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Predicted Benign and Synonymous variants in CYP11A1 Causes Primary Adrenal Insufficiency through Missplicing

--Manuscript Draft--

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Author Comments:	The authors reports no conflicts of interest in this work.
Abstract:	Primary adrenal insufficiency (PAI) is a potentially life-threatening condition that can present with non-specific features and can be difficult to diagnose. Here, we undertook next-generation sequencing in a cohort of children and young adults with PAI of unknown etiology from around the world and identified a heterozygous missense variant (rs6161, c.940G>A, p.Glu314Lys) in CYP11A1 in 19 individuals from 13 different families (allele frequency within undiagnosed PAI in our cohort 0.102 vs 0.0026 in gnomAD, p<0.0001). Seventeen individuals harbored a second heterozygous rare disruptive variant in CYP11A1 and two patients had very rare synonymous changes in trans (c.990G>A, Thr330=; c.1173C>T, Ser391=). Although p.Glu314Lys is predicted to be benign and showed no loss-of-function in an E. coli assay system, in silico and in vitro studies revealed that the rs6161/c.940G>A variant, plus the c.990G>A and c.1173C>T changes, affect splicing and that p.Glu314Lys produces a non-functional protein in mammalian cells. Taken together, these findings show that compound heterozygosity involving a relatively common and predicted “benign” variant in CYP11A1 is a significant contributor to PAI of unknown etiology, especially in European populations. These observations have implications for personalized management and demonstrate how variants that might be overlooked in standard analyses can be pathogenic when in combination with other very rare disruptive changes.
Additional Information:	
Question	**Response**
PRECIS: The precis is a brief description of your paper that will appear on the Table of Contents underneath your article title, should your paper be accepted (see the current issue of JES for examples). The description should be **no longer than 200 characters, including spaces,** and should briefly explain what was done in your study and what was concluded. Please ensure that the precis does not simply repeat the article title.	We demonstrated that two extremely rare synonymous variants in CYP11A1 and one more prevalent variant (rs6161), previously designated benign, give rise to adrenal insufficiency by missplicing of RNA.
DATA REPOSITORIES AND DATA REGISTRATION: I have read and agree to take appropriate action to comply with the following Data Repositories and Data Registration	Not Applicable

guidelines and confirm that I have included the appropriate registration numbers / information in the text of the manuscript being submitted.	
CELL LINE AUTHENTICATION: I have read and understood the Cell Line Authentication policy and describe my submission as follows:	Not applicable to my manuscript.
STEROID HORMONE MEASUREMENT: I have read and understood the Steroid Hormone Measurement policy and describe my submission as follows:	My manuscript includes steroid hormone assays and meets the standards described in the Steroid Hormone Measurement policy.
SPECIAL REQUESTS: Enter specific comments or requests to the editors here.	We previously submitted the paper to JCEM and received good reviews however, it was felt to be too genetic. We subsequently tried genetic journals but these felt it to be too endocrine. We have now rewritten it to simplify it, added another case and submit it for consideration for publication in the Journal of the Endocrine Society. We feel that JES will reach the best translational audience as the paper has important messages both clinically and in terms of genetic analysis and mechanisms.
Funding Information:	Medical Research Council (MR/K020455/1) — Dr Louise A Metherell Wellcome Trust (098513/Z/12/Z, 209328/Z/17/Z) — John Achermann Great Ormond Street Hospital Charity (V2518) — John Achermann National Institute for Health Research (IS-BRC-1215-20012) — John Achermann National Institutes of Health — Richard Auchus Mater Medical Research Institute — Mark Harris

Dear Editor

We thank the reviewers for their constructive comments. We have addressed the majority of these and revised the manuscript accordingly.

In particular, we have provided more extensive details about the cohorts studied so that the prevalence of our findings can be seen within the context of undiagnosed primary adrenal insufficiency and also within the context of our total cohort numbers. We include these new data in Supplementary Table 2 and have re-written the relevant sections of the manuscript. We feel this approach strengthens the significance of our findings considerably.

As requested, we have also undertaken further functional studies of the CYP11A1 variant in a mammalian cell system (HEK cells). These new data suggest the L335P and R460W variants along with the E314K have minimal enzyme activity.

Reviewer Comments:
Reviewer 1: Maharaja et al describe novel CYP11A1 variants in 19 patients manifesting with primary adrenal insufficiency (PAI). Novel aspect is that they show that a variant previously predicted as benign and synonymous variants in the CYP11A1 sequence were found to be disease-causing through misplicing. Importantly, these variants were found quite often in their unique cohort of unsolved PAI (explaining about 10%).

1. The study is performed in a convincing way regarding genetic work-up. For functional work-up the only missing experiment is the demonstration that the variants transfected into a cell system (e.g. HEK-293 cells) have lower or lost enzyme activity. This would be particularly interesting for the p.Glu314Lys mutation, which is shown to be degraded in cells, but has similar activity to wild-type when tested as recombinant protein expressed in E. coli.

We have now performed the functional studies of mutations E314K, L335P, and R460W in V79 cells, which are preferable to HEK293 cells for mitochondrial P450 enzyme activity assays. The R460W mutation has <1% of wild-type activity in this system, whereas the E314K and L335P mutations have no demonstrable activity, based on lack of time-dependent increase in pregnenolone formation. These data and the related methods are now included in the manuscript.

2. Why did these patients having cortisol deficiency have a non-ketotic hypoglycemia (manuscript text e.g. line 406). Usually, cortisol and growth hormone deficiency manifest with ketotic hypoglycemia, while insulin excess causes a non-ketotic hypoglycemia. In Table 1, ketotic hypoglycemia is noted with several patients - please check for this inconsistency.

We thank the reviewer for noticing this error. It should be ketotic hypoglycaemia, not non-ketotic. We have corrected this in lines 345 and 447.

3. For Table 1: It would be interesting to give not only the age at presentation, but also the age when the clinical and biochemical characteristics as shown were assessed (or does it anyhow reflect the same time point?)

In Table 1 we have focussed on the “Age at Diagnosis” as this is the most reliable time point that can be defined, especially as several children had their diagnosis facilitated once adrenal insufficiency was diagnosed in a sibling (as noted in the “Presentation” column; 5B, 10A, 11B , 11C and also relevant for 8A and 8B).

The biochemical data primarily relate to this time point, unless otherwise indicated in parentheses, and we have clarified this in a footnote.

The age when gonadal function was assessed is also shown in parentheses.

Clearly the onset of symptoms may have a somewhat indolent course with hyperpigmentation or failure to thrive as is typical for adrenal insufficiency, but this is more difficult to define. Of interest, subject 5B had mild progression and did not start treatment until 24 years of age as shown in the table. This is clarified in a footnote too.

We have clarified the results to read "Diagnosis occurred at variable ages…" (line 342, instead of "Presentation..") and stated the number of children who's diagnosis was aided because of family history: "In six children or young people (6/19, 32%) the diagnosis was facilitated because of a history of PAI in their siblings" (line 343-4)

Reviewer 2:
The authors present a CYP11A1 variant, which appears to be present in a significant number of cases with mild to moderate P450scc deficiency. The paper provides some explanation of the underlying mechanism causing P450scc deficiency. There are, however, experiments lacking which investigate the impact on CYP11A1 activity in cell culture.

The authors claim their findings will have implications for personalised management. They fail, however, to specify how their molecular findings will improve management in patients. There are serious doubts that medical management of adrenal insufficiency has been changed after the molecular diagnosis has been achieved. They should provide evidence for improved outcomes.

We believe there are concrete examples of where the molecular diagnosis of adrenal insufficiency has implications for the management of an individual and their family.

Examples include

1) withdrawing mineralocorticoids in children with classic FGD due to MC2R mutations, who were misdiagnosed with adrenal hypoplasia;

2) investigating for associated features that may not be immediately apparent and could be treated earlier (e.g. gonadotropin insufficiency and spermatic defect in X-linked adrenal hypoplasia; renal dysfunction and associated anomalies in SGPL1; being aware of other features and myelodysplastic syndrome risk in SAMD9/MIRAGE syndrome; monitoring for progressive gonadal dysfunction and offering semen analysis in non classic congenital lipoid adrenal hyperplasia due to partial STAR defects; and diagnosing X-linked adrenal leukodystrophy with the potential option of stem cell transplant to prevent neurological deterioration)

3) defining the inheritance pattern and identifying individuals in a presymptomatic state before the onset of adrenal insufficiency; knowing the molecular genetic basis is important as these conditions can be X-linked, recessive, dominant or imprinted.

In this study, defining the molecular basis lead to an earlier diagnosis in 6 of the 19 patients (6/19, 32%) and the potential effect of partial CYP11A1 deficiency on gonadal function means that they will be monitored in the long-term for sex steroid and gonadotropin levels, replaced if necessary, and early reproductive interventions such as sperm banking offered.

It would be useful to insert subheadings into the results and discussion. The paper can be shortened.

We have inserted subheadings into the results section. We believe that subdividing the Discussion is not house style for JES but would do so if requested by the Editors.

We appreciate that the paper is long but have included full experimental details, as this is an online journal with fewer print restrictions.

Page 4: The cohort has not been specified. It would be vital to understand the overall number of patients of this cohort and the overall distribution of other gene defects in relation to the described CYP11A1 variants. Thus, the overall relevance to ALL patients with PAI remains questionable.

Thank you for this comment. This is a very valid point as data were not presented clearly in the original submission. We have reanalysed all data from our cohorts and now include this in the manuscript.

Our original manuscript focused on PAI of unknown etiology and clear inclusion and exclusion criteria were given. However, total numbers of individuals (and families) were not given clearly, so we have provided these data in the relevant methods and results sections, and in new Supplementary Table 2. The relevant sections of the manuscript have been re-written. Overall, these data have strengthened our argument and more than 20% of children with PAI of *unknown etiology* have this partial CYP11A1 c.940G>A diagnosis.

We have also analysed the data for our cohorts as a whole and provide these findings in Supplementary Table 2. When we include those children with a genetic diagnosis of PAI due to other forms of FGD/adrenal hypoplasia, the partial CYP11A1 c.940G>A diagnosis still accounts for more than 4%. We hope we have clarified the importance of this finding now.

Line 226: Please introduce Adx.

