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

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**ABSTRACT**

**Objective:** NNT (nicotinamide nucleotide transhydrogenase), one of 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 one hypertrophic cardiomyopathy. Intrafamilial phenotype heterogeneity was 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 can occur: isolated mineralocorticoid deficiency, isolated glucocorticoid deficiency, or combined mineralocorticoid and glucocorticoid deficiency (global adrenal insufficiency). Glucocorticoid deficiencies, also called 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. 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. Over the last three years, thanks to whole exome sequencing, three more causative genes have been discovered: MCM4 (mini chromosome maintenance deficient 4 homologue), NNT (Nicotinamide Nucleotide Transhydrogenase) and TXNRD2 (Thioredoxin reductase 2). As these genes encode proteins that work together for reactive oxygen species (ROS) detoxification or DNA replication, the spectrum of pathogenic mechanisms causing PAI is not limited to genes involved in adrenal development and steroidogenesis.

The incidence of NNT gene mutations in Clark et al’s FGD cohort was around 10% (15 families) and no predominant mutation was reported (21 private mutations). Twelve more families have been reported (12 additional mutations), some with mineralocorticoid defects. NNT encodes an integral protein of the inner mitochondrial membrane that acts as a proton pumping transhydrogenase. 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 always a homodimer of approximately 220 kDa. All NNTs show a similar structure with three major domains. Domain I contains the hydrophilic NAD(H) binding site and domain III, 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. 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\textsuperscript{19-22} (Figure 1).

Meimaridou \textit{et al} showed reduced, basal and ACTH-stimulated corticosterone, revealing impaired steroidogenesis in C57BL/6J mice with a spontaneous \textit{NNT} mutation (an in-frame 5 exon deletion). Furthermore, they showed increased ROS levels in an \textit{NNT} knock-down human adrenocortical cell line\textsuperscript{9}. 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\textsuperscript{23}. At present, in humans, \textit{NNT} mutations are known to be associated with adrenal insufficiency. Additionally, relationships between decreased \textit{NNT} activity, modified mitochondrial redox regulation and cardiac failure have been recently reported\textsuperscript{24-26}.

The aim of our study was to screen for \textit{NNT} mutations in fifty 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 thirteen families (eighteen patients) with \textit{NNT} mutations: thirteen patients were diagnosed with FGD and five with global adrenal insufficiency at onset. A range of functions, i.e., 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 fifty patients with primary adrenal insufficiency 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, Chalfont Saint Giles, Buckinghamshire, UK). 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 twenty 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, CA, USA). Sequence
variants were designated according to the Human Genome Society recommendations
(www.hgvs.org/rec.html) using the NCBI reference sequences NC_000005.9, NM_012343.3 and
NP_036475 built on the GRCh37/hg19.

Massive parallel sequencing (MPS) or next generation sequencing (NGS)

DNAs were tested using an amplicon-based library preparation. A custom panel targeting 57 genes,
involved in adrenal insufficiency and disorders of sex development, including NNT, was designed
using Ion AmpliSeq designer software (Life Technologies) (coding regions ± 50bp) (article underway,
list available on request). The library preparation was done according to the manufacturer’s
instructions with the Ion AmpliSeq Library Kit v2.0 (Life Technologies). Enrichment and
quantification of target DNA were validated on the Caliper LabChip-GX using the high sensitivity
The patients were barcoded and pooled by 140 groups of eight to get a sufficient depth of coverage (>100X) at sequencing. For the sequencing step, 141 enriched template-positive Ion PGM spheres were prepared by emulsion PCR with the Ion OneTouch 142 2 System (Life Technologies). The resulting live Ion Sphere Particles (ISPs) were loaded on an Ion 143 316 Chip. Sequencing was done on the Ion Torrent Personal Genome Machine (PGM) with the PGM 144 Sequencing 200 Kit. The bioinformatics pipeline used was the Torrent Suite software implemented 145 with the sequencer and with the default parameters. NNT mutations were validated by Sanger 146 sequencing.

