**NNT** mutations: a cause of primary adrenal insufficiency, oxidative stress and extra-adrenal defects

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**Abstract**

**Objective:** Nicotinamide nucleotide transhydrogenase (**NNT**), one of the several genes recently discovered in familial glucocorticoid deficiencies (FGD), is involved in reactive oxygen species detoxification, suggesting that extra-adrenal manifestations may occur due to the sensitivity to oxidative stress of other organs rich in mitochondria. Here, we sought to identify **NNT** mutations in a large cohort of patients with primary congenital adrenal insufficiency without molecular etiology and evaluate the degree of adrenal insufficiency and onset of extra-adrenal damages.

**Methods:** Sanger or massive parallel sequencing of **NNT** and patient monitoring.

**Results:** Homozygous or compound heterozygous **NNT** mutations occurred frequently (26%, 13 unrelated families, 18 patients) in our cohort. Seven new mutations were identified: p.Met337Val, p.Ala863Glu, c.3G>A (p.Met1?), p.Arg129*, p.Arg379*, p.Val665Profs*29 and p.Ala704Serfs*19. The most frequent mutation, p.Arg129*, was found recurrently in patients from Algeria. Most patients were diagnosed belatedly (8–18 months) after presenting severe hypoglycemia; others experiencing stress conditions were diagnosed earlier. Five patients also had mineralocorticoid deficiency at onset. One patient had congenital hypothyroidism and two cryptorchidism. In follow-up, we noticed gonadotropic and genitalia impairments (precocious puberty, testicular inclusions, interstitial Leydig cell adenoma, azoospermia), hypothyroidism and hypertrophic cardiomyopathy. Intrafamilial phenotype heterogeneity was also observed.

**Conclusions:** **NNT** should be sequenced, not only in FGD, but also in all primary adrenal insufficiencies for which the most frequent etiologies have been ruled out. As **NNT** is involved in oxidative stress, careful follow-up is needed to evaluate mineralocorticoid biosynthesis extent, and gonadal, heart and thyroid function.
Introduction

Primary adrenal insufficiency (PAI) is a life-threatening disorder. Three types of PAI can occur: isolated mineralocorticoid deficiency, isolated glucocorticoid deficiency or combined mineralocorticoid and glucocorticoid deficiency (global adrenal insufficiency). Glucocorticoid deficiencies, also called adrenocorticotrophic hormone (ACTH) resistance syndromes, are autosomal recessive disorders. They include familial glucocorticoid deficiency (FGD) (OMIM#202200) and triple A syndrome (AAAS) (OMIM#231550), also known as Allgrove syndrome (1, 2). Patients present episodes of hypoglycemia in the neonatal period or early childhood with low or unquantifiable cortisol, elevated ACTH levels, and normal aldosterone and plasma renin measurements. Until 2012, only a half of FGD cases could be explained by homozygous or compound heterozygous mutations in genes involved in the steroidogenic pathway: MC2R (25%), MRAP (20%), STAR (5%), and more rarely CYP11A1 (3, 4, 5, 6, 7). Over the last 3 years, thanks to whole exome sequencing, CYP11A1 mutations were identified in 50 families with PAI with no identified molecular etiologies and to perform a careful follow-up so as to identify any extra-adrenal defects. We found 13 families et al. (3) was around 10% (15 families) and no predominant mutation was reported (21 private mutations) (9). Twelve more families have been reported (12 additional mutations), some with mineralocorticoid defects (11, 12, 13, 14, 15, 16).

NNT encodes an integral protein of the inner mitochondrial membrane that acts as a proton pumping transhydrogenase (17). In prokaryotic cells, the enzyme is composed of two or three different subunits, whereas in eukaryotic cells, it is usually composed of a single subunit. The active form of the enzyme is a homodimer of ~220 kDa. All NNTs show a similar structure with three major domains. Domain I contains the hydrophilic NAD(H) binding site and domain III contains the hydrophilic NADP(H) binding sites. Domain II constitutes the hydrophobic transmembrane part of the enzyme that connects domains I and III and forms the proton channel (18). NNT supplies the high concentrations of NADPH needed for glutathione and thioredoxin antioxidant systems involving enzymes such as GPX1 (glutathione peroxidase 1), TXNRD2 and PRDX3 (peroxiredoxin 3). NADPH is a cofactor of P450 enzymes, notably in steroidogenesis (19, 20, 21, 22) (Fig. 1).