"Adx" has been changed to "adrenodoxin (Adx, ferredoxin)" now at line 246

Line 297: Subjects have not been described in detail.

We have re-written this section (lines 325-337) based on a better description of the cohort in the methods and inclusion and exclusion criteria (lines 110-129).

Lines 310-316: Please provide a better description including number of patients with specific features.

We have provided most details of clinical features in Table 1 rather than as individual case histories. However, we have included specific numbers of children with each feature as requested (lines 342-349). We agreed this section was too vague before and is more robust with numbers. This paragraph has been re-written to state:

"Clinical details for affected individuals are shown in Table 1. Diagnosis occurred at variable ages between 6 months and 16 years. In six children or young people (6/19, 32%) the diagnosis was facilitated because of a history of PAI in their siblings. Hyperpigmentation was often present at the time of diagnosis (13/19, 68%), and many children had a history of ketotic hypoglycemia or hypoglycemic convulsions (10/19, 53%). All children were treated with glucocorticoid replacement and almost half of them had received mineralocorticoid replacement (8/19, 42%). Most children developed normally in puberty although four boys experienced pubertal delay (4/9 postpubertal males, 44%) and mildly elevated gonadotropin concentrations were found in several adults. "

Page 14: It remains unclear, why the CYP11A1 enzyme activity has not been assessed in HEK cells. This would have provided direct evidence if the enzymatic function is impaired in a cell culture-based assay.
The treatment with cycloheximide is a very crude method. Ideally a pulse chase assay should have been performed. It is also unclear why the protein used for the activity assays has not been tested for its stability.

To address this question, we have performed the activity assays in V79 hamster lung cells as described in the responses to point 1 for reviewer 1.

Lines 373-374: This sentence appears confusing. PAI commonly requires mineralocorticoid replacement and only glucocorticoid therapy.

We have rephrased this to read: "Glucocorticoid replacement is central to the management of PAI, with some patients also requiring mineralocorticoid replacement"

Lines 375-376: Please specify the percentage of unknown cases in the overall cohort and the proportion of the novel CYP11A1 variant.

These data have now been included (see above) and are shown in Supplementary Table 2.

Line 395: It would be crucial to understand the relation of the stated 10%. Are these unsolved cases or the overall cohorts of all investigators?

We agree this was unclear. We have reanalysed our cohorts and now provide exact figures. In our total cohort the prevalence of PAI due to this variant was 20.8% (16/77) in individuals with PAI of unknown etiology and 4.1 % (16/395) overall once those with an established genetic diagnosis were included. These figures were higher when individuals of European ancestry were considered.

Lines 435-440: The conclusions are not backed by the data. The problems with prediction software are well recognised.

We conclude that the rs6161 change in conjunction with another loss-of-function allele is responsible for a significant proportion of unsolved PAI especially in European populations. This is backed by the data.

'Indeed, many of the subjects reported here had a diagnostic odyssey with some taking many years to receive a genetic diagnosis, possibly due to their non-classical presentations.' We have removed this sentence.

The problems with prediction software are well recognised in some circles but there is an overreliance on common prediction tools such as SIFT and PolyPhen to predict the consequence of discovered variants. We have altered the sentence to read

"This study highlights the limitations of relying on common in silico prediction tools and the necessity for individual assessment of a polymorphism. This assessment should include testing not only the protein function in a suitable cell line and assay system but also the consequence(s) of the change at nucleic acid level."

Table 1: Please provide the glucocorticoid replacement doses.
The glucocorticoid replacement doses were mostly within the standard range of 10-15 mg/m2/day but sometime on the higher side if there was an attempt to supress the ACTH (up to18 mg/m2/day). A footnote to this effect has been added to Table 1.

Figure 1 is of poor quality and not required.

Figures were incorporated into a pdf generated from a word document. The TIF versions are of much higher quality.

Figure 1 was requested by a previous reviewer, however, we are happy to be guided by the editors as to its inclusion.

Figure 2, A and C are of very low quality and cannot be reviewed.

We have altered Figure 2, improving part 2A and removing the low quality sequence data in 2C.

Figure 4: The kinetic parameters have not been determined as claimed in the figure legend. Activities have been measured at different substrate concentrations; however, a proper kinetic analysis has not been conducted.

We regret the errors in the legend for Figure 4. Formal kinetic parameters are misleading for the CYP11A1 enzyme because the substrates are not truly soluble at these concentrations, and the transfer process to the inner mitochondrial membrane is bypassed. Hence, these activities are at best estimates, and the purpose of this experiment was to show that the purified E314K enzyme was similar to wild-type CYP11A1. We were not intending to imply quantitatively identical activities or generate imprecise kinetic parameter estimates. We have therefore amended the figure legend appropriately.

RESEARCH ARTICLES

Predicted Benign and Synonymous variants in *CYP11A1* Causes Primary Adrenal Insufficiency through Missplicing

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Nantes, F-44000 France. [16]Pediatric Endocrinology Unit, Children´s Hospital, IBIMA, Málaga, Spain. [17]Department of Endocrinology and Diabetes, Royal Brisbane and Women's Hospital. [18]Institute of Health and Biomedical Innovation, Faculty of Health, Queensland University of Technology. [19]Faculty of Medicine, University of Queensland. [20]Department of Paediatrics, University College London Hospitals, 250 Euston Road, London NW1 2BU, UK. [21]Section of Child Health, Glasgow University School of Medicine, Glasgow G12 8QQ, United Kingdom. **denoting equal contribution, [#] denoting equal contribution.*

Precis:

We demonstrated that two extremely rare synonymous variants in *CYP11A1* and one more prevalent variant (rs6161), previously designated benign, give rise to adrenal insufficiency by missplicing of RNA.

Maharaj *et al*.

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Disclosure. **The authors report no conflicts of interest in this work.**

Abstract: Primary adrenal insufficiency (PAI) is a potentially life-threatening condition that can present with non-specific features and can be difficult to diagnose. Here, we undertook next-generation sequencing in a cohort of children and young adults with PAI of unknown etiology from around the world and identified a heterozygous missense variant (rs6161, c.940G>A, p.Glu314Lys) in *CYP11A1* in 19 individuals from 13 different families (allele frequency within undiagnosed PAI in our cohort 0.102 vs 0.0026 in gnomAD, p<0.0001). Seventeen individuals harbored a second heterozygous rare disruptive variant in *CYP11A1* and

two patients had very rare synonymous changes in *trans* (c.990G>A, Thr330=; c.1173C>T, Ser391=). Although p.Glu314Lys is predicted to be benign and showed no loss-of-function in an *E. coli* assay system, *in silico* and *in vitro* studies revealed that the rs6161/c.940G>A variant, plus the c.990G>A and c.1173C>T changes, affect splicing and that p.Glu314Lys produces a non-functional protein in mammalian cells. Taken together, these findings show that compound heterozygosity involving a relatively common and predicted "benign" variant in *CYP11A1* is a significant contributor to PAI of unknown etiology, especially in European populations. These observations have implications for personalized management and demonstrate how variants that might be overlooked in standard analyses can be pathogenic when in combination with other very rare disruptive changes.

Keywords: Addison's disease, silent variant, side chain cleavage enzyme, cytochrome p450scc, CYP11A1

Introduction

The first step in steroidogenesis involves cleavage of cholesterol to pregnenolone by the cytochrome P450 side chain cleavage enzyme (CYP11A1). CYP11A1 is encoded by *CYP11A1* and is expressed in key steroidogenic tissues such as the adrenal gland and gonads. Further tissue-specific enzymatic steps lead to production of all other steroid hormones. In the adrenal gland, this ultimately results in production of glucocorticoids (cortisol) and mineralocorticoids (aldosterone) and weak androgens; and in the gonads, production of sex steroids (estrogen and testosterone). [1]

Complete loss of CYP11A1 prevents biosynthesis of all steroid hormones and was predicted to be incompatible with life due to the inability of the placenta to maintain a pregnancy without progesterone production from fetally-derived tissue. [2] However, it has become clear that biallelic mutations in *CYP11A1* are compatible with survival to term. Defects in *CYP11A1* can cause a range of phenotypes: from classical CYP11A1 deficiency with severe disruption of adrenal and gonadal steroidogenesis, causing salt-losing adrenal insufficiency and gonadal hormone deficiency, to very mild phenotypes where only glucocorticoids are affected (OMIM 613743). [3-14, Supplementary Table 1]

Massively parallel sequencing (MPS) technologies have expedited discovery of disease-causing variants. However, assigning causality to identified variants can be complex. When filtering for causal variants,

synonymous changes (which do not affect amino acid coding) may be discarded, without considering their allele frequency and variants predicted 'benign' at the protein level may be deselected too. Further, splice site changes may only be considered if they alter the canonical GT…AG motifs bordering introns. Such stringency may result in pathogenic and clinically relevant variants being missed.

Here, we have investigated a large cohort of children and young people with primary adrenal insufficiency (PAI) of unknown etiology. We show that compound heterozygous variants in *CYP11A1* involving rs6161 (c.940G>A; p.Glu314Lys) are surprisingly common, and that altered splicing needs to be considered when predicted benign or very rare synonymous changes are found.

Material and Methods

A. Subjects and Sequencing

The main focus of this study was to assess *CYP11A1* in subjects with primary adrenal insufficiency (PAI) of *unknown etiology*.

The inclusion criteria included evidence of low cortisol, attenuated cortisol response on cosyntropin stimulation testing, and elevated adrenocorticotropic hormone (ACTH), with clinical signs of cortisol insufficiency and hyperpigmentation (Table 1). Some subjects also had elevated plasma renin activity, low aldosterone and/or electrolyte disturbances (hyponatremia, hyperkalemia) consistent with mineralocorticoid insufficiency.

The exclusion criteria were any individuals with an established biochemical and/or genetic diagnosis, such as other forms of congenital adrenal hyperplasia (e.g. 21-hydroxylase deficiency, 11-beta hydroxylase deficiency, 3-beta hydroxysteroid-dehydrogenase deficiency type 2), autoimmune adrenalitis, metabolic disorders or physical cause of adrenal insufficiency (e.g. hemorrhage, infection), or known genetic causes of familial glucocorticoid deficiency or adrenal hypoplasia. Individuals with isolated hypospadias, 46,XY disorders of sex development and intrauterine growth restriction (<2SDS) with associated adrenal insufficiency were also excluded.

