Loss-of-function NNT mutations impair antioxidants mechanisms and decrease cortisol secretion in patients with familiar glucocorticoid deficiency

Bodoni AF[1], Coeli-Lacchini FB[2], Sobral LM[5], Gebenlian JL[1] , Leoplodino AM[5], Moreira AC[2], Elias LL[3], Silva WAJr[4], Castro M[2] and Antonini SR[1]

Departments of Pediatrics[1], Internal Medicine[2], Physiology[3] and Genetics[4], Ribeirao Preto Medical School and Department of Clinical Analyses, Toxicology and Food Sciences School of Pharmaceutical Sciences of Ribeirao Preto[5]. University of Sao Paulo, Brazil

Background

- Mitochondrial Nicotinamide Nucleotide Transidrogenase (NNT) is essential in the antioxidant defense mechanisms (**Figure 1**).
- Recently, mutations in the *Nicotinamide Nucleotide Transidrogenase* (*NNT)* gene were described in few Familial glucocorticoid deficiency (FGD) patients.

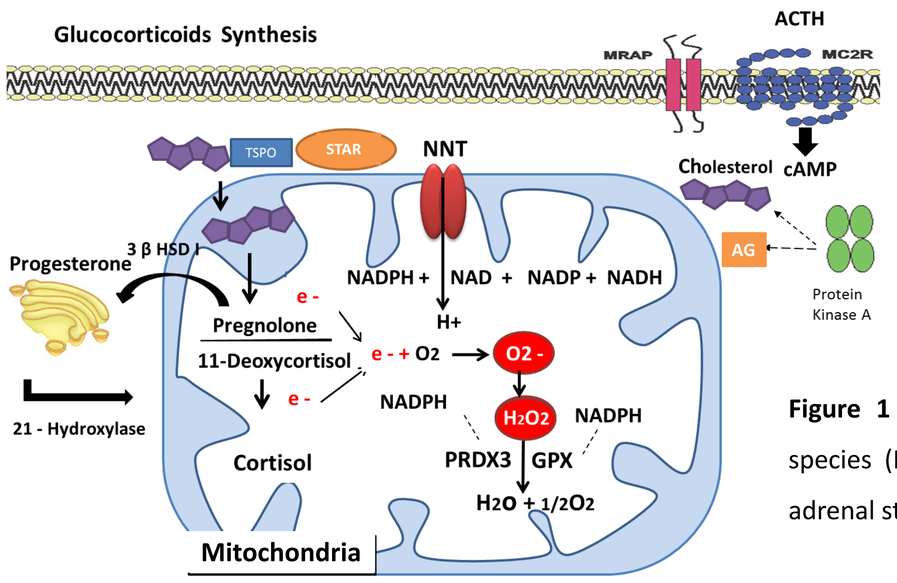


Figure 1 . Reactive oxygen species (ROS) production in adrenal steroidogenesis.

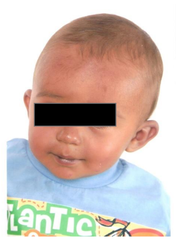
Aims

To characterize how mutations in NNT gene impair adrenal steroidogenesis resulting in familial glucocorticoid deficiency

Patient

- **Case Report:** A boy with 18-months old boy presenting with:

	Unit	Level
ACTH	pg/ml	> 1,250
Cortisol	ug/ml	< 1.2
17- OH progesterone	ng/dl	< 3.9
Androstenedione	ng/d	< 3,9
Testosterone	ng/dL	30
Plasma Renin Activity	ng/mL/h	4.2



- severe hypoglycemia
- seizures
- skin hyperpigmentation

- WES analysis revealed few final candidate genetic variants, including a homozygous exon 17 transition (c.2597G>A; p.G866D) in *NNT* gene.

Methods

Molecular Analysis

Genomic DNA was evaluated by whole exome sequencing (WES).

Candidate genetic variants were analyzed in silico and confirmed by Sanger sequencing.

Functional *in vitro Genotype-phenotype (p.G866D)*

Basal and 5h H_2O_2 stimulated ROS in mononuclear blood cells

- ROS intracellular production (DCFDA)
- Reduced glutathione (GSH; GSH-Glo Assay)
- Mitochondrial Mass (Mitotraker)

Adrenal Effect

H295 cell line

siRNA *NNT* gene knockdown

- ROS intracellular production (DCFDA)
- Mitochondrial Mass (Mitotraker)
- Cortisol secretion (RIA).

Results

- WES analysis revealed few final candidate genetic variants, including a homozygous exon 17 transition (c.2597G>A;p.G866D) in NNT gene.
- The novel mutation p.G866D, was validated by direct sequencing (Sanger; **Figure 2**).

