MIM 118485) can sometimes present with a phenotype resembling FGD [4,5]. It has recently been reported that mutations in NNT (nicotinamide nucleotide transhydrogenase; MIM 607878) and TXNRD2 (Thioredoxin Reductase 2; MIM 606448) can also result in FGD [3,6]. While mutations in MC2R, MRAP, STAR, and MCM4 account for ~50% of FGD patients, NNT mutations are found in 5-10% of FGD patients [3,7]. Mutations in TXNRD2, has been reported in only one Kashmiri family yet [2]. Mutations in MC2R, MR-AP, STAR and NNT have been found in patients from different ethnic origins. In contrast, only one private mutation for MCM4 has been reported, in patients from an Irish travelling community [8,9] who presented with a more complex phenotype, including natural killer cell deficiency, a DNA repair disorder, and FGD (MIM 609981).

When ACTH resistance is associated with alacrima and achalasia of the cardia resulting in dysphagia, this constitutes a separate condition known as triple A syndrome (AAAS or Allgrove syndrome, MIM 231550) which is caused by mutations in AAAS (MIM 605378).

The NNT gene is located at chromosome 5p12. It consists of 22 exons and codes for a 1086 amino acid protein. NNT is a highly conserved enzyme integrated in the inner mitochondrial membrane. It comprises three domains: two mitochondrial matrix domains and one transmembrane domain (including 14 α-
helixes) that spans the mitochondrial inner membrane (see Fig. 1). The mitochondrial matrix domains 1 and 2 contain the binding sites for NAD/NADH and NADP/NADPH, respectively, and protrude from the inner membrane into the mitochondrial matrix [10]. NNT utilizes the electrochemical proton gradient across the mitochondrial inner membrane to produce high concentrations of NADPH from NADH. NADPH is needed by glutathione peroxidase for the detoxification of reactive oxygen species (ROS) in mitochondria. Thus, NNT deficiency results in decreased NADPH production and impaired ROS detoxification [3,6]. Knockdown of NNT in a human adrenocortical cell line results in increased apoptosis and lowered glutathione peroxidase function. Lower levels of NNT thus make the adrenal cortex more vulnerable to ROS damage caused by defective ROS detoxification.

Here we report on a consanguineous Dutch family in whom a novel homozygous mutation in NNT led to FGD. We then summarize the pathogenic mutations reported in NNT thus far, their localization within the gene and protein domains, and their clinical effect.

Materials and methods

Clinical report

Our patient, the son of consanguineous Caucasian parents (his paternal great-grandmother was a sister of his maternal great-grandfather, see Figure 2A), was born in breech presentation, after an uneventful pregnancy, at a gestational age of 39 weeks. His birth weight was 3104 grams and birth length 48 cm. In the first postnatal months, he had feeding difficulties and vomited frequently. From the age of 4 months, his skin pigmentation increased. His psychomotor development was within the normal range.

At the age of 12 months, he had a long-lasting seizure and a blood glucose level of 1.6 mmol/l. Physical examination revealed mild plagiocephaly, hyperpigmented skin and thin, light blonde hair. He cried with tears, excluding triple A syndrome. His height (78 cm; +0.56 SD), weight (9.7 kg; -1 SD) and head circumference (47.8 cm; +0.4 SD) were within the normal range. He had dark pigmentation of the nipples and external genitalia, a penile length of 5.2 cm (+1.8 SD) and normal testes (2 ml).

Blood sampling for biochemical and endocrinological studies was performed in the morning and revealed normal sodium and potassium concentrations (Na 139 mmol/l, K 4.5 mmol/l), normal urine sodium and potassium levels (Na 17 mmol/l, N 33 mmol/l; measured in a normal hydration state), a low cortisol level (5 nmol/l, normal 50-800 nmol/l) and an elevated ACTH level (> 270 ng/l, normal < 46 ng/l).
Figure 1: Schematic representation of the NNT protein. Amino acid positions and predicted protein domains have been adapted from UniProtKB database (Q13423). Purple circles represent the transit peptide. Blue circles represent NADH- and green circles NADPH-binding sites. Red circles indicate the amino acids where mutations have been reported. Truncating mutations are shown in red boxes and non-truncating mutations in black boxes.