Patients were recruited from three main cohorts: 1) The "Barts/Royal London Hospital/QMUL" included 43 individuals with PAI of unknown etiology who were assessed by exome sequencing, targeted panel testing

or direct Sanger sequencing; 2) the “UCL/GOSH” cohort included 25 individuals with PAI of unknown etiology who were assessed by targeted panel testing; and 3) the “Turkish” cohort included 9 individuals with PAI of unknown etiology who were assessed by targeted panel and exome sequencing. Using this approach the prevalence of *CYP11A1* c.940G>A as a cause of PAI in a cohort (n=77) with no current diagnosis could be determined (Supplementary Table 2)

In order to establish the prevalence of CYP11A1 c.940G>A as a cause of PAI in these cohorts in general, total numbers of individuals recruited over the years were calculated (n=395). Although there is considerable overlap in clinical features, the “Barts/Royal London Hospital/QMUL” cohort (total n=256) focused more on classic “familial glucocorticoid deficiency” (FGD) (e.g. *MC2R*, *MRAP*, *NNT*, *AAAS*, *STAR*, *MCM4*) whereas the “UCL/GOSH” cohort (total n=57) was more focused on adrenal hypoplasia (e.g. *NR0B1*, with potential non-classic *STAR*, *NNT*, and severe *MC2R* amongst the diagnosis; almost 80% of children receiving mineralocorticoid replacement). The “Turkish” cohort (total n=82) included a range of diagnosis published recently (e.g. *MC2R*, *CYP11A1*, *MRAP*, *NNT*), and represented individuals and families with high consanguinity. [3] For this analysis individuals with hypospadias or 46,XY,DSD were excluded and children with classic forms of CAH (e.g. 21-hydroxylase), autoimmune disorders or physical causes would not have been referred.

Finally, one family (3 individuals) from Australia was included as the diagnosis had been reached by exome sequencing. This kindred were not included in prevalence figures as they did not form part of a cohort.

a) **DNA Samples**

Studies were performed with the approval of local ethics committees; Outer North East London Research Ethics Committee, reference number 09/H0701/12; London-Chelsea NRES Committee, reference number 13/LO/0224; London-Bloomsbury NRES Committee, reference number 07/Q0508/24; the Mater Hospital Ethics Committee, reference number 1931C. Following ethical approval and with informed consent, genomic DNA was extracted from whole blood of affected individuals, plus parents and unaffected siblings where available.

b) **Sequencing**

Whole exome sequencing was utilized for subjects 1, 11A, 11B, 11C; *CYP11A1* alone was sequenced for subjects 18 & 19; whilst all other subjects were analyzed by HaloPlex targeted capture array (Agilent, Santa Clara, CA) [3, 15] (see methods below). Sanger sequencing was employed to confirm segregation of the variants in kindreds where parents and/or unaffected siblings were available.

c) **Exome Sequencing**

Subject 1 was sequenced as described previously. [15] For individuals 11A, B and C, exome sequencing libraries were constructed using the Nextera Rapid Capture Exome (Illumina, San Diego, CA) according to the manufacturer's recommendations. Briefly, 50ng of genomic DNA was tagmented (fragmented and adapter sequences added) by the Nextera transposomes. Tagmented samples were purified and the fragment size confirmed using Agilent Technologies 2100 Bioanalyser. Libraries were denatured into single-stranded DNA and biotin-labelled probes specific to the target regions were used for the Rapid Capture hybridization. The pool was enriched for the desired regions by adding streptavidin beads that bind to the biotinylated probes. Biotinylated DNA fragments bound to the streptavidin beads were magnetically pulled down from the solution. The enriched DNA fragments were then eluted from the beads and hybridized for a second Rapid Capture. A second magnetic bead clean up was performed. The final libraries were analyzed on the 2100 Bioanalyzer and DNA 1000 chip kit (Agilent) to determine the quantity and size of the enriched fragments.

Massive parallel sequencing was performed with six samples per flow cell lane via the Illumina HiSeq2000 platform and version 3 SBS reagents to generate 100 bp paired-end reads. After demultiplexing, the Illumina Data Analysis Pipeline software (CASAVA v.1.8.2) was used for initial base calling. Sequence data were aligned to the current build of the human genome (UCSC Genome Browser, hg19, released February 2009) via the Novoalign alignment tool (v.2.08.02 1); sequence alignment files were converted by SAMtools (v.0.1.14) and Picard tools (v.1.42). SNPs and indels were called with the Genome Analysis Toolkit (GATK v.5506) and annotated by ANNOVAR.

Further analysis of sequence data was performed with custom scripts employing R and Bioconductor. We retained good-quality SNPs and indels (minimum depth of coverage for SNP calling: >10-fold for

homozygous SNPs, >15-fold for heterozygous SNPs). Additionally, we used variants that passed GATK Variant Quality Score Recalibration.

Remaining SNPs and indels were assessed according to prediction of potentially damaging consequence (‘‘nonsynonymous SNV’’ ‘‘splicing,’’ ‘‘frameshift substitution,’’ ‘‘stopgain SNV,’’ ‘‘stoploss SNV’’) by using both RefSeq and UCSC transcripts. Further filtering excluded SNPs with a minor allele frequency (MAF) > 0.01 observed in NCBI dbSNP (release 137), 1000 Genomes, 1000 Genomes small indels (called with the DINDEL program), the SNPs of 46Genomes release by Complete Genomics, and other whole exomes from more than 3000 control samples run internally by similar capture technology. Variants not present in any of these databases were considered novel.

d) **HaloPlex Targeted Gene Panel and Next Generation Sequencing**

A custom HaloPlex DNA target enrichment panel (Agilent Technologies Inc) was designed (SureDesign) to capture 160 known and candidate genes involved in adrenal development and function. All coding exons and 100 base pairs of intronic flanking sequence were included. The panel covered known genes potentially causing PAI, congenital adrenal hyperplasia-related genes, potential syndrome-related genes and candidate genes based on data from biochemical/biological pathways, mouse models of adrenal dysfunction, and gene expression. [3]

Sequence capture was performed according to the HaloPlex Target Enrichment Protocol version D.5 (Agilent Technologies Inc) for Illumina sequencing. Patient gDNA aliquots (225 ng) were processed in batches of 24 samples at a time with an enrichment control DNA sample as a positive control. Sequencing was performed on a MiSeq next-generation sequencer (Illumina Inc).

Sequence alignment and variant calling were performed using SureCall (version 2.0) software (Agilent Technologies Inc).

e) **Sanger Sequencing**

Potential disease causing variants were confirmed by PCR and Sanger sequencing. *CYP11A1* exons of interest including intronic boundaries were amplified by PCR using specific primers (Supplementary Table 3). The reaction mixture contained 100 ng DNA template, 1 x PCR buffer, 200 µM each dNTP, 200 nM each primer and 1 U Taq DNA polymerase (Sigma-Aldrich). Cycling conditions were: 95˚C for 5 mins (1 cycle);

95˚C for 30s, 55˚C for 30s, and 72˚C for 30s (30 cycles); and 72˚C for 5 mins. PCR products were visualized on 1% agarose gel and sequenced using the ABI Prism Big Dye sequencing kit and an ABI 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions.

f) **Sequence Interpretation**

Variants were considered highly likely to be pathogenic if they segregated with the phenotype with an appropriate inheritance pattern within families, were determined damaging or likely damaging by several bioinformatic prediction models (Ensembl Variant Effector Predictor; SIFT; PolyPhen2; and Mutation Taster – see URLs) and/or if they had been reported in association with adrenal insufficiency previously. In addition, novel missense and synonymous changes with a minor allele frequency less than 3% in the Exome Aggregation Consortium (ExAC) browser (ExAC; Cambridge, MA, http://exac.broadinstitute.org; accessed February 2018) were considered.

***g) In silico* Analysis of Variants**

Minor allele frequencies (MAF) were determined from Exome Aggregation Consortium (ExAC) and the Genome Aggregation Database (gnomAD) browsers. The functionality of the variants was assessed at all levels by variant effect predictor (VEP) and MutationTaster. Splice function was assessed using Human splicing finder 3.0, SpliceAid and ESEfinder.

h) **Statistical Analysis**

The difference in allele frequencies between subjects (counting one proband per family to avoid cascade testing bias) and controls (MAF from gnoMAD non-Finnish Europeans, determined from ExAC data) was measured by the Chi-square test with Yates' correction.

B In Vitro Splicing Assay

An *in vitro* splicing assay was designed using the commercially obtained Exontrap cloning vector pET01 (MoBiTec GmbH, Göttingen, Germany) containing an intronic sequence interrupted by a multiple cloning site. DNA fragments of interest were amplified using standard PCR protocol and specifically designed primers (Supplementary Table 4) containing a restriction enzyme target site for *Xba*I. Cycling conditions were as follows: 95°C for 5 minutes, 15 x (95°C for 30 seconds, 70°C for 30 seconds (-1°C per cycle), 72°C

for 30 seconds), 30 x (95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds) and 72°C for 5 minutes. PCR products were sequenced as above, column purified using the QIAquick® PCR Purification Kit according to the manufacturer's protocol and cloned into the Exontrap cloning vector pET01 (MoBiTec GmbH, Göttingen, Germany). Cloned sequences were verified by Sanger sequencing to ensure the fragment was in the correct orientation using pET01 specific primers (ET PRIM 06 (Forward) GCGAAGTGGAGGATCCACAAG and ET PRIM 07 (Reverse) ACCCGGATCCAGTTGTGCCA). Wild-type (pET01-WT) or mutant (pET01-MUT) plasmids were transfected into HEK293 cells using Lipofectamine® Reagent. Total RNA, obtained from cells 24h after transfection, was subjected to reverse transcription-PCR (RT-PCR) generating cDNA with primer GATCCACGATGC (MoBiTec®) and amplifying with primers within the 5' and 3' exons in the pET01 vector; primer 02 GAGGGATCCGCTTCCTGGCCC) and primer 03 (reverse sequence CTCCCGGGCCACCTCCAGTGCC) (Supplementary Table 5). Amplification products were assessed on a 2% agarose gel.