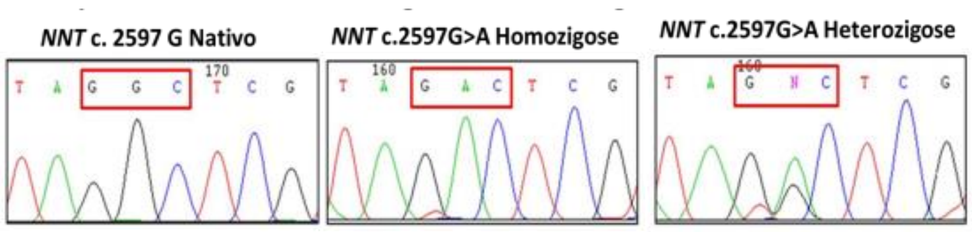
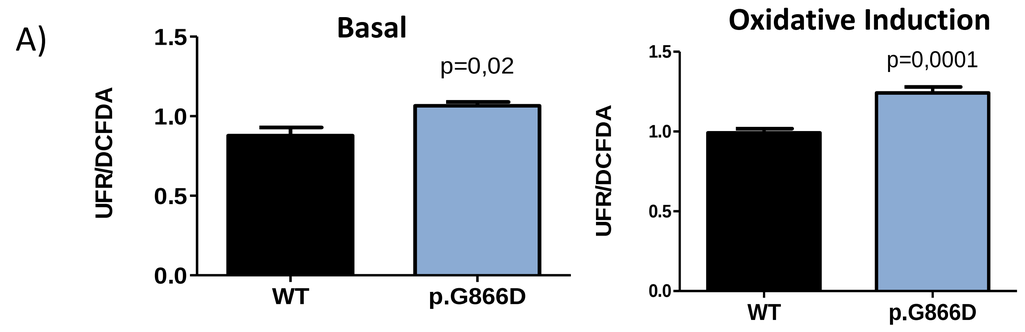


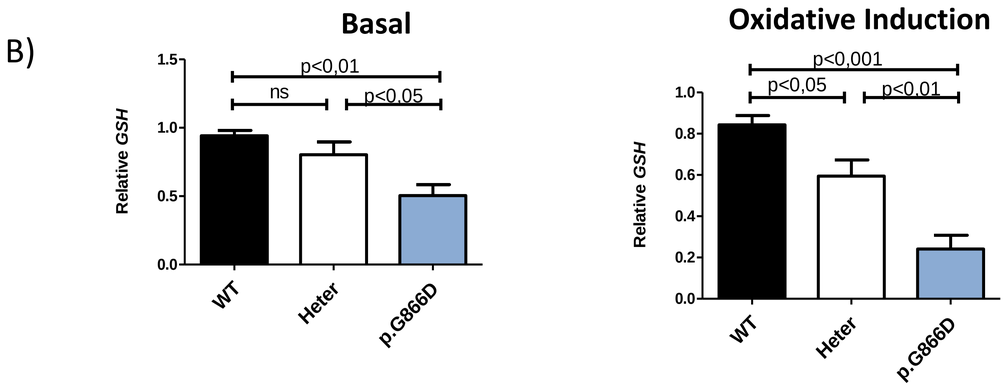
Figure 2. Family pedigree analysis confirmed segregation of this homozygous variant c.2597G>A (ENST00000264663 - p.G866D) with the phenotype and asymptomatic parents and his younger brother were heterozygous carriers. c.2597G>A (ENST00000264663 - p.G866D).

Genotype-Phenotype Association

Increased ROS intracellular production



Reduced glutathione



Mitochondrial Mass

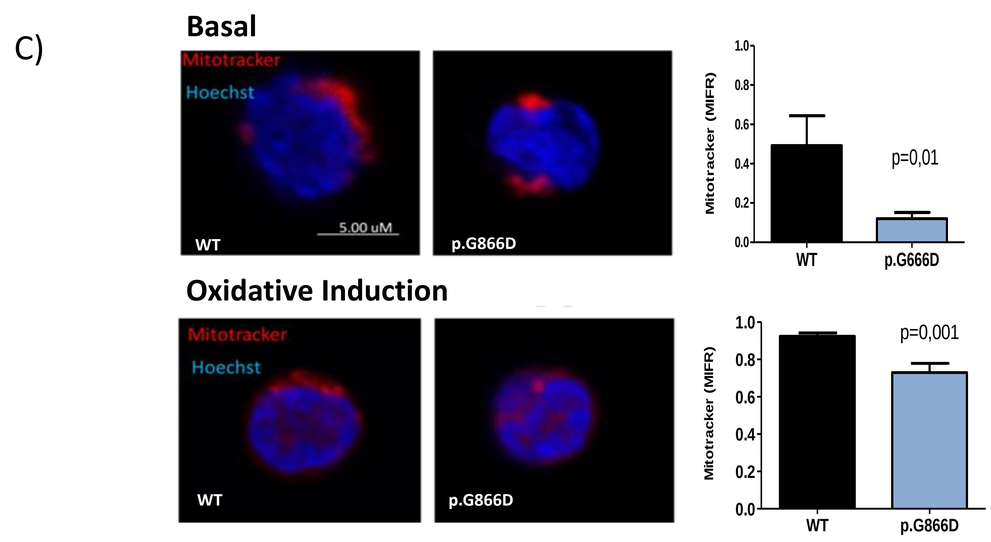
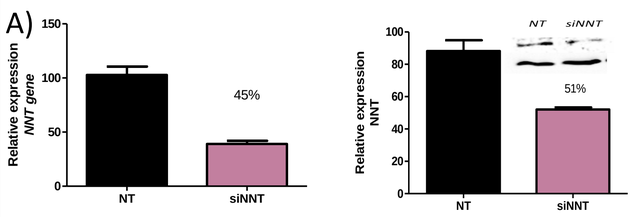