Serum 17-hydroxyprogesterone (0.6 nmol/l, normal 0.5-10 nmol/l) and androstenedione concentrations (0.1 nmol/l, normal < 1 nmol/l) were low-normal, while the plasma renin activity was within the normal range (3.9 nmol/l/h, normal 0.8-4 nmol/l/h). Urinary steroid analysis detected no tetrahydrocortisone and no abnormal steroid metabolites. No antibodies against the adrenal cortex could be detected and serum very long-chain fatty acids were normal. An abdominal ultrasound showed normal adrenal glands for the boy’s age. He was treated with hydrocortisone 13-15 mg/m²/day to normalize his serum ACTH concentrations. He became more active and had no more signs of hypoglycemia or seizures.

At the start of this study, the NNT gene was not firmly associated with FGD and Sanger sequencing in the proband had failed to identify a pathogenic mutation in the MC2R and MRAP genes. Therefore, we performed a SNP analysis to identify regions of identity-by-descent, followed by exome sequencing with special attention paid to variants in the homozygous regions we had identified.
**Molecular studies**

Genomic DNA was extracted from peripheral blood samples from the patient and his parents after informed consent was obtained. SNP array analysis was performed using the HumanCytoSNP-12 v2.1 DNA BeadChip (Illumina, San Diego, CA, USA) and analyzed using GenomeStudio Data Analysis Software and the cnvPartition v3.1.6 algorithm DNA samples were enriched using the SureSelect Human All Exon V2 Kit (Agilent Technologies, Inc., Santa Clara, CA, USA) according to the manufacturer’s instructions and sequenced on an Illumina HiSeq2000. Alignment to the human genome reference (UCSC, hg 19 version, build 37.1) was done using SOAP aligner [11]. Duplicated reads were removed and an average coverage of 71.85 was obtained for the target regions. The Soapsnp software [12] was used to assemble the consensus sequence and to call genotypes in target regions and flanking regions. For insertions or deletions (indels) in the target regions, reads were aligned to the reference genome via the Burrows-Wheeler Aligner [13] and indels were identified by Genome Analysis Toolkit [14]. Variants were annotated using a BGI internal pipeline (BGI, Shenzhen, China). We excluded synonymous variants or those in the 3'-UTR, 5'-UTR, intronic and intergenic regions. Under a recessive model, we retained homozygous variants and compound heterozygous variants in the patient. Finally, we excluded common variants (minor allele frequency \( \geq 0.5\% \)) in both the 1000 Genomes Project and ESP6500). The homozygous variant we identified in the NNT gene was validated by amplification of the relevant part of exon 9 using 5'-GTGAACATAGGGTGATAGAC-3’ and 5’-TCAGCATAAAGCTGGGCATAC-3’ primers and sequencing using standard Sanger sequencing protocols.

**Review of the literature**

All NNT mutations and their clinical phenotypes reported in the literature up to October 12, 2015 were retrieved from PubMed using NNT [Title/Abstract] AND mutation [Title/Abstract] as a query. Subsequently, papers were selected describing patients with adrenal insufficiency due to NNT mutations. Additionally, the ClinVar database (see Web Links) was searched for pathogenic mutations in NNT. Fig. 1 shows the reported disease-causing mutations in NNT and their localization within the protein domains using information adapted from the UniProtKB database (Q13423). One of the reported mutations, c.1A>G, was re-analyzed using ORF Finder (see Web Links). This program predicted that the mutation affected NNT function (supplement figure A).

As part of our review analysis, the age of disease onset among three groups with a different number of truncating alleles in NNT were compared with Kruskal–
Wallis ANOVA. For the two sib-pairs [15] described in Table 1, their mean age of onset was used in this analysis. Truncating mutations were defined as mutations causing a premature termination of transcription, i.e. nonsense and frameshift mutations, while missense mutations were classified as non-truncating. Splice site mutations were categorised depending on the predicted effect (see table 1).