Meimaridou et al. (9) showed reduced, basal and ACTH-stimulated corticosterone, revealing impaired steroidogenesis in C57BL/6J mice with a spontaneous NNT mutation (an in-frame 5 exon deletion). Furthermore, they showed increased ROS levels in an NNT knockdown human adrenocortical cell line. Oxidative stress impedes steroidogenesis, which in turn induces more oxidative stress resulting from electron leaks throughout the steroidogenic pathway. Why it affects adrenal hormone production preferentially remains unknown. All tissues rich in mitochondria may be affected, resulting in a wide spectrum of diseases. Phenotypically, C57BL/6J mice do not have adrenal defects but show glucose intolerance and impaired insulin secretion (23). At present, in humans, NNT mutations are known to be associated with adrenal insufficiency. Additionally, relationships between decreased NNT activity, modified mitochondrial redox regulation and cardiac failure have been recently reported (24, 25, 26).

The aim of our study was to screen for NNT mutations in 50 families with PAI with no identified molecular etiologies and to perform a careful follow-up so as to identify any extra-adrenal defects. We found 13 families
(18 patients) with NNT mutations: 13 patients were diagnosed with FGD and 5 with global adrenal insufficiency at onset. A range of functions, such as adrenal/mineralocorticoid, puberty, fertility, heart, pancreatic, thyroid and growth, were subjected to long-term monitoring.

**Patients and methods**

**Patients**

The NNT gene was analyzed in 50 patients with PAI with no molecular diagnosis. Informed consent was provided by all enrolled patients and the study was conducted in accordance with the principles of the Declaration of Helsinki. Very long-chain fatty acids in boys and 17-hydroxyprogesterone in all patients were either within normal limits or low, excluding adrenoleukodystrophy and 21-hydroxylase deficiency, and adrenal autoantibodies were negative, excluding an autoimmune disorder. Mutations in STAR, CYP11A1, MC2R and MRAP were excluded by Sanger sequencing, as were those in NR0B1 for boys.

**Molecular genetic analysis of the NNT gene**

Genomic DNA was extracted from EDTA-preserved whole blood using the Nucleon BACC3 kit (GE Healthcare). Sanger sequencing was done for 47 patients and massive parallel sequencing (MPS) for three (patients 11, 12 and 13 in Table 1).

**Sanger sequencing**

Selective amplification of the 21 coding exons of the NNT gene was performed in 20 fragments by PCR using specific primers (available on request). Conventional dideoxy sequencing of exons and exon-intron boundaries was done using Big-Dye Terminators. Sequencing products were loaded on an ABI-3730XL and analyzed using SeqScape software v2.5 (Life Technologies). Sequence variants were designated according to the Human Genome Society recommendations (www.hgvs.org/rec.html) using the National Center for Biotechnology Information (NCBI) reference sequences NC_000005.9, NM_012343.3 and NP_036475 built on the GRCh37/hg19.

**Array comparative genomic hybridization and long-range PCR**

To confirm a deletion, array comparative genomic hybridization (aCGH) or chromosomal microarray (CMA) was performed according to the manufacturer’s instructions, using the Agilent SurePrint G3 Human CGH Microarray 4x180K AMADID 022060 (Agilent Technologies). This was followed by long-range PCR using the Qiagen LongRange PCR Kit (Qiagen) according to the supplier’s recommendations. Conventional dideoxy sequencing of the PCR product was done as described in the section ‘Sanger sequencing’ (primers available on request).