Array comparative genomic hybridization (aCGH) and long range PCR

To confirm a deletion, aCGH or chromosomal microarray (CMA) was performed according to the 149 manufacturer’s instructions, using the Agilent SurePrint G3 Human CGH Microarray 4x180K 150 AMADID 022060 (Agilent Technologies, Inc, Santa Clara, CA). This was followed by long-range 152 PCR using the Qiagen LongRange PCR kit (Qiagen, Hilden, Germany) according to the supplier’s 153 recommendations. Conventional dideoxy sequencing of the PCR product was done as described in the 154 paragraph “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 158 structurally conserved regions and to predict putative effects of missense mutations. The sequences 159 were found in the Uniprot database (http://www.uniprot.org/), aligned with ClustalW 160 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) using default parameters, displayed and then edited using 161 Genedoc (http://www.psc.edu/index.php/user-resources/software/genedoc).

Software and databases

For each new missense mutation, pathogenicity was predicted in silico using several programs: align 164 GVGD, Polyphen 2, SIFT and Mutation taster. The Grantham score was calculated to predict the 165 effect of substitutions between amino acids. This score looks at chemical properties to define a score 166 range between 0 and 215. Higher scores indicate greater differences in the chemical properties 167 between two amino acids (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 thirteen families (eighteen patients) (Table 1, Figure 2). Seven of them were new mutations: two nonsense (p.Arg129*, p.Arg379*), two missense (p.Met337Val, p.Ala863Glu), two frameshift (p.Val665Profs*29, p.Ala704Serfs*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 eight of the thirteen families and homozygous mutations were found in eleven 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 (Table 1)

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\(^18, 27\) (Figure 3).

The p.Ala863Glu mutation is located in the transmembrane helix 14 (H14) of domain II and is highly conserved between species (Figure 3). H14 appears to 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 one hundred French Caucasian healthy controls. As expected, the parents of patient 7 were heterozygous as were his two healthy brothers.

The missense mutation, p.Met337Val, was identified in the NAD(H) binding domain near the NAD(H) binding site. It should inhibit the hydride transfer from NADH to NADP⁺. The residue is highly conserved between species (Figure 3). The mutation was predicted to be deleterious by all of the mutation prediction tools mentioned above, despite a Grantham score of 21. This variation was not listed in the databases (dbsNP, EVS or ExAC browser) and not found in one hundred healthy controls from the Maghreb.

**Clinical data at onset and follow-up (Table 2)**

Table 2 presents the clinical data and follow up for the eighteen 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, upon inquiry, was often reported to have been present before the hypoglycemia. Five out of the thirteen 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 had a global adrenal insufficiency (patients 2b, 6, 8) with SW. The median age at onset in our cohort was 11.5 months (min-max: 1.5 months-4 years) (Table 2, Figure 4A). 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-value =0.03379, **Figure 4B**) and our cohort aggregated with the data available in the literature (Kruskal-Wallis test; p-value =0.003705, **Figure 4 C**). 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-value =0.2172).

**Follow-up**

At study end, the age of the 16 patients ranged from 4 to 57 years old, 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.

- **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 five, 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 GnRH analog therapy was discontinued and the adenoma removed. Thereafter, testosterone remained at pre-pubertal 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 FSH (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.
  - Patient 1 had a testicular biopsy at age 31 for a left varicocele with epididymitis.

- **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 basilar septum: 36 mm). 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 six 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 hypothyroidism with low free T4 at age five and elevated TSH with no goiter at age seven.