Results
Molecular studies
Genome-wide SNP analysis did not reveal any putative disease-associated copy number variants in the patient. However, we identified 25.7 Mb (~0.8%) copy-neutral regions of homozygosity, including a 6.67 Mb region on chromosome 5 located at 39548842-46228333 bp, between SNP markers rs6870776 and rs7293466 (GRCh19/hg37), and harboring the NNT gene (Fig. 2B). Subsequent exome-sequencing revealed a novel homozygous frameshift mutation in NNT (NM_012343.3: c.1259dupG). This mutation was then confirmed by Sanger sequencing and both parents were found to be heterozygous carriers (Fig. 2C). The mutation is located in exon 9 and creates a frameshift starting at codon His421, leading to a premature stop-codon (p.His421Serfs*4). This particular mutation has not been annotated in dbsNP or mutation databases, including those of the 1000 Genomes Project, the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project, nor the Genome of the Netherlands (see Web Links).

Review of the literature
Our literature review yielded 29 NNT disease-causing mutations in 23 patients with FGD, including our patient, two reported sib-pairs and one unrelated patient with a recurrent mutation, shown to be a founder mutation by Weinberg-Shukron et al. 2015). Fifteen patients from 14 families had homozygous mutations and eight patients from seven families had compound heterozygous mutations (Table 1). Remarkably, almost all mutations are unique and there is a preponderance of missense mutations: 17 missense, 7 frameshift (or predicted-to-be frameshift), and 5 nonsense mutations. For one mutation (c.1A>G; first patient in Table 1), a substitution at the initial methionine, the exact effect is unknown. Applying ORF Finder showed there is no ATG codon upstream of the one used normally in the primary NNT transcript, whereas the closest in-frame ATG codon is located downstream at position c.394, and if used, would result in in-frame translation (supplement figure A). However, if this is the case, the first 131 amino acids of NNT, including the signal peptide (amino acids 1-43), will be missed. As this signal peptide is necessary for importing NNT into the mitochondrial membrane, its
Glucocorticoid deficiency and NNT mutations

absence may result in no functional NNT in mitochondria. Moreover, the first translation-initiating methionine in NNT is an evolutionarily conserved amino acid among human, chimpanzee, rat, mouse, cow, chicken, frog and tetraodon (Alamut Interactive Biosoftware, supplement figure B). Therefore, we categorized the c.1A>G mutation as truncating.

The 29 mutations are scattered over the gene. Figure 1 visualizes their location in the NNT protein. Twenty mutations are located in the mitochondrial matrix domains (the largest part of the protein), seven in the transmembrane regions and two in the transit peptide. None of the 13 mutations in mitochondrial matrix domain 1 are located at the NAD binding site, while two out of the five mutations in mitochondrial matrix domain 2 are at the NADP binding site.

The clinical features of all the reported patients, including ours, are summarized in Table 1. Information on the size of the adrenal gland was only known for three reported patients, all being normal like in our patient. In three of the reviewed families the combination of FGD with mineralocorticoid deficiency occurred. The sib-pair of a consanguineous Palestinian family has a homozygous NNT mutation (c.598 G>A, p.Gly200Ser). The same authors describe a non-related patient with an identical homozygous mutation and shared haplotype, indicating a founder mutation. Combined mineralocorticoid and glucocorticoid deficiency was documented in all three affected individuals [16]. Additionally, combined adrenal failure, precocious puberty and testicular adrenal rest tumor was reported in a patient with another homozygous NNT mutation (c.1163 G>A, p.Tyr388Ser) [17].

For 21 patients, belonging to 19 families, the age of disease presentation was known (Figure 3). The median age of onset was 12 months in the 19 families with a range of 3 days to 39 months in the 21 individual patients. In four out of the 16 families with isolated glycocorticoid deficiency, hyperpigmentation of the skin was reported as the first clinical presentation. The median age of onset in the six families with two truncating mutations was 9 months (range 4-18 months, n=7 patients), in the four families with one truncating and one non-truncating mutation it was 13.5 months (range 7-29 months, n=4 patients), and in the nine families with two non-truncating mutations it was 12 months (range 3 days to 39 months, n=10 patients) (figure 3). These differences are not statistically significant (p=0.5147).