**Pathogenicity prediction**

**Multiple sequence alignment**

Multiple sequence alignment of NNT protein sequences from different species was used to analyze structurally conserved regions and to predict putative effects of missense mutations. The sequences were found in the Uniprot database (http://www.uniprot.org/), aligned with ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default parameters, displayed and then edited using Genedoc (http://www.psc.edu/index.php/user-resources/software/genedoc).
### Table 1  
**NNT mutations in 13 families with PAI.**

<table>
<thead>
<tr>
<th>Nucleotide change (NM_012343.3)</th>
<th>Exon</th>
<th>Protein change (NP_036475)</th>
<th>Protein consequence</th>
<th>Domain</th>
<th>Predictive software</th>
<th>Mutation Taster</th>
<th>dbSNP ID</th>
<th>ESP</th>
<th>ExAC</th>
<th>Allele count</th>
<th>Family number</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.3G&gt;A</td>
<td>2</td>
<td>p.M17</td>
<td>Start loss</td>
<td></td>
<td>Pre-sequence</td>
<td>NA</td>
<td>0/13006</td>
<td>0/121286</td>
<td>1</td>
<td>4, 12</td>
<td>(9, 14)</td>
<td></td>
</tr>
<tr>
<td>c.211 C&gt;T</td>
<td>3</td>
<td>p.R71*</td>
<td>Premature truncation at amino acid 71. NMD?</td>
<td></td>
<td></td>
<td>NA</td>
<td>0/13006</td>
<td>0/121286</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.385 C&gt;T</td>
<td>4</td>
<td>p.R129*</td>
<td>Premature truncation at amino acid 129. NMD?</td>
<td></td>
<td>dl</td>
<td>NA</td>
<td>0/13002</td>
<td>0/121286</td>
<td>2, 3, 4, 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1009A&gt;G</td>
<td>8</td>
<td>p.M337V</td>
<td>Missense mutation at amino acid 337 in the DH binding domain protein</td>
<td></td>
<td>dl</td>
<td>Less likely Deleterious Probably damaging Disease causing</td>
<td>0/13006</td>
<td>0/121286</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1135C&gt;T</td>
<td>9</td>
<td>p.R379*</td>
<td>Premature truncation at amino acid 379. NMD?</td>
<td></td>
<td>dl</td>
<td>NA</td>
<td>0/13006</td>
<td>0/121286</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>c.1310C&gt;T</td>
<td>10</td>
<td>p.P437L</td>
<td>Missense mutation at amino acid 437 Role in dI-dII/dIII communication</td>
<td></td>
<td>dl</td>
<td>Less likely Deleterious Probably damaging Disease causing</td>
<td>0/13006</td>
<td>1/120146</td>
<td>10</td>
<td>(9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.1992_2005del</td>
<td>14</td>
<td>p.V665P6*29</td>
<td>Frameshift: premature truncation at amino acid 694</td>
<td></td>
<td>dl TMH7</td>
<td>NA</td>
<td>0/13006</td>
<td>0/121286</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2106_2109dup</td>
<td>15</td>
<td>p.A704Sf*19</td>
<td>Frameshift: premature truncation at amino acid 723</td>
<td></td>
<td>dl TMH9</td>
<td>NA</td>
<td>0/13006</td>
<td>0/121286</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.2588C&gt;A</td>
<td>17</td>
<td>p.A863E</td>
<td>Missense mutation at amino acid 863 in the transmembrane domain (helix 14)</td>
<td></td>
<td>dl TMH14</td>
<td>Most likely Deleterious Probably damaging Disease causing</td>
<td>0/13006</td>
<td>0/121286</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c.(-51+-53-1) (381=1_382-1)del</td>
<td>2-3</td>
<td>p.0?</td>
<td>Start loss. Absence of protein</td>
<td></td>
<td></td>
<td></td>
<td>0/121286</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

d, domain; TMH, transmembrane helix; NA, not applicable; NMD, nonsense-mediated decay.
Software and databases

For each new missense mutation, pathogenicity was predicted in silico using several programs: Align-GVGD, Polyphen-2, SIFT and Mutation Taster. The Grantham score was calculated to predict the effect of substitutions between amino acids. This score looks at chemical properties to define a score range between 0 and 215. Higher scores indicate greater differences between two amino acids in the chemical properties (i.e. polarity and molecular volume) and may indicate a stronger (negative) effect on protein structure and function. The dbSNP, EVS and ExAC browser databases were searched to determine if variants had already been reported.