C Fibroblasts Isolation, Culture and Expansion

Fibroblast isolation and culture was performed as described previously. [17] Briefly, a 4mm punch biopsy from the upper arm (patient 1 and his parents) was immediately incubated with isolation medium (DMEM supplemented with 10% (vol/vol) FBS and 1% penicillin/streptomycin [P/S]) in a 15 ml-falcon tube. 1 square mm cubes of skin sample were digested using DMEM/high glucose, 20%FBS (vol/vol), 0.25% collagenase type-I, 0.05% DNAse-I and 1% P/S (Sigma-Aldrich) at 37°C overnight in a 15 ml-falcon tube. After centrifugation for 5 min at 1000 rpm, the pellet was resuspended in 5 ml of isolation medium before plating in gelatin coated T25 flasks. Cells were kept in human fibroblast media (DMEM/High glucose with sodium pyruvate and L-glutamine, 20% FBS (vol/vol) and 1% P/S).

D RNA Extraction and PCR Analysis.

Total RNA was purified with a RNeasy Mini Kit (Qiagen, 74106). RNase-Free DNase Set (Qiagen, 79254) was used to eliminate gDNA contamination. 50ng of RNA was incubated with 60ng/µl of random primers for 5 min at 70^{o}C followed by incubation with 500 µM dNTPs, 40 U of RNAse inhibitor, 200 U of M-MuLV RT and 1X M-MuLV buffer for 10 min at 25^{o}C, 90 min at 42^{o}C and 15 min at 70^{o}C to generate cDNA. PCR

amplifications were performed using 2 ng of cDNA, dNTPs 200 µM and Q5 DNA polymerase (NEB, M0491) using primers in exons 3 and 6 of *CYP11A1* (Supplementary Table 6).

E Protein Expression in Escherichia coli and Purification.

The F2 construct of human *CYP11A1*, adrenodoxin (Adx, ferredoxin), and ferredoxin reductase was kindly provided by Professor Walter L. Miller, University of California, San Francisco. [18] From this three different cDNAs were constructed separately by PCR; A cDNA encoding human *CYP11A1* in pcDNA3 was amplified by PCR to remove 39-amino acid N-terminal mitochondria-targeting sequence and to add a His6-tag at the C-terminus. The primers were: 11A1-sense 5'-ATACATATGGCGTCTACCCGTTCTCCT-CGCCCCTTCAATGAGAT-3'; 11A1-antisense 5'-AGAATTCTCAGTGATGGTGATGGTG-ATGCTGCTGGGTTGCTTCCT-3'. The PCR products were then cloned into pET-17b vector via *Nde*I/*Eco*RI restriction sites. The cDNA for ferredoxin reductase was amplified by PCR to include His6-tag at the C-terminus and cloned into pET-17b via *Nde*I/*Eco*RI restriction sites. The primers were: FdR-sense 5'-ATACATATGGCGTCTACCCAGGAAAAGACCCCACAG-3'; and FdR-antisense 5'-AGAATTCTCAATGGTGATGGTGATGGTGGTGGCCCAGGAGGC-GCA-3'. The sequence coding for mature human Adx (amino acids 62-184) was amplified by PCR to include His6-tag at the N-terminus and cloned into pLW01 via *Nco*I/*Eco*RI restriction sites. The primers were: Adx-sense 5'-TATACCATGGCACACCATCACCATCACCATTCATCAG-AAGATAAAATAACAGTC-3', and Adx-antisense 5'-ATGAATTCTCAGGAGGTCTTGCCC-AC-3'. QuikChange Lightning kit site-directed mutagenesis (Agilent Technologies, Santa Clara, CA) was used to generate the p.Glu314Lys variants for *CYP11A1* in both pcDNA3 and pET-17b. The primers were: sense 5'-GCAAGATGTCCTTCAAGGACATCAAGGCC-3'; antisense 5'-GGCCTTGATGTCCTTGAAGGACATCTTGC-3'. Sanger sequencing of plasmid constructs confirmed the intended site-specific mutations and ensured lack of other substitutions. Wild-type (WT) and Glu314Lys plasmid DNAs were isolated using a Qiagen (Valencia, CA) maxi prep kit.

Human Adx was expressed in *E. coli* strain C41(DE3) and purified as described. [19] Human ferredoxin reductase [20], *CYP11A1* WT [19] and Glu314Lys were expressed in C41(DE3) with pGro7 (Thermo Fisher Scientific) and purified as described previously. [21] Briefly, Fernbach flasks containing 1 liter of Terrific Broth (supplemented with 0.5 mM 5-aminolevulinic acid for *CYP11A1* [Sigma]) with 100 µg/ml ampicillin (and 20 µg/ml chloramphenicol for pGro7) (Sigma) were inoculated with 20 ml of an overnight pre-culture. The

cells were grown at 37°C with shaking at 250 rpm until the A_{600} reached 1.0-1.4 AU, at which time the culture was induced with 0.4 mM IPTG (supplemented with 4 g/L arabinose for ferredoxin reductase and P450s) and grown for 20-48 h at 28°C. After cell lysis with French press in buffer A (sterile phosphate-buffered saline containing 20% glycerol for ferredoxin reductase and *CYP11A1*), the recombinant P450 proteins were solubilized using 1% cholate (Chem-Impex International) and 0.5% NP-40. After centrifugation at 70,000 x g for 18 min, the supernatant was mixed with 3-5 ml Ni-NTA affinity resin, and polyhistidine-tagged proteins were eluted with 10 ml 250 mM imidazole, followed by buffer exchange using PD-10 columns. Purified *CYP11A1* preparations showed a specific content of 8–12 nmol P450/mg protein with 3-10% P420.

F Reconstituted Enzyme Assays.

In a 2 ml polypropylene tube, purified human CYP11A1 (10 pmol, WT or Glu314Lys) was mixed with equal amount of ferredoxin reductase, 40-fold molar excess of Adx, and dilauroylphosphatidyl choline in <10 µL volume and incubated for 5 min. The reaction mixture was then diluted to 0.2 ml with 50 mM HEPES buffer (pH = 7.4), 4 mM $MgCl_2$, 0.2% Tween 20, and substrates 22*R*-hydroxycholesterol (in ethanol) or cholesterol (in methyl-P-cyclodextrin inclusion complexes). The resulting mixture was pre-incubated at 37 °C for 3 min before adding NADPH (1 mM) and incubating at 37 °C for another 20 min. The reaction mixture was extracted with 1 ml dichloromethane, and the organic phase was dried under nitrogen flow. The steroids were reconstituted in 70 µl of methanol, and a solution of 0.1 ml 100 mM potassium phosphate buffer (pH 7.4) containing 10 µl of cholesterol oxidase (100 units/ml) was added with 3 µl internal standard (1 mM dehydroepiandrosterone). The mixture was incubated at 30°C for 6 h to convert the product pregnenolone to progesterone and the internal standard to androstenedione. The mixture was extracted with 1 ml dichloromethane, and the organic layer was dried under nitrogen flow.

a) Chromatography, Data Acquisition, and Determination of Kinetic Constants.

Reaction products were analyzed using an Agilent 1260 Infinity HPLC system with UV detector. Extracted steroid products were dissolved in 20 µl of methanol, and 5 µl injections were resolved with a 50 × 2.1 mm, 2.6 µm, C_8 Kinetex column (Phenomenex, Torrance, CA), equipped with a guard column at a flow rate of 0.4 ml/min. A methanol/water linear gradient was used: 27% methanol from 0 to 0.5 min, 39% to 16 min, 44% to 20 min, 60% to 22 min, 71% to 30 min, 75% to 30.5 min, 27% to 33 min. Steroids progesterone and

androstenedione were identified by retention times of external standards chromatographed at the beginning and ends of the experiments, and the data were processed with Laura4 software (LabLogic) and graphed with GraphPad Prism 6 (GraphPad Software, San Diego, CA).

b) Immunoblotting

One day prior to transfection, HEK-293T cells were plated in 6 well plates at 50% confluency. The following day, cells were transiently transfected with 1 µg plasmid DNA/well and 3 µl FuGENE 6 transfection reagent/well (Promega, Madison, WI). Transfected cells were treated with 25 µM CHX for the specified amount of time where indicated, and whole cell protein extracts were collected 48 hours after transfection using RIPA buffer (Sigma-Aldrich) supplemented with mini protease inhibitor tablet (Roche). Lysates were briefly sonicated for ~10 seconds, centrifuged to remove cellular debris, and resolved (25 µg total protein) on a 4-20% SDS-PAGE gel (Novex). The proteins were transferred to a PVDF membrane (Millipore, Billerica, MA), blocked with 5% fat-free milk in Tris-buffered saline with 0.1% Tween-20, and detected by rabbit anti-CYP11A1 at 1:5000 dilution (RRID: AB_2747382; generous gift from Walter Miller [22]) while mouse anti-GAPDH at 1:10000 dilution (RRID: AB_2107426) served as a loading control. Immunoreactive bands were visualized on film using HRP-conjugated mouse or rabbit secondary antibodies (1:5000, RRIDs AB_330924 and AB_2099233 respectively) in combination with SuperSignal West chemiluminescence substrate (Pierce/ThermoFisher).

c) Activity assay in intact cells

V79 hamster lung cells were plated in 12-well plates at ~70% confluency. The next day, cells were transiently transfected with 1 µg/well pcDNA3-*CYP11A1* plasmids (WT or mutations) in triplicate using TransIT-LT1 transfection reagent (Mirus) according the manufacturer's instructions. At 24 h post-transfection, cells were incubated with 1 ml of serum-free medium containing 22*R*-hydroxycholesterol (5 µM) and DHEA (control steroid, 1 µM). Aliquots of medium were removed at 6 and 24 h, and 0.1 ml of each sample was mixed with deuterium-labeled internal standards and extracted with 1 ml methyl tert-butyl ether. Pregnenolone product was converted to the oxime with hydroxylamine in aqueous ammonia and quantified using tandem mass spectrometry as described [23].