Not surprisingly, the age of onset was earlier in those patients presenting with a combined adrenal insufficiency. However, if we exclude these patients from our analysis the differences in age of onset (9, 13.5 and 15.5 months, respectively) was still not significant (p=0.2161)(see Figure 3, dashed line).
Figure 2: (A) Partial pedigree of our proband with FGD, demonstrating his consanguineous parents. (B) SNP array data showing a 6.67 Mb region of homozygosity in chromosome 5 at chr5: 39548842-46228333 bounded by SNP markers rs6870776 and rs7293466 (GRCh19/hg37). (C) Sequence chromatograms of part of NNT exon 9 obtained by Sanger sequencing and showing a homozygous insertion in the proband. The parents both carry a heterozygous insertion at the same position.
Table 1: All reported NNT mutations and their phenotypes

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Predicted protein effect</th>
<th>Exon/Splice site</th>
<th>Protein domain</th>
<th>Age of onset (months)</th>
<th>Other phenotypic features</th>
<th>Aldosterone level</th>
<th>Renin level</th>
<th>Cortisol mmol/L</th>
<th>ACTH pg/mL</th>
<th>(Ref)</th>
<th>Patient no.</th>
</tr>
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<tbody>
<tr>
<td>c.1A&gt;G hzm</td>
<td>p.Met1?</td>
<td>2</td>
<td>IniMet*</td>
<td>12</td>
<td>U + Febrile convulsions</td>
<td>N</td>
<td>N</td>
<td>Und</td>
<td>1139</td>
<td>(Meimaridou et al. 2012)/8</td>
<td></td>
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<td>c.63delG; c.1864-1G&gt;T</td>
<td>p.Ser22Profs6;  p.Ile622Aspfs*1f</td>
<td>2; intron 13</td>
<td>[Tr;pept]; [TM]</td>
<td>14</td>
<td>U</td>
<td>N</td>
<td>N</td>
<td>&lt;11</td>
<td>&gt;1000</td>
<td>(Meimaridou et al. 2012)/12</td>
<td></td>
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<tr>
<td>c.211C&gt;T</td>
<td>p.Arg71*</td>
<td>3; intron 20</td>
<td>MM1; MM2</td>
<td>6</td>
<td>+ + Coma triggered by infection (at 12 and 21 month age)</td>
<td>↓N</td>
<td>↓</td>
<td>30</td>
<td>109</td>
<td>(Novoselova et al. 2015)/1</td>
<td></td>
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<tr>
<td>c.2996-1186_2996-1183dupAGTA</td>
<td>p.Asp999Glyfs*2f</td>
<td>3; intron 20</td>
<td>MM1; MM2</td>
<td>4</td>
<td>- + Endocrinologically monitored from birth, treated from age 8 months</td>
<td>N</td>
<td>N</td>
<td>38</td>
<td>115</td>
<td>(Novoselova et al. 2015)/2</td>
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<td>p.Ser193Asn</td>
<td>4</td>
<td>MM1</td>
<td>29</td>
<td>U</td>
<td>N</td>
<td>N</td>
<td>1.2</td>
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<td>p.Gly200Ser</td>
<td>4</td>
<td>MM1</td>
<td>3 days</td>
<td>+ + Hypoponeraemia, hyper-kalaemia, Addisonian crisis</td>
<td>↓↑↑</td>
<td>0.55</td>
<td>&gt;1251</td>
<td>(Weinberg-Shukron et al. 2015)/1</td>
<td></td>
<td></td>
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<tr>
<td>c.598 G&gt;A hzm</td>
<td>p.Gly200Ser</td>
<td>4</td>
<td>MM1</td>
<td>27 days</td>
<td>U + Hypoponeraemia, hyper-kalaemia, Addisonian crisis</td>
<td>↓↑↑</td>
<td>&lt;0.69</td>
<td>&gt;1251</td>
<td>(Weinberg-Shukron et al. 2015)/2</td>
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<td></td>
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<tr>
<td>c.644T&gt;C hzm</td>
<td>p.Phe215Ser</td>
<td>5</td>
<td>MM1</td>
<td>11</td>
<td>U + Hypoponeraemia, hyper-kalaemia, Addisonian crisis, Hypotension Very mild secondary and transient mineralo-corticoid disturbance, normalized after gluco-corticoid treatment</td>