Results

NNT gene sequencing

Ten different NNT mutations, scattered throughout the gene, were found in 13 families (18 patients) (Fig. 2 and Table 1). Seven of them were new mutations: two nonsense (p.Arg129* and p.Arg379*), two missense (p.Met337Val and p.Ala863Glu), two frameshift (p.Val665Profs*29 and p.Ala704Sersfs*19), and one start loss (c.3G>A (p.Met1?). The p.Arg129* mutation was found in four families, all of Algerian origin (Table 2). Consanguinity was present in 8 of the 13 families and homozygous mutations were found in 11 families. No consanguinity was found in the other homozygous families (1, 5 and 10), but the parents in family 10 were from the same small village in France. The patients of families 4 and 12 were compound heterozygotes. Patient 12 was first thought homozygous for the mutation p.Arg71*. However, the mother did not carry the mutation and thus a deletion was suspected and thereafter confirmed by aCGH analysis and long-range PCR sequenced step by step. For the three patients studied by MPS, all variants found in other genes were benign.

Pathogenicity prediction

The p.Pro437Leu and p.Arg71* mutations have already been described (9, 15). For the frameshift or nonsense mutations, the consequences should be premature truncated proteins or an absence of protein due to intervention of the nonsense-mediated decay system. The new mutation, c.3G>A (p.Met1?), affecting the translation initiation site, should switch this latter to an in-frame downstream methionine at codon 192. In the absence of its N-terminal part, the resulting NNT should be non-functional.

To predict the pathogenicity of missense mutations, multiple alignments of NNT proteins were done in order to locate the changed residue in the protein structure and identify conservation between species (18, 27) (Fig. 3). The p.Ala863Glu mutation is located in the transmembrane helix 14 (H14) of domain II and is highly conserved between species (Fig. 3). H14 appears to