- Three patients had recurrent urinary tract infections (4b, 11a, 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 \textit{NNT} mutations identified in eighteen patients, eleven with FGD and seven with global adrenal insufficiency, who were members of thirteen families, i.e., 26\% of the fifty 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 \cite{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, i.e., intercurrent infections, suggesting the need of a stress to trigger the disease (Figure \textbf{4A}). This is in accordance with the literature where the minimum age at diagnosis is three days \cite{12} and the median age at onset is 12 months for the 29 patients for whom data are available \cite{9,11-13,15,16}. Unlike those of Jazayeri \textit{et al}, our data suggest earlier onset in patients with global adrenal deficiency compared to those with isolated glucocorticoid deficiency (Figure \textbf{4B and C}). The phenotypic variability between patients having a same mutation or within a 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 fourteen of our patients, five families were recently described with mineralocorticoid deficiency present at onset and three others with elevated renin or electrolytes imbalance \cite{11-13,16}. Our patient 3 had salt wasting at age 15, although aldosterone requirements normally decrease throughout life \cite{30}. We observed phenotypic heterogeneity even within a 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 \textit{NNT} mutations have disorganized zonae fasciculata with higher levels of apoptosis \cite{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 mtDNA 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 five
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 either to 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 concluded that chronic excessive ACTH levels may stimulate Leydig cells
and lead to gonadotropin-independent precocious puberty \(^{32}\), a view toward which Hershkovitz et al’s
case \(^{13}\) argues as well. In contrast, the testicular inclusions of our patient 12, associated with
azoospernia 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. In comparison, the suppression of NNT in zebrafish results in ventricular malformations and contractile dysfunctions. 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. 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. However, Nickel et al 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.

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. The biosynthesis of thyroid hormones (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 significant amount of ROS, thyroid peroxidase and iodide organification are inhibited. Another argument is the prevalence of thyroid dysfunction in patients with Down syndrome who are under unusual increased oxidative stress. NNT mutations may disturb the balance between H₂O₂ produced for TH biosynthesis and anti-oxidants 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. Increased ROS usually plays a role in innate immunity against bacterial cyto-invasion.

Despite that, three of our patients experienced recurrent urinary tract infections. We thus feel that
additional studies are necessary to further investigate renal function.

In conclusion, we report 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 methods section, with a large panel of genes including NNT, appears to be the most efficient for
genetic diagnosis. The analysis of more than one gene at a time is a powerful way to reach a
diagnosis in diseases with phenotype heterogeneity. We note that more and more “atypical” cases of
PAI are being described, for example STAR and CYP11A1 mutations in boys with PAI with or
without DSD.

DISCLOSURE

Declaration of interest

The authors declare that there are no conflicts of interest that could prejudice the impartiality of the
research reported.

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transhydrogenase on mitochondrial DNA integrity in a case of familial glucocorticoid


**TABLE AND FIGURES LEGENDS**

**Table 1**: NNT mutations in thirteen families with PAI. d: domain, TMH: transmembrane helix, -: not applicable, NMD: nonsense-mediated decay

**Table 2**: Clinical characteristics and follow-up of the patients with NNT mutations. -: not determined, M: male, F: female, NL: normal, *: deceased, yrs: age in years, ttt: treatment, MC: mineralocorticoid, GC: glucocorticoid, SW: salt wasting, ↘: decreased, ↗: increased, LVEF: left ventricular fraction, ENT: ear, nose, throat. Major abnormalities are in bold. For two patients (4a and 9) the age of diagnosis was late despite an earlier onset.

**Figure 1**: Role of NNT in free radical metabolism in the mitochondria ETC: electron transport chain GSSG: glutathione disulfide; GSH: glutathione; GPX: glutathione peroxidase; GR: glutathione reductase; TXNRD2: thioredoxin reductase; PRDX3: peroxiredoxin 3

**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.

**Figure 3**: Partial multiple amino acid alignment of NNT in human, bovine, mouse, *Caenorhabditis elegans*, *Escherichia coli* and *Acetabularia acetabulum*. Alignment was performed in clustalW and edited with Genedoc. The mutant residues p.M337V and p.A863E and corresponding amino acids are shaded and show the conservation across all species.