<td>↓↑↑</td>
<td>&lt;27.6</td>
<td>&gt;5000</td>
<td>(Weinberg-Shukron et al. 2015)/3</td>
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<tr>
<td>c.600_601delG hzm</td>
<td>p.Tyr201Lysfs*1f</td>
<td>intron 4</td>
<td>[MM1]</td>
<td>18</td>
<td>+ +</td>
<td>N</td>
<td>N</td>
<td>&lt;5</td>
<td>1460</td>
<td>(Meimaridou et al. 2012)/2</td>
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<tr>
<td>Mutation</td>
<td>Predicted protein effect</td>
<td>Protein domain</td>
<td>Hypophysectomy</td>
<td>Hypopituitarism</td>
<td>Other phenotypic features</td>
<td>Aldosterone level</td>
<td>Initial Ref.</td>
<td>Cortisol level</td>
<td>ACTH level</td>
<td>(Ref) / Patient no.</td>
<td></td>
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<tr>
<td>c.1069A&gt;G; c.2637delA</td>
<td>p.Thr357Ala; p.Met880*</td>
<td>8; 18</td>
<td>MM1; [MM2]</td>
<td>7</td>
<td>+</td>
<td>Seizure following a cold</td>
<td>N</td>
<td>N</td>
<td>&lt;0.6</td>
<td>3412 (Meimaridou et al. 2012)/13</td>
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<tr>
<td>c.1094A&gt;C hnz</td>
<td>p.His365Pro</td>
<td>8</td>
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<td>12</td>
<td>U</td>
<td>U</td>
<td>Hypotonia, febrile</td>
<td>N</td>
<td>N</td>
<td>4.6</td>
<td>654 (Meimaridou et al. 2012)/6</td>
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<tr>
<td>c.1107_1110delTCA; c.3027T&gt;G</td>
<td>p.His370*; p.Asn1009Lys</td>
<td>9; 21</td>
<td>[MM1]; NADP</td>
<td>12</td>
<td>U</td>
<td>U</td>
<td>Exhausted, feeding difficulties</td>
<td>N</td>
<td>N</td>
<td>310</td>
<td>††</td>
</tr>
<tr>
<td>c.1163A&gt;C hnz</td>
<td>p.Tyr388Ser</td>
<td>9</td>
<td>MM1</td>
<td>10</td>
<td>U</td>
<td>+</td>
<td>Hyponatremia, hyper-kalaemia, vomiting, Addisonian crisis, testicular adenoma, rest tumor, precocious puberty</td>
<td>Und*</td>
<td>N*</td>
<td>&lt;17</td>
<td>1030 (Hershkovitz et al. 2015)</td>
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<tr>
<td>c.1259dupG hnz</td>
<td>p.His421Serfs*4</td>
<td>9</td>
<td>[MM1]</td>
<td>4</td>
<td>+</td>
<td>Feeding difficulties, vomiting, seizure</td>
<td>U</td>
<td>N</td>
<td>5</td>
<td>&gt;270 Present patient</td>
<td></td>
</tr>
<tr>
<td>c.1310C&gt;T; c.1669C&gt;T</td>
<td>p.Pro437Leu; p.Gln557*</td>
<td>10; 12</td>
<td>MM1; [TM1]</td>
<td>15</td>
<td>+</td>
<td>+</td>
<td>N</td>
<td>N</td>
<td>††</td>
<td>(Meimaridou et al. 2012)/4</td>
<td></td>
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<tr>
<td>c.1355delA hnz</td>
<td>p.Gln452Argfs*45</td>
<td>10</td>
<td>[MM1]</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>N</td>
<td>N</td>
<td>††</td>
<td>(Meimaridou et al. 2012)/10</td>
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<tr>
<td>c.1598C&gt;T hinz</td>
<td>p.Ala533Val</td>
<td>11</td>
<td>TM</td>
<td>9</td>
<td>+</td>
<td>+</td>
<td>N</td>
<td>54</td>
<td>624 (Meimaridou et al. 2012)/1</td>
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<tr>
<td>c.2930T&gt;C hnz</td>
<td>p.Leu977Pro</td>
<td>20</td>
<td>MM2*</td>
<td>U</td>
<td>U</td>
<td>U</td>
<td>N</td>
<td>N</td>
<td>††</td>
<td>(Meimaridou et al. 2012)/3</td>
<td></td>
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<tr>
<td>c.3022G&gt;C hnz</td>
<td>p.Ala1008Pro</td>
<td>21</td>
<td>NADP</td>
<td>39</td>
<td>+</td>
<td>U</td>
<td>Seizures</td>
<td>N</td>
<td>N</td>
<td>Und</td>
<td>&gt;1000 (Meimaridou et al. 2012)/14</td>
</tr>
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</table>