Figure 2

NNT mutations. Comparison between the domain structure of NNT protein and NNT exons in humans. Above: nonsense or frameshift mutations; below: missense mutations. New mutations indicated by rectangles. Underlined mutation is probably a splicing mutation. A full colour version of this figure is available at http://dx.doi.org/10.1530/EJE-16-0056.
<table>
<thead>
<tr>
<th>Family</th>
<th>Origin</th>
<th>Consanguinity</th>
<th>NNT mutation</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Clinical data at age of diagnosis</th>
<th>Mineralocorticoid defect</th>
<th>Gonads</th>
<th>Heart</th>
<th>Thyroid</th>
<th>Related death</th>
</tr>
</thead>
<tbody>
<tr>
<td>2a</td>
<td>Algerian</td>
<td>Yes</td>
<td>p.R129*/p.R129*</td>
<td>M</td>
<td>32</td>
<td>Hypoglycemic convulsions, SW</td>
<td>MC ttt at 3 years (∆ renin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2b</td>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>15</td>
<td>Hypoglycemic convulsions, SW</td>
<td>MC ttt, SW at 12 years after an attempt to stop MC ttt</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Algerian</td>
<td>Yes</td>
<td>p.R129*/p.R129*</td>
<td>F</td>
<td>19</td>
<td>Hypoglycemia, asthenia, melanoderma</td>
<td>No MC ttt but 4 adrenal crises with ∆ renin \ aldosterone (4, 7, 8 and 10 years) SW at 15 and 18 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td>French/ Algerian</td>
<td>No</td>
<td>p.R71*/p.R129*</td>
<td>F</td>
<td>30</td>
<td>Hypoglycemic convulsions following gastroenteritis</td>
<td>MC ttt at 2 years (∆ renin)</td>
<td></td>
<td>Menarche (11 years), two children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4b</td>
<td></td>
<td></td>
<td>p.R71*/p.R129*</td>
<td>F</td>
<td>23</td>
<td>Hypoglycemic convulsions following gastroenteritis</td>
<td>No MC ttt but ∆ renin</td>
<td></td>
<td>-</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>French</td>
<td>No</td>
<td>p.V665Pf*/p.V665Pf*</td>
<td>M</td>
<td>14</td>
<td>Hypoglycemic convulsions following gastroenteritis</td>
<td>No</td>
<td></td>
<td>Onset of puberty at 12 years, low testosterone at 13 years</td>
<td>Ni imaging (11 years)</td>
<td></td>
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<tr>
<td>6</td>
<td>Turkish</td>
<td>Yes</td>
<td>p.A7045fs*/p.A7045fs*</td>
<td>F</td>
<td>18</td>
<td>Hypoglycemia, asthenia, melanoderma, weight loss, fever, SW</td>
<td>MC ttt</td>
<td></td>
<td>Menarche (12.5 years)</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>7</td>
<td>Moroccan</td>
<td>Yes</td>
<td>p.A863E/p.A863E</td>
<td>M</td>
<td>13</td>
<td>Hypoglycemic convulsions</td>
<td>No</td>
<td></td>
<td>Precocious puberty with 3 nodular testis</td>
<td>Ni imaging (4 years)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Mauritian</td>
<td>yes</td>
<td>p.M17/p.M17</td>
<td>F</td>
<td>8</td>
<td>Hypoglycemic coma, SW</td>
<td>MC ttt</td>
<td></td>
<td>No pubertal symptoms</td>
<td>-</td>
<td>No</td>
</tr>
<tr>
<td>Family</td>
<td>Origin</td>
<td>Consanguinity</td>
<td>NNT mutation NP_036475</td>
<td>Sex</td>
<td>Age (years)</td>
<td>Age at diagnosis (months or years where indicated)</td>
<td>Clinical data at age of diagnosis</td>
<td>Mineralocorticoid defect</td>
<td>Gonads</td>
<td>Heart</td>
<td>Thyroid</td>
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</tr>
<tr>
<td>9</td>
<td>Algerian</td>
<td>Yes</td>
<td>p.R379*/p.R379*</td>
<td>M</td>
<td>10</td>
<td>22 (onset)/8 years</td>
<td>Misdiagnosed at 22 months (several hyperthermic convulsions, psychomotor retardation, sodium valproate ttt), all symptoms improved after glucocorticoid therapy initiated at 8 years</td>
<td>No</td>
<td>Leydig cell adenoma (5 years) following by precocious puberty</td>
<td>–</td>
<td>–</td>
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<tr>
<td>10a</td>
<td>French</td>
<td>No</td>
<td>p.P437L/p.P437L</td>
<td>M</td>
<td>57</td>
<td>4 years</td>
<td>Melanoderma, asthenia Salt craving, MC ttt</td>
<td>NI puberty</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10b</td>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>51</td>
<td>18</td>
<td>Melanoderma, asthenia Salt craving, MC ttt</td>
<td>NI testicular function at 49 years</td>
<td>–</td>
<td>–</td>
<td>NI imaging (51 years)</td>
</tr>
<tr>
<td>10c</td>
<td></td>
<td></td>
<td></td>
<td>F</td>
<td>4*</td>
<td>16</td>
<td>Familial story deceased at 4 years</td>
<td>MC ttt</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11a</td>
<td>Algerian</td>
<td>Yes</td>
<td>p.R129*/p.R129*</td>
<td>M</td>
<td>6</td>
<td>2</td>
<td>SW (2 months, 4 years), MC ttt</td>
<td>No</td>
<td>Cryptorchidism (surgery)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11b</td>
<td></td>
<td></td>
<td></td>
<td>M</td>
<td>8</td>
<td>4 years</td>
<td>Hypoglycemia (NI at 3 years)</td>
<td>SW, MC ttt</td>
<td>Cryptorchidism (surgery)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>12</td>
<td>French</td>
<td>No</td>
<td>p.R71*del ex2-3</td>
<td>M</td>
<td>35</td>
<td>10</td>
<td>SW</td>
<td>No</td>
<td>Cryptorchidism (surgery)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>Algerian</td>
<td>Yes</td>
<td>p.M337V/ p.M337V</td>
<td>F</td>
<td>9</td>
<td>8</td>
<td>Melanoderma, asthenia i↑ renin, ↘ aldosterone (16 months, 12 years), salt, no MC ttt</td>
<td>No</td>
<td>No pubertal symptoms</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Major abnormalities are in bold. For two patients (4a and 9), the age of diagnosis was late despite an earlier onset. –, not determined; M, male; F, female; NI, normal; *, deceased; ttt, treatment; MC, mineralocorticoid; GC, glucocorticoid; SW, salt wasting; ↘, decreased; i↑, increased; LVEF, left ventricular fraction; ENT, ear, nose, throat.
indirectly facilitate proton translocation by influencing the centrally located H9, H10 and H13, in which the proton channel is assumed to be located. In *Escherichia coli*, mutations of certain residues in these regions result in intermediate inhibitory effects (28). This mutation may disrupt the conformational changes responsible for interconversion of the open and occluded states (29). It may also play a role in coupling between the redox state of the nucleotide and the proton movement in the protein, as it is near the NADP(H) binding domain. In *in silico* predictions, this mutation was most likely pathogenic using Align-GVGD class, probably damaging using Polyphen-2, deleterious using SIFT, and disease causing using Mutation Taster. The high Grantham score of 107 is also concordant. This mutation is not reported in dbSNP, EVS or ExAC browser databases and has not been found in 100 healthy controls from the Maghreb (Table 1).