**Figure 4**: A. Age at presentation for the patients of our cohort GD: glucocorticoid deficiency, SW: salt wasting. B&C. Difference in age at presentation (Kruskal-Wallis test, *: significance at p<0.05) between the group of patients with glucocorticoid deficiency and the group with salt wasting +/-
glucocorticoid deficiency within our cohort (p-value =0.03379*) (B); our data aggregated with the data available in the literature (p-value =0.003705*) (C).
Table 1: NNT mutations in thirteen families with PAI

<table>
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<th>Nucleotide change (NM_012343.3)</th>
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<th>Ref.</th>
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<tbody>
<tr>
<td>c.3G&gt;A</td>
<td>2</td>
<td>p.M1?</td>
<td>Start loss</td>
<td>Pre-sequence</td>
<td>-</td>
<td>0/13006</td>
<td>0/121286</td>
<td>9,14</td>
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<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>dI</td>
<td>-</td>
<td>0/13006</td>
<td>0/121286</td>
<td>1,4,12</td>
<td>15</td>
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<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>dI</td>
<td>-</td>
<td>0/13002</td>
<td>0/121286</td>
<td>2,3,4,11</td>
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<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>dI</td>
<td>less likely</td>
<td>Deleterious</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>0/13006</td>
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<tr>
<td>c.1135C&gt;T</td>
<td>9</td>
<td>p.R379*</td>
<td>Premature truncation at amino acid 379. NMD?</td>
<td>dI</td>
<td>-</td>
<td>0/13006</td>
<td>0/121286</td>
<td>9</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>dI</td>
<td>less likely</td>
<td>Deleterious</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>0/13006</td>
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<tr>
<td>c.2106_2109dup</td>
<td>15</td>
<td>p.A704S6*19</td>
<td>Frameshift: premature truncation at amino acid 723</td>
<td>dII TMH9</td>
<td>-</td>
<td>0/13006</td>
<td>0/121286</td>
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<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>dII TMH14</td>
<td>Most Likely</td>
<td>Deleterious</td>
<td>Probably damaging</td>
<td>Disease causing</td>
<td>0/13006</td>
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<tr>
<td>c.(+51+1_53-1)_381+1_382-1del</td>
<td>2-3</td>
<td>p.0?</td>
<td>Start loss. Absence of protein</td>
<td>dI</td>
<td>-</td>
<td>0/121286</td>
<td>12</td>
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</table>