Patients in italic are sib pairs, Patients in **bold** have combined gluco- and mineralocorticoid deficiency. *The position of each mutation is given as the number of bases from the start codon of the transcript NM_012343.3; **predicted NNT protein domains according to UniProtKB database (Q13423), between square brackets: truncated transcript, most likely resulting in nonsense mediated decay, for explanation of domains (see Fig. 1); † Levels before treatment, normal reference values: for aldosteron and renin dependent on age of the patient and assay used, therefore interpretation of original authors is indicated (arrows), for cortisol >200 nmol/l, for ACTH 0-50 pg/ml; ‡ predicted effect not known; § hyperpigmentation of the skin reported as first clinical feature; †† predicted result if exon skipped; ‡‡ plasma renin activity and aldosterone levels measured after steroid withdrawal under intravenous fluid administration; ** between two protein parts of the NADP binding site (see Fig. 1). Abbreviations: hnz=homozygous, MM=molecular matrix, N=normal, NADP=NADP binding site, TM=transmembrane domain, U=unknown, Und = undetectable.
Discussion
We identified a novel frameshift mutation in NNT in a Dutch patient with FGD. The patient showed hypoglycemia and hyperpigmentation, similar to what is observed in other patients with a NNT mutation (see Table 1) [6]. DNA diagnostics for NNT was at that time not available in the Netherlands. However, the mutation could easily be identified thanks to the combination of SNP haplotyping and whole exome sequencing in this distantly-related family. Especially in clinical phenotypes with heterogeneous causes, whole exome sequencing has proven to be a very powerful diagnostic approach [18,19].

The question is whether based on phenotype a clinical distinction can be made between the different causes of FGD. Clinically, cortisol deficiencies caused by mutations in MCM4 and AAAS are not pure FGD syndromes. They both have additional features: natural killer cell deficiency and short stature in the MCM4-related syndrome, and alacrima with achalasia in the AAAS-related syndrome. Patients with a disease-causing mutation in STAR usually present with lipoid congenital adrenal hyperplasia (LCAH, MIM 201701), which is a more severe phenotype than FGD. These patients have adrenal and gonadal insufficiency, with high ACTH and renin levels, and low cortisol and aldosterone levels. As a consequence, a failure of androgenization occurs in patients with a 46,XY karyotype, resulting in complete or partial sex reversal. Atypical or mild LCAH may present as FGD [4,20]. Similar to nonclassic LCAH, a partial defect in CYP11A1 may also lead to misdiagnosis of FGD [5,21].