Results

A Prevalence of the CYP11A1 c.940G>A variant in PAI

Initially we undertook whole exome or targeted panel sequencing in 77 individuals or family members with PAI of unknown etiology from the three main cohorts studied (see Subjects and sequencing and Supplementary Table 2). Sixteen individuals from twelve different families were found to harbor the rs6161 variant in *CYP11A1* (chr15:74635368C>T; c.940G>A) together with another very rare heterozygous variant (Table 2 families 1 to 10; 12, 13), giving an overall prevalence of *CYP11A1* c.940G>A associated with PAI of unknown etiology of 20.8% (16/77) and a prevalence of 4.1% (16/395) in our cohorts of adrenal insufficiency patients overall (Supplementary Table 2). Even when only one proband was counted per family to avoid cascade testing bias, the MAF of rs6161 was enriched in our cohort with a frequency of 0.102 (12 out of 59 compared to a minor allele frequency (MAF) of 0.0026 across all gnomAD populations or 0.0042 in non-Finnish Europeans)(Chi-Square p<0.0001).That means approximately 1 in 200 of the population (and 1 in 120 Europeans) are heterozygous carriers of this variant, compared to 1 in 5 of the undiagnosed PAI study group. Finally, a further family from Australia with three affected individuals, was identified to have this variant together with a second change and was included in our series (family 11, Table 1). Consequently, compound heterozygosity of rs6161 with another disruptive variant in *CYP11A1* was identified in 19 individuals from 13 families in total. Validation of the variants and segregation with disease was confirmed by Sanger sequencing in these patients, and in family members where possible, showing that the variants occurred in *trans*. [Table 2, Figure 1].

Clinical details for affected individuals are shown in Table 1. Diagnosis occurred at variable ages between 6 months and 16 years. In six children or young people (6/19, 32%) the diagnosis was facilitated because of a history of PAI in their siblings. Hyperpigmentation was often present at the time of diagnosis (13/19, 68%), and many children had a history of ketotic hypoglycemia or hypoglycemic convulsions (10/19, 53%). All children were treated with glucocorticoid replacement and almost half of them had received mineralocorticoid replacement (8/19, 42%). Most children developed normally in puberty although four boys experienced pubertal delay (4/9 postpubertal males, 44%) and mildly elevated gonadotropin concentrations were found in several adults.

The protein change resulting from the recurrent rs6161, p.Glu314Lys, is predicted benign by commonly used algorithms such as SIFT and PolyPhen, but 'disease causing' by MutationTaster2. Furthermore, the nucleotide change, in exon 5, is predicted to affect splicing (HSF3.0, ESEfinder) (Supplementary Table 7). The *trans* variants were all extremely rare with MAF ranging from zero to 2.2 $x10^{-5}$ in gnomAD (Table 2). In many cases the variant resulted in an obviously deleterious effect; either an early stop gain mutation (p.Arg120Ter, p.Arg439Ter and p.Arg424Ter), a deletion or insertion causing frameshift and premature stop codon (c.566dupC, c.835delA and c.790_802del) or a canonical splice site change likely to cause skipping of the adjacent exon giving rise to a prematurely truncated mRNA (c.1158-2A>G and c.426-2A>G). These alleles are liable to be destroyed by nonsense-mediated mRNA decay (NMD). The missense variants p.Leu335Pro, seen in two siblings (Family 5) and the p.Arg460Trp, seen in family 13 are in conserved residues and, on analysis, were predicted deleterious (SIFT) or probably damaging (PolyPhen). However, most intriguingly, two very rare synonymous variants were observed (c.990G>A and c.1173C>T), with no predicted amino acid change (Thr330= and Ser391=) in exons 5 and 7 respectively (Figure 1). Both were designated 'disease-causing' by MutationTaster2 and predicted to alter splicing (HSF 3.0 results in Supplementary Tables 8 & 9).

B Investigation of Splicing

To investigate potential splicing effects of these variants, a series of functional studies were undertaken. SNP rs6161 (c.940G>A) is within exon 5, 51bp upstream of the natural splice donor site of intron 6, c.990G>A occurs at the last base of exon 5 and c.1173C>T is within exon 7, 16bp downstream of natural acceptor site of intron 6 (Figure 1). Utilizing minigene constructs (Figure 2A), the wild-type allele (c.940G) showed inclusion of exon 5 but the variant allele (c.940A), caused exon skipping (Figure 2B). Similarly, we showed that variant c.990A caused exon 5 skipping, while variant c.1173T caused the complete skipping of exon 7 (Figure 2B). Of note, the wild-type exon 7, c.1173C, is also reported to show a degree of exon skipping *in vitro*, consistent with the skipping of this exon in certain transcripts (CD013982) reported in Jin *et al*. [24] In each instance exon skipping would result in a frameshift and premature translation-termination codon, p.Ala277AspfsTer11 (for exon 5 variants) and p.Leu387HisfsTer29 (for exon 7 variant), if the message is not destroyed *in vivo* by nonsense-mediated mRNA decay. Sanger sequencing of cDNA

fragments confirmed the variant caused the skipping of its containing exon in each case, with no evidence of cryptic splice site usage (data not shown).

In order to investigate potential splice effects *in vivo*, we studied genomic DNA and RNA-derived cDNA in fibroblasts from Subject 1 and his parents. By PCR of exon 5 in genomic DNA it was confirmed that he had inherited the c.940G>A variant from his mother and the c.990G>A variant from his father (Figure 3A). PCR of genomic DNA demonstrated that both alleles were equally represented (data not shown). However, at the RNA level there was complete absence of the c.990A allele, with no exon skipped RNA detected by an RT-PCR from exon 4-6 (Figure 3A) and both patient and father's sequence showing wild-type c.990G only (Figure 3B). This finding would be consistent with NMD of the variant RNA species. In contrast, the c.940A allele showed evidence of skipping of exon 5 alongside production of normally spliced, exon 5 containing, transcript; in the subject and his mother's RNA two bands were seen, the upper corresponding to exons 4-5-6 and the lower consisting of a transcript lacking exon 5 (bands at 384 and 223bp respectively in Figure 3A) (confirmed by Sanger sequencing, data not shown). As expected, the patient's RNA contains only the mutant c.940A transcript at this position as the paternal "wild-type" allele has presumably been degraded by NMD. In the mother both c.940A and c.940G transcripts are detected with the c.940A being less abundant than the wild-type, consistent with exon skipping and NMD for the majority of the variant allele (see the relative height of the peaks for the mutant 'A' and wild-type 'G' seen in the mother's RNA) (Figure 3B arrowed).

C Assessment of Protein Function

Given the likelihood therefore that some of the c.940A (rs6161) transcripts will be translated into protein, we evaluated the function of the resultant mutant p.Glu314Lys protein. The mutation was recreated by site-directed mutagenesis, expressed in *E. coli*, and the catalytic activity of CYP11A1 measured by assays of cholesterol and 22*R*-hydroxycholesterol conversion to pregnenolone. Despite the substitution of negatively-charged Glu314 with a positively-charged lysine residue, the mutant enzyme exhibited similar side-chain cleavage activities to wild-type CYP11A1 with either substrate (Figure 4A, B). However, when cDNA encoding p.314Lys was transfected into HEK-293 cells the protein was truncated (degraded), and the half-life after cycloheximide (CHX) treatment was much shorter than for WT (Figure 4C). The truncated protein was consistent in size with potential proteolytic cleavage of the protein around the p.Glu314Lys change and,

taken together with the splicing results, suggests relatively little functional CYP11A1 would be produced from this allele. To ascertain whether enough protein escapes degradation in intact cells to produce pregnenolone and to test for activity in the other two identified missense mutations, V79 cells were transfected with pcDNA3 expression plasmid encoding wild-type CYP11A1 or the three mutations. Pregnenolone formation from 22*R*-hydroxycholesterol was measured with tandem mass spectrometry. Mutation p.Arg460Trp demonstrated <1% of wild-type activity, whereas mutations p.Glu314Lys and p.Leu335Pro yielded no time-dependent pregnenolone formation (Table 3).

Discussion

Primary adrenal insufficiency is a life-threatening condition. Glucocorticoid replacement is central to the management of PAI, with some patients also requiring mineralocorticoid replacement. Approximately twenty different genetic causes of PAI have been identified to date (www.icped.org), but in a substantial proportion of individuals the cause is not currently known.

The enzyme CYP11A1 plays a key role in the initial steps of steroidogenesis by catalyzing the cleavage of cholesterol to pregnenolone. Children with marked disruption of this enzyme typically present with severe salt-losing adrenal insufficiency in the first few days of life and a complete block in gonadal steroidogenesis, with female-typical external genitalia in 46,XY infants or a lack of puberty in 46,XX girls. [3-5, 9-14] More recently, partial loss-of-function of CYP11A1 has been reported in children with delayed-onset adrenal insufficiency and hypospadias, or glucocorticoid insufficiency alone. [6-8] The glucocorticoid pathway seems especially vulnerable to loss of CYP11A1 activity, potentially due to the much higher molar concentrations of cortisol that are required, and even low levels of residual enzyme activity appear to be sufficient for fetal gonadal steroidogenesis (Supplementary Table 1. [6-8]. Similar findings have been shown for steroidogenic acute regulatory protein (*STAR*), a related protein involved in facilitating cholesterol transfer into mitochondria, where complete disruption causes congenital lipoid adrenal hyperplasia affecting adrenal and sex hormones, but partial dysfunction is associated with predominant glucocorticoid insufficiency. [25, 26]

This current study included individuals or families with primary adrenal insufficiency where the current cause was unknown. In thirteen families (19 affected individuals) massively parallel sequencing revealed the rs6161 (c.940G>A, p.Glu314Lys) variant in *CYP11A1* in compound heterozygosity with another very rare and/or deleterious variant. We showed that the rs6161 variant and two rare synonymous variants (Thr330=

and Ser391=) can cause missplicing, giving rise to absent or dysfunctional proteins thereby contributing to the pathogenesis in primary adrenal insufficiency. In our cohort, compound heterozygosity for the c.940G>A with a second disruptive variant accounted for more than 20% of undiagnosed primary adrenal insufficiency and was one of the most common causes of familial PAI. Our observations are supported by the recent case report of compound heterozygosity for c.940G>A with a disruptive splice variant (c.425+1G>A) in a boy with an indolent presentation of primary adrenal insufficiency from 3 years of age and a history of hypospadias. [14] Indeed, the relatively high MAF of rs6161 in the population (approximately 1 in 120 Europeans are carriers) suggests that there is a natural “pool” of this variant in this population and can manifest a phenotype when in combination with a very rare disruptive variant in *trans*. Four individuals in gnomAD (out of 138,595 individuals, or 277190 alleles) are homozygous for rs6161 (c.G>940A) but it is not known whether this is associated with any adrenal phenotype.