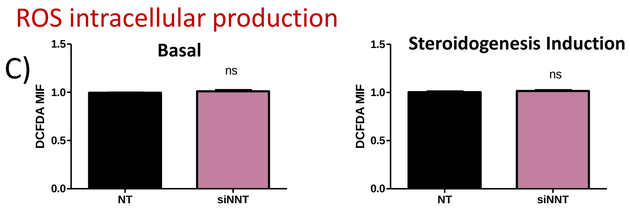
Figure 3. A) ROS production, homozygote for the G866D mutation in the NNT gene, show increase in ROS production in basal conditions and after oxidative stress induction in the homozygous. B) GSH content in mononuclear blood cells, Homozygote for the G866D mutation in the NNT gene, show decrease in GSH in basal conditions and after estress oxidative induction. C) Mitochondrial activity in mononuclear blood cells stained with MitoTracker Red. Representative images are shown. Scale bars, 5 µm. Mitochondrial mass was significantly reduced in NNT p.G866D homozygous cells when compared to WT, both in basal and after oxidative stress induction (p=0.01 and p=0.001).

Adrenal Effect (NCI-H295 Adrenal Cell line)

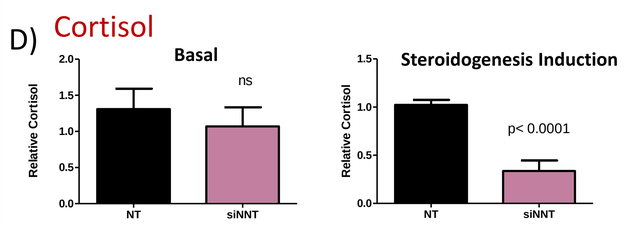
Knockdown Validation



ROS intracellular production



Cortisol



Mitochondrial Mass

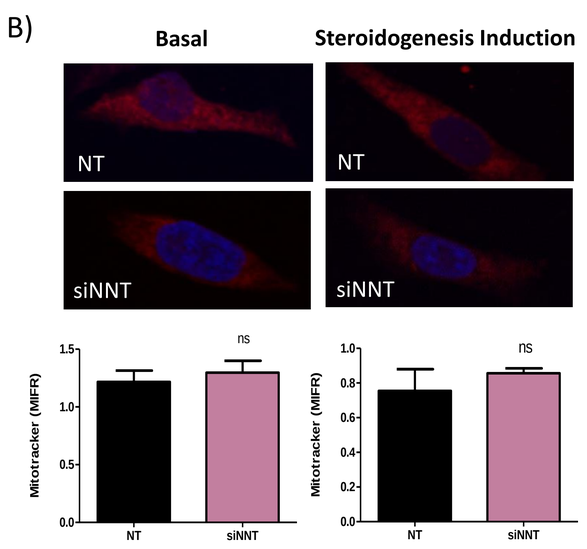


Figure 4. The knockdown of the *NNT* gene in the H295 showed a 55% reduction in *NNT* RNAm and 50% reduction in protein expression. B) ROS production was not altered. C) Mitochondrial mass in H295 stained with MitoTracker Red. Representative images are shown. Scale bars, 5 µm. Mitochondrial mass was preserved after reduction NNT protein. D) Cortisol secretion was preserved after reduction of the NNT protein in basal condition. However, after 24h of treatment with 10uM forskolin, a potent stimulator of steroidogenesis, there was a marked decrease of cortisol production (p<0.0001).

Conclusion

This study confirms the association of the homozygous NNT p.G866D variant with the phenotype of FGD.

In vitro, this loss-of-function NNT variant significantly impairs antioxidants mechanisms and affects the glutathione reductase systems resulting in increased ROS accumulation. In adrenal cells, NNT impairment results in significant reduction of the steroidogenesis, as shown by decreased production of cortisol.

Financial Support: FAPESP CNPq CAPES