FGD due to MRAP and MC2R mutations is clinically indistinguishable from FGD due to NNT mutations, although it has been suggested, but not yet confirmed, that obesity may be part of the phenotype in MRAP mutations [22]. Obesity was not reported in the patients with NNT or MC2R mutations.

Chung et al. [23] showed that MRAP-related FGD (n=22) usually has an earlier age of onset (median 1 month; range birth – 1.6 years) than FGD due to MC2R mutations (n=40, median 2 years, range 1 week – 16 years). In the 21 patients with NNT mutations and an available age of onset presented here, the median age of onset was 12 months (range 3 days – 3.25 years), thus falling in-between the MRAP- and MC2R-related phenotypes.
Type of mutations in NNT-related FGD

Following the observed 31% residual NNT activity in a Japanese patient due to a homozygous non-truncating mutation (c.644 T>C; F215S) we studied the relationship between age of disease onset and type of NNT mutations. Since truncating mutations are most likely to result in no protein being produced at all, it was not surprising that they were located throughout the NNT gene. The NNT non-truncating mutations, however, were often located in or near important domains that were likely to affect the function of the NNT enzyme (four out of 14 were near binding sites and four were within the transmembrane domain). However, the age of disease onset between truncating and non-truncating NNT mutations was not statistically significant (Figure 3) and it was not possible to determine a reliable correlation between the type of NNT mutations and cortisol or ACTH levels. Although the observed residual enzyme activity in the Japanese patient who carried a homozygous non-truncating mutation may support the idea that non-truncating mutations delay the age of disease presentation, we cannot extend this finding to all non-truncating mutations. A possible explanation for this is that the amount of residual NNT activity might be highly dependent of the location of the mutation within the protein. The age of disease onset may also depend on additional comorbidity, such as the combined mineralocorticoid insufficiency, usually resulting in symptoms occurring earlier in life, as illustrated in Figure 3. The missense mutations resulting in mineralocorticoid deficiency were located in the mitochondrial matrix I domain and both resulted in a Serine amino acid. However, a similarly located missense mutation resulting in a Serine residue was found in a patient without mineralocorticoid deficiency (age of onset 19 months).

A limiting factor of our study is the small patient cohort and larger groups of patients are needed to investigate whether the type of NNT mutation indeed is correlated with age of disease onset.

Phenotypic heterogeneity

Among the reviewed patients are four patients from three families with two different homozygous missense mutations and the combination of mineralocorticoid and glucocorticoid deficiency [16-17]. Mineralocorticoid deficiency was excluded in our patient (plasma renin and sodium were last checked at the age of 6 years) and not reported in any of the other patients.
Glucocorticoid deficiency and NNT mutations

Figure 3: NNT mutations and age of disease presentation.
Age of onset of disease in patients with (AA) two truncating mutations (●), (AB) one truncating and one non-truncating mutation (■), and (BB) two non-truncating mutations (▲). The solid symbols represent patients with isolated glucocorticoid deficiency, while the open symbols (∆) are patients with combined glucocorticoid and mineralocorticoid deficiency. The solid horizontal line represents the median age in each group (for sib pairs the mean age was used when calculating the median age of onset). In column BB, the dashed line represents the median age when patients with combined adrenal deficiency were excluded from the analysis. The difference in age of presentation between groups (AA, AB and BB) were not significant according to Kruskal-Wallis test with and without combined mineralocorticoid and glucocorticoid deficiency; the p-values were 0.51 and 0.22, respectively.

in any of the other patients (see Table 1). As explained above, we could not identify a genetic explanation for this broader adrenal phenotype, e.g. the missense mutations were not located at a specific functional domain. The patient reported by Hershkovitz also had a testicular adrenal rest tumor in combination with testicular enlargement, precocious virilization and skin hyperpigmentation. The symptoms regressed after intensification of glucocorticoid treatment. It should be noted that chronic elevation of ACTH may result in adrenal rest tumors, which may regress when glucocorticoid therapy is intensified. Although this is not specific for FGD, it is worth mentioning and has implications for the surveillance of patients with an NNT mutation.