**Clinical data at onset and follow-up**

Table 2 presents the clinical data and follow-up for the 18 patients reported. Only the predominant features are presented in the text.

**Clinical presentation at age of diagnosis**

Severe hypoglycemia, sometimes leading to coma, was the main symptom at age of diagnosis in all but two patients (numbers 11a and 12). That symptom was often associated with infections and melanoderma. This latter symptom, upon inquiry, was often reported to have been present before the hypoglycemia. Five out of the 13 families had experienced multiple deaths of other children; although not diagnosed at the time, those deaths too were probably due to adrenal insufficiency and severe hypoglycemia. Patient 11a and 12 experienced salt wasting (SW) at onset without hypoglycemia and three other patients (patients 2b, 6, and 8) with SW. The median age at onset in our cohort was 11.5 months (min–max: 1.5 months–4 years) (Fig. 4A and Table 2). Most cases were diagnosed belatedly around the first year of life (8–18 months), but some involving stress conditions were diagnosed earlier. A difference in age at onset was detected between the subgroup with isolated glucocorticoid deficiency and that with global adrenal insufficiency. This was the case for both our cohort alone (Kruskal–Wallis test: \( P = 0.03379 \), Fig. 4B) and our cohort aggregated with the data available in the literature (Kruskal–Wallis test: \( P = 0.003705 \), Fig. 4C). However, no difference in age at onset was found between the subgroup homozygous for non-truncated mutations and that homozygous for truncated mutations (Kruskal–Wallis test: \( P = 0.2172 \)).

**Follow-up**

At study end, the age of the 16 patients ranged from 4 to 57 years, permitting a long patient follow-up.

- Mineralocorticoid function:
  - Patient 3 had SW at age 15 then recurrence at 18, illustrating the importance of follow-up. Moreover, eight other patients (1, 2a, 4a–b, 10a–c, 13) had elevated renin and/or low aldosterone and needed mineralocorticoid or salt therapy.
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Gonadotropic/genitalia function:
- Patients 11a and 11b, both presented with cryptorchidism and underwent surgery for ectopic testes.
- Two patients (7 and 9) had precocious puberty at age 5, associated with testicular nodules, low or undetectable gonadotropins and high testosterone. Patient 9 had surgery revealing an interstitial Leydig cell adenoma. In this patient, a short growth hormone-releasing hormone (GNRH) analog therapy was discontinued and the adenoma removed. Thereafter, testosterone remained at prepubertal values.
- For patient 12, testicular inclusions were detected at age 18 during imaging studies for azoospermia and were consistent with testicular adrenal rest tumor (TART). His azoospermia was associated with elevated follicle-stimulating hormone (FSH) (luteinizing hormone (LH): 9 mUI/mL, FSH: 18 mUI/mL), but normal testosterone (5.8 nmol/L). His karyotype was normal(46,XY) with no Y chromosome microdeletion. Increasing the dose of his glucocorticoid replacement therapy did not reduce the testicular inclusion and had no effect on spermatogenesis.

Heart function:
- A transthoracic echocardiography in patient 1, at age 23, showed a typical and severe asymmetrical left ventricular hypertrophy (maximal wall thickness measured at the basal septum: 36mm). The resting left ventricular outflow gradient was measured at 15 mmHg. There was no mitral regurgitation. The left atrium was dilated (25 mm²).
- Patient 9 at age 6 had normal heart function, but with a left ventricular ejection fraction of 75%.