d:domain, TMH: transmembrane helix, -: not applicable, NMD nonsense-mediated decay
<table>
<thead>
<tr>
<th>Family</th>
<th>Origin</th>
<th>Consanguinity</th>
<th>NNT mutation</th>
<th>Age at diagnosis (months or yrs where indicated)</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>
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<tbody>
<tr>
<td>1</td>
<td>French</td>
<td>no</td>
<td>p.R71*/p.R71*</td>
<td>M 43 14 hypoglycemia, melanoderma</td>
<td>thirst for salt, MC ttt (↗ renin)</td>
<td>onset of puberty at 12 yrs, NI testicular function, surgery for varicocele</td>
<td>hypertrophic myocardopathy</td>
<td>NI</td>
<td>Nl</td>
<td>brother at 2 yrs</td>
</tr>
<tr>
<td>2a</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>M 32 6 hypoglycemic convulsions, delay of diagnosis due to GC therapy for asthma</td>
<td>MC ttt at 3 yrs (↗ renin)</td>
<td>onset of puberty at 13 yrs 9 months</td>
<td>-</td>
<td>NI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2b</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>M 15 3 hypoglycemic convulsions, SW</td>
<td>MC ttt, SW at 12 yrs after an attempt to stop MC ttt</td>
<td>onset of puberty at 12 yrs, low testosterone at 15 yrs</td>
<td>NI imaging (12 yrs)</td>
<td>congenital hypothyroidism with thyroid gland in place</td>
<td>sister at 7 months</td>
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</tr>
<tr>
<td>3</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>F 19 15 hypoglycemia, asthenia, melanoderma</td>
<td>no MC ttt but 4 adrenal crises with ↗ renin ↓ aldosterone</td>
<td>menarche (11 yrs), NI menstrual cycle</td>
<td>-</td>
<td>NI</td>
<td>Nl</td>
<td>-</td>
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<tr>
<td>4a</td>
<td>French/ Algerian</td>
<td>no</td>
<td>p.R71*/p.R129*</td>
<td>F 30 1.5 (onset) /12 hypoglycemic convulsions following gastroenteritis</td>
<td>MC ttt at 2 yrs (↗ renin)</td>
<td>menarche (11 yrs), two children</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>4b</td>
<td>Algerian</td>
<td>no</td>
<td>p.V665F<em>29/p.V665F</em>29</td>
<td>F 23 10 hypoglycemic convulsions following gastroenteritis</td>
<td>no MC ttt but ↗ renin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>no</td>
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<tr>
<td>5</td>
<td>French</td>
<td>no</td>
<td>p.V665F<em>29/p.V665F</em>29</td>
<td>M 14 19 hypoglycemia convulsions on ENT infections</td>
<td>no</td>
<td>onset of puberty at 12 yrs low testosterone at 13 yrs</td>
<td>NI imaging (11 yrs)</td>
<td>-</td>
<td>Nl</td>
<td>-</td>
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<tr>
<td>6</td>
<td>Turkish</td>
<td>yes</td>
<td>p.A704S<em>19/p.A704S</em>19</td>
<td>F 18 8 hypoglycemia, asthenia, melanoderma, weight loss, fever, SW</td>
<td>MC ttt</td>
<td>menarche (12.5 yrs)</td>
<td>-</td>
<td>transient subclinical hypothyroidism (TSH: 6.8 mU/L at 5yrs)</td>
<td>no</td>
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<tr>
<td>7</td>
<td>Moroccan</td>
<td>yes</td>
<td>p.A863E/p.A863E</td>
<td>M 5 13 hypoglycemic convulsions</td>
<td>no</td>
<td>precocious puberty with 3 nodular testis</td>
<td>NI imaging (4 yrs)</td>
<td>-</td>
<td>Nl</td>
<td>no</td>
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<tr>
<td>8</td>
<td>Mauritian</td>
<td>yes</td>
<td>p.M1?/p.M1?</td>
<td>F 8 9 hypoglycemic coma, SW</td>
<td>MC ttt</td>
<td>no pubertal symptoms</td>
<td>-</td>
<td>NI</td>
<td>Nl</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R379*/p.R379*</td>
<td>M 10 22 (onset) /8yrs</td>
<td>Misdiagnosed at 22 months (several hyperthermic convulsions, psychomotor retardation, sodium valproate ttt), all symptoms improved after glucocorticoid therapy initiated at 8 yrs</td>
<td>no</td>
<td>Leydig cell adenoma (5 yrs) following by precocious puberty</td>
<td>subNI imaging at 6 yrs (LVEF at 75%)</td>
<td>subclinical hypothyroidism (TSH: 3.5 mU/L at 5 yrs and 10.5 yrs), thyroid hormone treatment</td>
<td>no</td>
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<tr>
<td>10a</td>
<td>French</td>
<td>no</td>
<td>p.P437L/p.P437L</td>
<td>M 57 4 yrs</td>
<td>melanoderma, asthenia</td>
<td>salt craving, MC ttt</td>
<td>NI puberty</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>10b</td>
<td>French</td>
<td>no</td>
<td>p.P437L/p.P437L</td>
<td>M 51 18</td>
<td>melanoderma, asthenia</td>
<td>salt craving, MC ttt</td>
<td>NI testicular function at 49 yrs</td>
<td>NI imaging (51 yrs)</td>
<td>-</td>
<td>no</td>
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<tr>
<td>10c</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>F 4* 16</td>
<td>familial story, deceased at 4 yrs</td>
<td>MC ttt</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>11a</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>M 6 2</td>
<td>SW</td>
<td>SW (2 months, 4 yrs), MC ttt</td>
<td>cryptorchidism (surgery)</td>
<td>NI imaging (2 yrs)</td>
<td>-</td>
<td>Brother at 8 months</td>
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<tr>
<td>11b</td>
<td>Algerian</td>
<td>yes</td>
<td>p.R129*/p.R129*</td>
<td>M 8 4 yrs</td>
<td>hypoglycemia (NI at 3 yrs)</td>
<td>no</td>
<td>cryptorchidism (surgery)</td>
<td>NI imaging (2 yrs)</td>
<td>-</td>
<td>Brother at 8 months</td>
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<tr>
<td>12</td>
<td>French</td>
<td>no</td>
<td>p.R71*/del ex2-3</td>
<td>M 35 10</td>
<td>SW</td>
<td>SW, MC ttt</td>
<td>bilateral TART, azoospermia</td>
<td>-</td>
<td>Nl</td>
<td>-</td>
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<tr>
<td>13</td>
<td>Algerian</td>
<td>yes</td>
<td>p.M337V/p.M337V</td>
<td>F 9 8</td>
<td>melanoderma, asthenia</td>
<td>↑ renin, ↑ aldosterone (16 months, 12 yrs), salt, no MC ttt</td>
<td>no pubertal symptoms</td>
<td>-</td>
<td>NI</td>
<td>Sister at 4 yrs</td>
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</tbody>
</table>