From recent reports it can be concluded that the phenotypic spectrum of NNT mutation is not restricted to FGD but may also include combined adrenal insufficiency and extra-adrenal manifestations like gonadal adrenal rest tumors. Moreover, an association with left ventricular noncompaction (LVNC) was recently reported for heterozygous NNT loss of function mutations [24]. The authors identified a single allele NNT mutation in two patients presenting with LVNC, without adrenal manifestations. They showed that suppression of Nnt in zebrafish caused early ventricular malformation and contractility defects. However, cardiac problems were not described in the patients listed in Table 1 who had biallelic mutations in NNT. Our patient had no features of a cardiac disease, a good physical
condition and a normal heart contour on an X-ray of the thorax at the age of 5 years. No cardiac disease has been documented in the families of the reviewed patients, however under-reporting is very likely. More clinical studies are needed to investigate the prevalence of LVNC in heterozygous carriers of an NNT mutation.

Altogether, these recent findings in NNT mutation patients expand our knowledge of the phenotypic spectrum of NNT mutations and this has implications for the surveillance of these patients. They should be closely monitored for combined adrenal insufficiency and extra-adrenal manifestations, e.g. precocious puberty, adrenal rest tumors, possibly cardiac manifestations and other symptoms we still may not be aware of being associated with NNT mutations. The diagnosis of FGD due to NNT mutations is also important for the family, to check newborns at an early age, preventing serious illness or death due to an Addisonian crise, or preventing cerebral damage due to hypoglycemia and hypotension.

**NNT pathomechanism in FGD**

Although NNT is widely expressed in humans – with its expression most readily detectable in adrenal, heart, kidney, thyroid and adipose tissues [6] – we do not know why NNT mutations apparently only affect adrenocortical cells. Production of ROS within mitochondria is one of the major internal triggers for apoptotic cell death [25]. Mice carrying Nnt mutations show slightly disorganized zona fasciculata with higher levels of apoptosis than wild-type mice [6]. As Yamaguchi et al. suggested, the adrenocortical cells of the zona fasciculata that produce a large amount of cortisol may be specifically vulnerable to oxidative stress caused by impaired redox potential and increased ROS [7]. Weinberg-Shukron et al. were able to provide patient-based evidence that NNT mutations indeed increase cellular oxidative stress and impair mitochondrial function and morphology [16]. They observed that ROS levels were 40% higher in NNT_p.Gly200Ser homozygous fibroblasts compared with control fibroblasts. They also found a significant reduction in ATP content (25% less than in healthy control). Additionally, in 50% of the patients’ cells the mitochondria had a pathological punctate appearance which is known to be related to various apoptotic stimuli [26]. It is possible that functional compensation by overlapping antioxidant defense mechanisms protects other cell types or tissues in patients with NNT mutations [27].

**Conclusion**

We have identified a novel NNT mutation in a Dutch patient with FGD, using SNP haplotyping and exome sequencing, an efficient approach in the heterogenous and
clinically not easily distinguishable group of FGD disorders. We also synthesized the, still limited, data on FGD caused by NNT mutations to date and noticed a recent broadening of the phenotype. As a consequence, patients who carry NNT mutations should be closely monitored for likely extra-adrenal manifestations. We showed that the type of mutation is not apparently correlated with age of disease onset. However, larger studies are needed to further investigate this.

**Web Links**

1000 Genomes Project: [http://www.1000genomes.org/](http://www.1000genomes.org/)
Genome of the Netherlands: [http://www.genoomvannederland.nl/](http://www.genoomvannederland.nl/)
References


Chapter 3


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Supplementary materials
**Supplement figure A:** ORF Finder result for NNT transcript (NM_012343.3). The yellow boxes display first ATG start codon and second in-frame potential ATG, respectively. The red boxes represent three out-of-frame potential translational start sites which might result in non-functional NNT. The green box shows a transit peptide (amino acids 1-43).

**Supplement figure B:** Evolutionary conservation of the M1 residue translation-initiating methionine in NNT is outlined in red (extracted from Alamut Interactive Biosoftware).