Clinically, the majority of affected individuals reported here were at the mild end of the spectrum, having been diagnosed with “familial glucocorticoid deficiency” or “Addison’s disease”. For most, onset occurred during childhood rather than in the neonatal period and hypoglycemic convulsions or ketotic hypoglycemia were a common feature. Others had a more indolent course during adolescence or were only diagnosed because of family history and mild symptoms, highlighting the importance of making this diagnosis and investigating “at risk” individuals. Glucocorticoid insufficiency was common to all affected individuals but approximately half needed mineralocorticoid (MC) replacement and disordered sex development was not seen. Three males had delayed puberty, but others progressed through puberty normally, and two older males have fathered children. However, monitoring of MC activity and gonadal function will be needed for long term management, and more detailed long-term studies will be important to understand the natural history of this condition. Of note, heterozygous parents appear unaffected, as is usual for disorders of steroidogenesis.

The rs6161 allele alters splicing of the pre-mRNA sequence with the result that many RNA transcripts do not include exon 5. The transcripts that do include exon 5 will give rise to a mutant protein with an amino acid change, glutamic acid to lysine, at residue 314. Although the mutation reverses the charge on this side chain from negative to positive, the residue is located on the surface of the protein in the I-helix facing solvent and has no effect on protein function. Given this ambiguity, we went on to show that the purified p.314Lys

mutation has normal spectral and catalytic properties in *E. coli* but gives rise to an unstable truncated protein of 30-35kDa when expressed in mammalian cells. *In vivo*, in affected individuals, it is likely that the combination of defective splicing and protein dysfuction will result in low levels of functional, full-length protein derived from this allele. This may be sufficient for gonadal steroidogenesis but insufficient for glucocorticoid production. Furthermore, our studies have only been possible in skin fibroblasts or non-steroidogenic cells, so it is possible that higher residual function occurs in the adrenal gland or gonad.

Tools for prediction of the functional effects of non-synonymous variants are well-established [27], but these may not predict the effect such changes have on splicing. In particular, variants causing benign amino acid changes may have their effect on mRNA processing or post-translational modification. Synonymous or 'silent' changes are often ignored and, when tested, commonly used prediction tools may prove inconclusive. Up to 45% of synonymous SNPs are likely to alter pre-mRNA splicing and regulatory information may be dispersed throughout nearly every nucleotide in an exon [28, 29], making it important to consider them in variant analyses, especially when very rare and discovered in known or likely causative genes.

Conclusion

We conclude that the rs6161 change in conjunction with another loss-of-function allele is responsible for a significant proportion of unsolved PAI especially in European populations. This study highlights the limitations of relying on common *in silico* prediction tools and the necessity for individual assessment of a polymorphism. This assessment should consider not only the protein function in a suitable cell line and assay system but also the consequence of the change at nucleic acid level.

Appendix

Web Resources;

Human Splicing Finder 3.0 http://www.umd.be/HSF3/

Mutation Taster http://www.mutationtaster.org/

PolyPhen-2 http://genetics.bwh.harvard.edu/pph2/

Sorting Intolerant from Tolerant (SIFT) http://provean.jcvi.org/genome_submit_2.php

dbSNP, www.ncbi.nlm.nih.gov/SNP/

Exome Aggregation Consortium (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org) [Accessed February 2018].

The Genome Aggregation Database, http://gnomad.broadinstitute.org/ [Accessed February 2018]

Variant effect predictor http://www.ensembl.org/Tools/VEP

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

The Human Gene Mutation Database: http://www.hgmd.cf.ac.uk/ac/index.php

Ingenuity variant analysis: http://www.ingenuity.com/

Otogenetics: http://www.otogenetics.com/

ESEfinder 3.0 http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home

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Figure Legends

Figure 1. Position of variants in CYP11A1 genomic/pre-mRNA sequence found in this series of PAI patients.

Boxed in bold the three predicted benign or synonymous variants assessed for their effect on splicing; SNP rs6161 (c.940G>A, p.Glu314Lys) is 110bp from the start and 51bp from the end of exon 5, c.990G>A (p.Thr330=) occurs at the last base of exon 5 and c.1173C>T (p.Ser391=) is 16bp from the start of exon 7.

Figure 2. An *in vitro* assay reveals aberrant splicing of variants c.940G>A, c.990G>A and c.1173C>T.

(A) Minigene construction. Diagram of exon 5 and parts of flanking introns 4 and 5, inserted into the multiple cloning site (MCS) of the pET01 construct (lower). In the pET01 construct, the intron containing the MCS is flanked by the 5'-donor and 3'-acceptor splice sites of pre-proinsulin 5' and 3' exons, respectively (green arrows) (http://www.mobitec.com/cms/products/bio/04_vector_sys/exontrap.html). The expression of this vector sequence was driven by the promoter present in the long terminal repeat (LTR) of Rous Sarcoma Virus followed by a short stretch of a eukaryotic gene (phosphatase). The sequences containing the mutations detected in *CYP11A1* exons 5 and 7 (MUT) or those that did not (WT) were cloned into the MCS of pET01. The primers used in the reverse transcription-PCR (RT-PCR) experiments within the preproinsulin 5' and 3' exons are indicated by the black arrows (Supplementary Table 5). (B) Representative results of RT-PCR analysis using HEK293 cells transfected with an empty pET01 vector (empty vector), the pET01 vector containing the wild-type exon or the mutant exon; on the left the c.940A in exon 5, in the middle the c.990A change in exon 5, on the right the C.1174T change in exon 7. A transcript of 225bp was observed in the empty and mutant vector RT-PCRs, for all variants investigated, corresponding to the two-exon amplification product resulting from splicing of the preproinsulin 5' and 3' exons from the vector. For wild-type vectors containing c.940G and c.990G, a 386 bp transcript was observed corresponding to the three-exon amplification product resulting from correct splicing of *CYP11A1* exon 5 between the two vector exons. The intermediate band for exon 5 constructs, at approximately 350bp (asterisked), was shown to be a mixture of sequence including both the 386 and 225 bp bands. For wild-type c.1173C, a 304bp transcript corresponding to the size of the three-exon amplification product resulting from correct splicing of *CYP11A1* exon 7 was observed. Sanger sequencing confirmed these findings (data not shown).

Figure 3. Sequence analysis of *in vivo* splicing in patient 1 and his parents. (**A)** RT-PCR amplification products utilising primers in exons 4 and 6 of *CYP11A1*. Lanes 1 and 2, patient 1 and his mother show two bands corresponding to transcripts containing exon 5 (upper band) and skipping exon 5 (lower band), this was confirmed by Sanger sequencing. In contrast, in lane 3 the father shows only the upper, exon 5 containing, transcript. **(B)** Partial chromatograms from Sanger sequencing of the upper bands in the patient, mother and father. For the c.990G>A change (on the right) the patient and father's sequence reveals only the wild-type c.990G (white arrows), suggesting that the c.990A variant results in exon skipping and the destruction of the truncated mRNA by NMD. For c.940G>A (on the left) the patient's sequence only has the mutant c.940A allele A inherited from his mother (black arrow), corroborating the NMD destruction of the c.990A allele inherited from his father, since if it were present it would give a heterozygous base at this position. In contrast the mother's sequence shows both wild type G and a small peak of the mutant A (black arrow) consistent with skipping of mutant exon 5 in most mutant transcripts but revealing the presence of some transcripts containing exon 5.

Figure 4. The p.314Lys protein evaluation. A. Side-chain cleavage activity of CYP11A1 mutant p.314Lys is unaltered in *E. coli*. Expressing the purified mutant protein in *E. coli*, showed comparable activity (black bars) as the wild-type enzyme (grey bars). The activity of the mutant p.314Lys was determined to be indistinguishable from wild-type protein whether the substrate was 22*R*-hydroxycholesterol (left panel) or cholesterol (right panel). **B.** The p.314Lys variant exhibits altered protein expression compared to wild-type when expressed in HEK-293T cells. HEK-293T cells were transiently transfected with either wild-type CYP11A1 or p.314Lys constructs and cultured for 48 hours. For the indicated times prior to protein collection, the cells were treated with 25 µM CHX. Whole cell lysates were analyzed by immunoblot and probed with anti-CYP11A1 and anti-GAPDH antibodies. For the wild-type, a full-length protein was observed at 60kDa, whereas for the p.314Lys mutant, the protein was truncated to 30-35kDa, consistent with a cleavage event around the site of the amino acid change. Cycloheximide treatment revealed the mutant protein also has a shorter half-life.

Table 1. Clinical presentation of 19 individuals with *CYP11A1* mutations

Subject	Sex	Age at diagnosis [a]	Presentation	Replacement	ACTH at presentation (pg/ml)	Cortisol at presentation (peak stimulated) (nmol/l)	Na/K (mEq/l)	Plasma renin activity (ng/ml/h unless stated)	Aldosterone (pmol/l)	Puberty	FSH (iU/L) /LH (iU/L) /testosterone (nmol/l)/oestradiol (pmol/l)	Comments
1	M	10y	Hypoglycemic convulsions, vomiting, hyperpigmentation	GC	149	234 (peak 118)	142/4.4	2.0 (<3.0)	300	Normal	FSH 3.4, LH 4.1, testosterone 14.8 (21y)	Originally diagnosed with ketotic hypoglycemia. Published in Chan *et al.* (15)
2	M	9m	Hypoglycemia (ketotic)	GC	1002	93	N	N	ND	Delayed then progressed	N	Fertile
3	F	11m	Failure to thrive, anorexia, hyperpigmentation	GC	>1500	190	N	ND	496	Normal	ND	Brother died age 3y with similar features
4	M	11m	Pneumonia, hypoglycemia, collapse	GC, MC	155 (had been on treatment)	90 (peak 264)	132/4.0	5.3	ND	Normal	FSH 9.0, LH 7.4, testosterone 20.3 (16y)	
5A	F	16y	Secondary amenorrhea/galactorrhea, pituitary corticotrope adenoma, hyperpigmentation	GC	3354	108 (peak 154)	ND	2.4	1136	Normal (secondary amenorrhea)	N	Pituitary macroadenoma, prolactinemia. Published in Benoit *et al.* (16)
5B	F	14y [b]	Investigated as sister diagnosed	GC (24y)	400	276 (peak 303)	ND	N	416	Normal	ND	-