Other functions:
- Two patients (2b and 9) had hypothyroidism with a thyroid gland in place. Patient 2b had congenital hypothyroidism and patient 9 had hypothyroidism with low free thyroxine (T₄) at age 5 and an elevated thyroid-stimulating hormone (TSH) with no goiter at age 7.
- Three patients had recurrent urinary tract infections (4b, 11a, and 13).
- We did not have information on social aspects for all the patients but four of them (patients 3, 5, 7, and 9) were experiencing poor academic performance or acquisition delays, possibly due to severe hypoglycemia.
- None of the patients presented pancreatic dysfunction or impaired glucose tolerance. There were no growth disorders for patients who reached adult age.

Discussion
Here, we report seven new NNT mutations identified in 18 patients, 11 with FGD and 7 with global adrenal insufficiency, who were members of 13 families, that is, 26% of the 50 families studied.

The mutations were distributed throughout the gene and most led to a premature truncated protein or an absence of protein. The most frequent mutation found in our cohort was p.Arg129*, which was identified in four Algerian families, suggesting the possibility of a founder mutation similar to p.Gly200Ser in Palestine (12). We also identified two novel missense mutations, which should be pathogenic. Our patients with NNT mutations displayed a severe phenotype, with adrenal insufficiency often revealed by hypoglycemic convulsions. Most of the cases were diagnosed belatedly, around the first year of life (8–18 months). Some, however, were discovered earlier if stress conditions had occurred, that is, intercurrent infections, suggesting the need of a stress to trigger the disease (Fig. 4A).

This is in accordance with the literature, where the minimum
age at diagnosis is 3 days (12) and the median age at onset is 12 months for the 29 patients for whom data are available (9, 11, 12, 13, 15, 16). Unlike the data by Jazayeri et al. (2015), our data suggest earlier onset in patients with global adrenal deficiency compared with those with isolated glucocorticoid deficiency (Fig. 4B and C). The phenotypic variability between patients having the same mutation or within the same family (family 2) suggests that there is no correlation between genotype and phenotype.

It is clear that NNT mutations can result in global adrenal deficiency. In the literature and comparably to 14 of our patients, 5 families were recently described with mineralocorticoid deficiency present at onset and 3 others with elevated renin or electrolyte imbalance (11, 12, 13, 16). Our patient 3 had salt wasting at age 15, although aldosterone requirements normally decrease throughout life (30). We observed phenotypic heterogeneity even within the same family. Patient 11a presented salt wasting, whereas patient 11b had no mineralocorticoid deficiency. This emphasizes the need for careful monitoring of this function, since some patients classified as FGD may also have a slight mineralocorticoid defect. It has been shown that C57BL6/J mice carrying NNT mutations have disorganized zonae fasciculata with higher levels of apoptosis (9). As aldosterone requirement decreases through life, the mineralocorticoid defect may be the consequence of extended damage to all adrenal zona.

NNT has a role in the oxidative stress response and mutations in it may thus affect all tissues rich in mitochondria. For this reason too, patients with NNT mutations need to be closely monitored. Two in vitro studies on the fibroblasts (12) and lymphocyte mitochondria (31) of patients homozygous for missense NNT mutations showed an increase in ROS levels, a decrease of ATP content, and impaired morphology of mitochondria with reduced mitochondrial mass and increased mitochondrial DNA deletion due to a lack of thymidylate biosynthesis (12, 31). The results from those two studies suggest that all tissues can be injured, as do our results from the follow-up of patients with extra-adrenal defects.

Although NNT is widely expressed in adrenal, heart, kidney, thyroid, and adipose tissues, the most affected tissue in our cohort appeared to be the gonads (9). Two of our patients had cryptorchidism (patients 11a and b) and two others (7 and 9) presented similar histories involving, both at about 5 years of age, the development of palpable nodules on the testicular surface or testicular enlargement followed by the onset of puberty with high testosterone levels. These last two cases are comparable to that reported by Hershkovitz et al. (13). For our patients 7 and 9, gonadotropins were in the normal pre-pubertal range and the increase in testosterone seemed to be due to secretion by autonomous nodules responsible for the onset of puberty. Since the regression of puberty for patient 9 was due to either the removal of the adenoma or the short GNRH analog treatment, we cannot pronounce as to the central, peripheral or mixed origin of the precocious puberty. Reporting on a boy with a mutation in DAX-1/NR0B1, Domenice et al. (32) concluded that chronic excessive ACTH levels may stimulate Leydig cells and lead to gonadotropin-independent precocious puberty, a view shared by Hershkovitz et al. (13) as well. In contrast, the testicular inclusions of our patient 12, associated with azoospermia but normal testosterone values at age 18, although not reduced by glucocorticoid therapy, should be TART, often found in congenital adrenal hyperplasia (33).