*: deceased, yrs: years, ttt: treatment, MC: mineralocorticoid, GC: glucocorticoid, SW: salt wasting, ↓: decreased, ↑: increased, LVEF: left ventricular fraction, ENT: ear, nose, throat. Major abnormalities are in bold. For two patients (4a and 9) the age of diagnosis was late despite an earlier onset.
GLUTATHIONE SYSTEM

[Diagram showing GR, GPX, NADPH, GSSG, GSH, H₂O, H₂O₂]

THIOREDOXIN SYSTEM

[Diagram showing TXNRD2, PRDX3, Trx(SH)₂, TrxS₂, NADPH, H₂O₂, H₂O]

Free Radical & Steroidogenesis

O₂ + e⁻ → O₂⁻ → MnSOD → H₂O₂
p.M337

<table>
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<tr>
<td>Bovine</td>
<td>ILFNKEMIESMKEGSVVVDLAAEAGGNFET</td>
</tr>
<tr>
<td>Mouse</td>
<td>VLFSDKEMIESMKEGSVVVDLAAEAGGNFET</td>
</tr>
<tr>
<td>C. elegans</td>
<td>ILITEEMIKSMKPGSVVVVLAAESGGNIAT</td>
</tr>
<tr>
<td>E. coli</td>
<td>KLITREMVDMSKAGSVIVDLAAQNGGNCEY</td>
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<tr>
<td>A. acetabulum</td>
<td>KLILKDMIESMKPVSVVVLAAENNGNIET</td>
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p.A863

<table>
<thead>
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<tr>
<td>Human</td>
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<tr>
<td>Bovine</td>
<td>EGFLLNNLLLTVGALIGSSGAILSYIMCV.</td>
</tr>
<tr>
<td>Mouse</td>
<td>EGFLLNNLLLTVGALIGSSGAILSYIMCV.</td>
</tr>
<tr>
<td>C. elegans</td>
<td>EGFMLDNSLLTVLGAIGSSGAILSHIMCK.</td>
</tr>
<tr>
<td>E. coli</td>
<td>AGFMLSNDLLIVTGALVGSSGAILSYIMCK.</td>
</tr>
<tr>
<td>A. acetabulum</td>
<td>G–SVLDNNLLLTVGALIGSSGAILSAIMCK.</td>
</tr>
</tbody>
</table>
Figure 4: A. Age at presentation for the patients of our cohort GD: glucocorticoid deficiency, SW: salt wasting. B&C. Difference in age at presentation (Kruskal-Wallis test, *: significance at p<0.05) between the group of patients with glucocorticoid deficiency and the group with salt wasting +/- glucocorticoid deficiency within our cohort (p-value =0.03379*) (B); our data aggregated with the data available in the literature (p-value =0.003705*) (C).