6	M	4y	Recurrent illnesses, hyperpigmentation	GC, MC	1147	252 (peak 79)	133/5.4	ND	ND	Delayed then progressed	FSH 18.3, LH 9.1 (16 years); FSH 9.0, LH 6.1, testosterone 13.2 (25y)	-
7	M	7y	Recurrent illnesses, hyperpigmentation	GC, MC	1091	31	N	89.4/202 mU/l	<100	N/A (prepubertal)	N/A	-
8A	M	9y	Convulsions, hyperpigmentation, younger brother diagnosed	GC, MC	6128 (23y)	339 (peak 389)	137/3.5	9.7 (23y); 83.5 miU/l (<40) (38y)	ND	Delayed then progressed	FSH 13.8, LH 5.8, testosterone 24.7 (38y)	-
8B	M	8y	Convulsions, hyperpigmentation, younger brother diagnosed	GC, MC	264 (17y) (on treatment)	278 (peak 301)	141/3.6	10.2 (21y)	ND	Delayed then progressed	FSH 9.3, LH 5.4, testosterone 33.3 (36y)	-
8C	M	1.5y	Febrile convulsions, hypoglycemia, hyperpigmentation	GC, MC	4356	174 (peak 178)	129/5.9	20.9 (<2.0) (17y); 119.2 miU/l (<40) (32y)	ND	Normal	FSH 41.2, LH 33.9, testosterone 27.2 (32y)	-
9	M	2y	Hypoglycemia, salt-wasting, hyperpigmentation	GC, MC	463	157	127/6.8	ND	<100	Normal	FSH 14, LH 15, total testosterone 7 ng/ml (15y)	-
10A	F	15y	Investigated as brother diagnosed	GC	67	129 (peak 279)	141/4.2	2.5 (0.5-3.1)	380	Normal	N	

10B	M	5y	Recurrent illnesses, ketotic hypoglycaemia, hyperpigmentation	GC	385	116 (172)	N (Na128 vomiting)	3.1 (<7.0)	140	N/A (prepubertal	N/A	
11A	F	4y	Hypoglycemic seizure, cardiac arrest, died	GC	>1250	73	ND	ND	ND	N/A (died)	N/A (died; normal ovaries on autopsy)	Autopsy: small adrenals with normal zona glomerulosa and atrophied zona fasciculata
11B	F	2y	Hyperpigmentation , elder sister diagnosed	GC	686	Peak 55	140/4.0	45 mU/l (3-35)	155	Normal	FSH 3.6, LH 3.2, estradiol 200 (14y)	Hypertension treated with captopril; small adrenal glands on CT
11C	M	11m	Investigated as sister diagnosed	GC	350	226 (276)	137/4.1	70 mU/l (3-35) (14y)	227 (14y)	Normal	FSH 14, LH 4.5, testosterone 14 (14y)	Hypertension treated with Ramipril. Also treated for steroid responsive nephrotic syndrome (minimal change disease)
12	F	6m	Hyperpigmentation	GC	2896	14 (14)	ND	54 ng/l (<150)	97	N/A (prepubertal)	N/A	
13	F	3y	Hypoglycaemia, hyperpigmentation , lethargy	GC, MC	1487	64 (with blood glucose 1.6mmol/l)	131/4.3	27.7	137	N/A (prepubertal)	N/A	

Notes: [a] Age of diagnosis is shown which corresponds to the age investigations were undertaken unless indicated. [b] Regular replacement therapy was initiated at a later age. ACTH pg/ml x 0.22 for pmol/l, normal range 10-60 pg/ml. Cortisol nmol/l x 0.036 for µg/dl, normal peak stimulated value > 550 nmol/l; plasma renin activity pmol/ml/hr x

1.3 for ng/ml/h; aldosterone pmol/l x 0.36 for pg/ml; testosterone nmol/l x 28.9 for ng/dl; estradiol pmol/l x 0.27 for pg/ml. Additional normal ranges are shown as well as age at sampling, Some variation may occur due to assay methods and age of the patient.

Abbreviations: M, male; F, female; y, years; m, months; GC, glucocorticoid; MC, mineralocorticoid; N, within normal range; ND, not done; N/A, not applicable

Table 2. Genetic diagnoses in 19 patients with *CYP11A1* mutations

Subject	Genomic co-ordinates and nucleotide change (Genome assembly GRCh37.p13)	cDNA position and nucleotide change (transcript NM_000781)	Protein (prediction)	dbSNP/HGMD (if annotated)	Number of alleles present In gnomAD/number sequenced in gnomAD MAF gnomAD	Predicted[#] and/or tested consequence	Segregation	Country	Sequencing method
rs6161 variant common to all									
All	74635368C>T	940G>A	E314K	rs6161	710/277190 (4 homozygotes) 0.002561	missense[#]/skipping of exon 5 = p.A277Dfs*11	Yes	various	various
Variants *in trans* with rs6161									
1	74635318C>T	990G>A	T330=	NA	not seen 0	silent[#]/skipping of exon 5 p.A277Dfs*11 and NMD	Yes	UK	WES
2	74635473delT	835delA	I279Yfs*10	rs757299093/ CD050132	7/277156 4.061e-6	early stop[#]	Yes	UK	HaloPlex
3	74631031G>A	1315C>T	R439*	rs755975808	2/277174 7.216e-6	early stop[#]	Yes	Spain	HaloPlex
4	74631641G>A	1173C>T (ND)	S391=	rs751829641	6/277178 2.165e-5	silent[#]/skipping exon 7 = p.L387Hfs*29	ND	UK	HaloPlex
5A	74632081A>G	1004T>C	L335P	NA	not seen 0	missense[#]	Yes	France	HaloPlex
5B	74632081A>G	1004T>C	L335P	NA	not seen 0	missense[#]	Yes	France	HaloPlex
6	74637586T>C	c.426-2A>G	exon 3 skip	rs754329273	2/245202 8.157e-6	skipping of exon 3[#] resulting in p.K142Nfs*3	ND	UK	HaloPlex
7	74631076G>A	1270C>T	R424*	rs762412759	5/277032 1.805e-5	truncation/NMD[#]	ND	UK	HaloPlex

8A	74636157_74636169del	c.790_802del	K264Lfs*5	NA	not seen 0	early stop[#]	Yes	UK	HaloPlex
8B	74636157_74636169del	c.790_802del	K264Lfs*5	NA	not seen 0	early stop[#]	Yes	UK	HaloPlex
8C	74636157_74636169del	c.790_802del	K264Lfs*5	NA	not seen 0	early stop[#]	Yes	UK	HaloPlex
9	74631658T>C	c.1158-2A>G	exon 7 skip	NA	not seen 0	skipping of exon 7 resulting in p.L387Hfs*29[#]	ND	Turkey	HaloPlex
10A	74640308G>A	c.358C>T	R120*	NA	2/246224 8.123e-6	early stop	ND	UK	HaloPlex
10B	74640308G>A	c.358C>T	R120*	NA	2/246224 8.123e-6	early stop	ND	UK	HaloPlex
11A	74635473delT	c.835delA	I279Yfs*10	rs757299093/CD050132	7/277156 4.061e-6	early stop[#]	Yes	Australia	WES
11B	74635473delT	c.835delA	I279Yfs*10	rs757299093/CD050132	7/277156 4.061e-6	early stop[#]	Yes	Australia	WES
11C	74635473delT	c.835delA	I279Yfs*10	rs757299093/CD050132	7/277156 4.061e-6	early stop[#]	Yes	Australia	WES
12	74637444dupG	c.566dupC	S191Lfs*10	NA	not seen 0	early stop	ND	Canada	*CYP11A1* sequencing
13	74630968G>A	c. 1378C>T	R460W	rs535782968	2/246170 8.124e-6	missense[#]	ND	UK	*CYP11A1* sequencing

Notes: #, predicted consequence of variant at protein level.

Abbreviations: NA, not applicable; ND, not determined, NMD, nonsense-mediated mRNA decay; WES, whole exome sequencing.

Table 3. CYP11A1 activity in transfected V79 cells

	Pregnenolone formed (pg/well)	
CYP11A1	*6h*	*24h*
Wild-type	23238 ± 3919	48113 ± 10369
R460W	105 ± 28	388 ± 226
L335P	84 ± 15	73 ± 19
E314K	83 ± 3	86 ± 30