To date, we have observed heart function impairment (progressive hypertrophic cardiomyopathy) in only one of our patients, but cannot exclude future cases because of the mean age of our cohort and the subnormal imaging of patient 9. This underlines the specific role of NNT in heart tissue. In B6J-Sod2−/− mice, the presence of a normal NNT allele preserves cardiac function, delays the onset of heart failure, and extends survival to the end of gestation (24). In comparison, the suppression of NNT in zebrafish results in ventricular malformations and contractile dysfunctions (34). Moreover, in humans, relationships between decreased NNT activity, modified mitochondrial redox regulation and cardiac failure have been reported. In the failing human heart, a partial loss of NNT activity adversely affects NADPH-dependent enzymes and the capacity to maintain membrane potential. This contributes to a decline in bioenergetic capacity, redox regulation and antioxidant defense, exacerbating oxidative damage to cellular proteins (26). A recent report of a heterozygous frameshift mutation of NNT in humans with left ventricular noncompaction supports the assumption that NNT plays a major role in myocardium (34). However, Nickel et al. (35) demonstrated a completely opposing view. They reported that during heart pressure overload, NNT adopts a reverse mode contributing to oxidative stress from which mice with mutation in NNT are protected. Those puzzling new insights may suggest that the functional mode (forward or reverse) of NNT is dependent on the metabolic state. Nevertheless, TXNRD2 is in the same pathway of ROS detoxification and TXNRD2 heterozygous mutations in humans have also been linked to dilated cardiomyopathy. Thus, for now, cardiac follow-up should be done (10, 36).

The thyroid gland, highly exposed to oxidative stress, was the third most-affected organ in our cohort. Beyond...
our two patients with hypothyroidism, probably due to some hormone synthesis defect, two other cases with subclinical hypothyroidism have been reported (16). The biosynthesis of thyroid hormone (TH) is an oxidative biochemical reaction that depends on the formation of peroxide. However, two studies have suggested that when thyroid cells are exposed to significantly increased oxidative stress, peroxide produced for TH biosynthesis and antioxidants to protect cells from H₂O₂-mediated oxidative damages, thus leading to TH formation inhibition. Nevertheless, our patients with hypothyroidism were consanguineous and we cannot exclude that another gene involved in the thyroid may be mutated.

Other functions in our patients were normal, especially growth and glucose metabolism. Glucose intolerance or diabetes in humans has not been reported in the setting of NNT mutation, although defects in mitochondrial energy metabolism have also been implicated in diabetes. This contrasts with the impaired insulin secretion observed in NNT mutant mice for which only the β-cells seemed sensitive (40). Increased ROS usually plays a role in innate immunity against bacterial cytotoxicity (41). Despite that, three of our patients experienced recurrent urinary tract infections. Thus, we feel that additional studies are necessary to further investigate renal function.

In conclusion, we reported here mutations in the NNT gene, which was one of the most frequent molecular etiologies in our ‘atypical’ congenital adrenal insufficiency cohort. Deducing from our results and those of other authors, mutations in NNT should be searched not only in FGD, but also in global adrenal insufficiency. Above all, careful follow-up, especially for mineralocorticoid, puberty, fertility, heart and thyroid function, must be maintained for all patients. The MPS approach described in the section ‘Patients and methods’, with a large panel of genes including NNT, appears to be efficient for genetic diagnosis (16, 42). The analysis of more than one gene at a time is a powerful tool to identify the causative mutation, although defects in mitochondrial energy metabolism have also been implicated in diabetes. This contrasts with the impaired insulin secretion observed in NNT mutant mice for which only the β-cells seemed sensitive (40). Increased ROS usually plays a role in innate immunity against bacterial cytotoxicity (41). Despite that, three of our patients experienced recurrent urinary tract infections. Thus, we feel that additional studies are necessary to further investigate renal function.

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