Figure 1

Click here to access/download;Figure;alternate fig 1.pptx

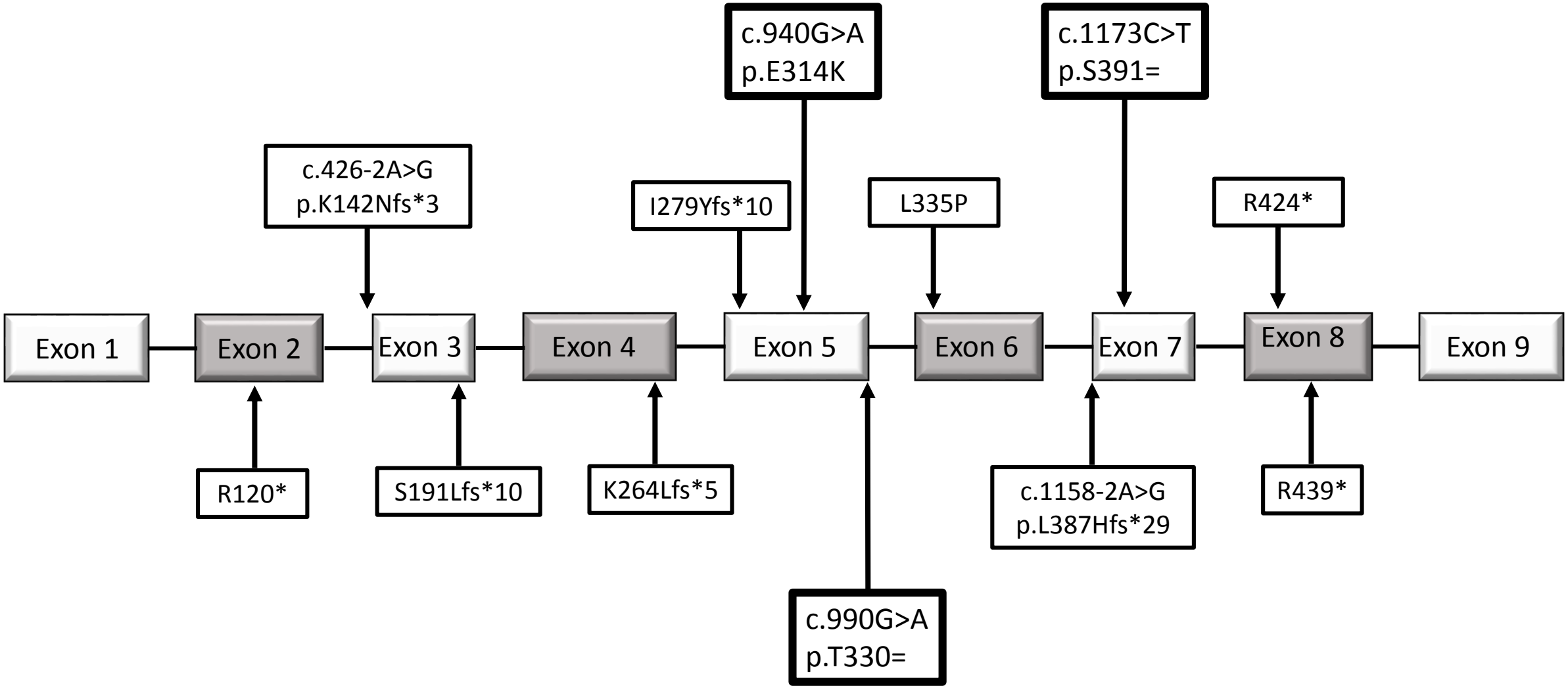


Figure 2

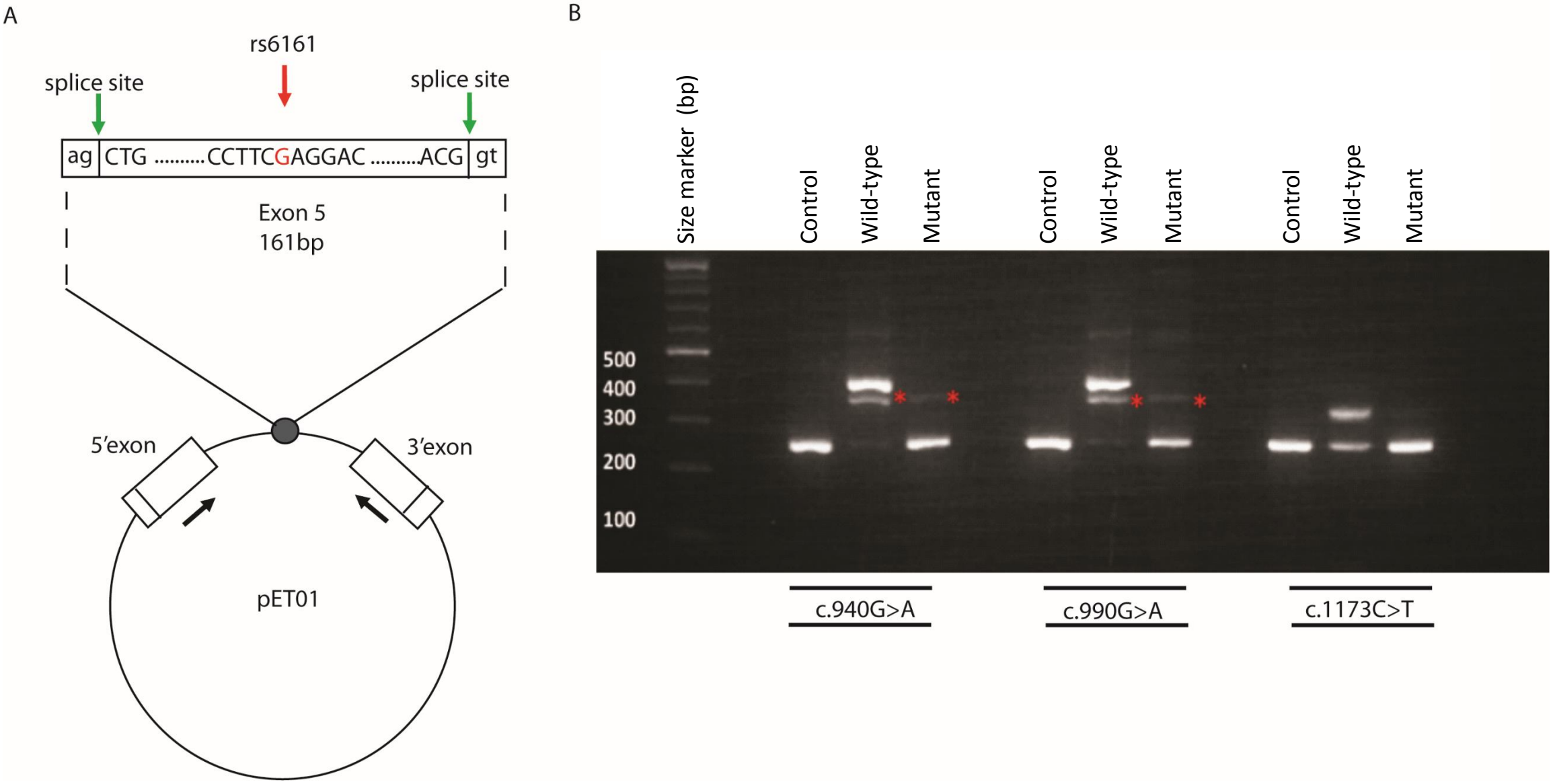
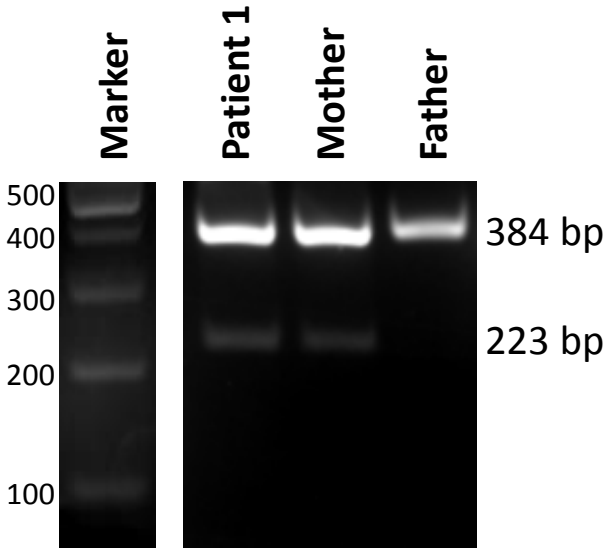


Figure 3

A



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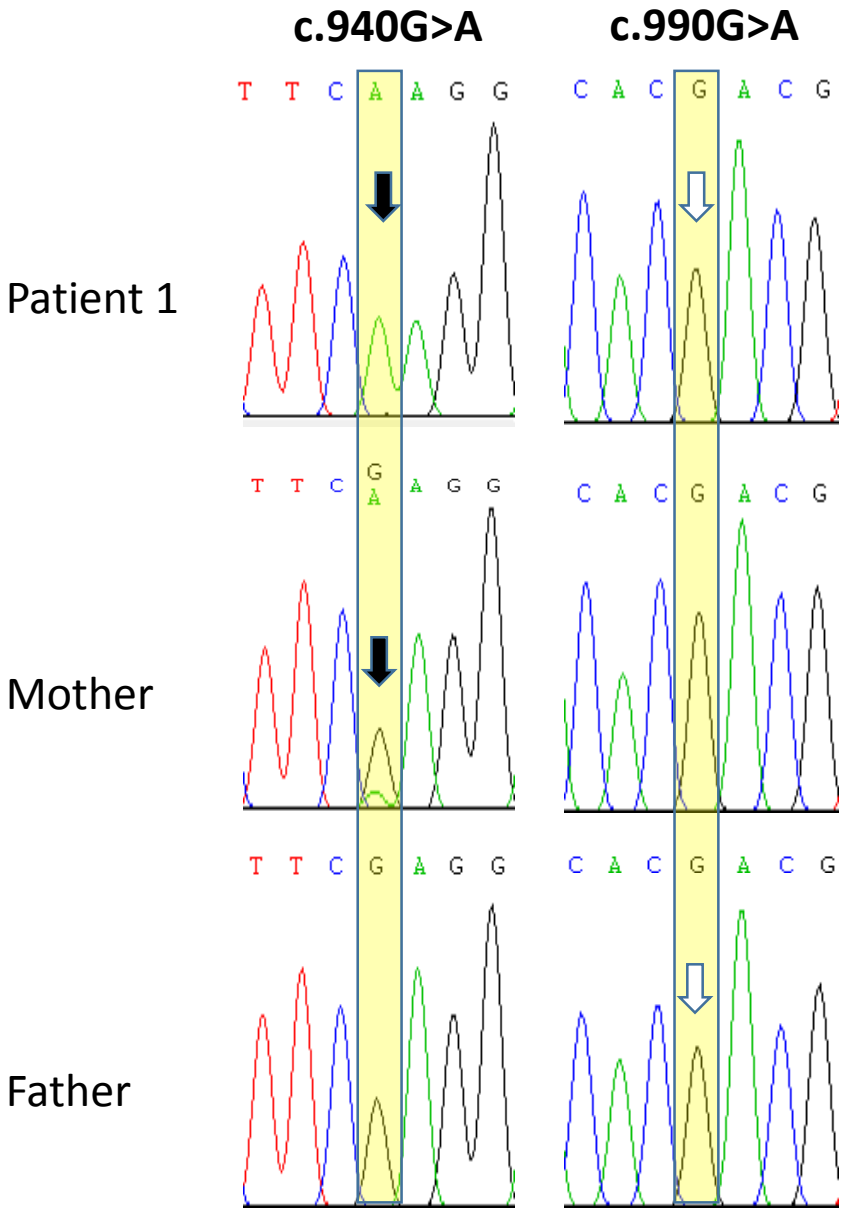

Figure 4