

## Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Noordam C, Dhir V, McNelis JC, et al. Inactivating *PAPSS2* mutations in a patient with premature pubarche. *N Engl J Med* 2009;360:2310-8.

## **SUPPLEMENTAL APPENDIX**

### **CASE REPORT**

Clinical studies in the patient were carried out at the Endocrine Unit of the Department of Pediatrics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. The patient's medical history was otherwise unremarkable. She had been delivered by Caesarean section at 38 weeks of gestation due to signs of intrauterine distress; however, postnatal Apgar Scores were 7/10 and 8/10 at 1 and 5 min, respectively, thus there were no signs of postnatal asphyxia. She had shown normal growth velocity with a constant height standard deviation score (SDS) of -1.0 from birth until her presentation at age 8 years.

Congenital adrenal hyperplasia due to 21-hydroxylase, 11 $\beta$ -hydroxylase, or 3 $\beta$ -hydroxysteroid dehydrogenase type 2 deficiencies was excluded by normal baseline and cosyntropin-stimulated values of cortisol, 17-hydroxyprogesterone, 11-deoxycortisol and 17-hydroxypregnenolone plasma levels. These findings were confirmed by 24-h urine steroid metabolite analysis showing normal excretion of pregnanetriol, 17-hydroxypregnanolone, pregnanetriolone, tetrahydro-11-deoxycortisol and 5-pregnenetriol; the ratio of (tetrahydrocortisol + allo-tetrahydrocortisol) / tetrahydrocortisone was also normal, excluding cortisone reductase deficiency. Thyroid function tests, serum prolactin and IGF-1 were normal at ages 8 and 12 years as were fasting plasma glucose and insulin at age 12 years. Magnetic resonance imaging of the adrenals at age 8 years revealed normal size, shape and location of both adrenal glands; ultrasound at 12 years of age showed no evidence of polycystic ovarian morphology.

## METHODS

### *DNA SEQUENCE ANALYSIS*

Genomic DNA was isolated from peripheral blood leucocytes. PCR amplification of the coding sequences of human *SULT2A1* (GenBank accession number NC\_000019.8, NM\_003167), *PAPSS1* (NC\_000004.10, NM\_005433) and *PAPSS2* (isoform a, NC\_000010.9, NM\_004670) including exon-intron boundaries was performed employing specific primers listed in **Supplemental Table 1**, followed by direct sequencing on an ABI3700 automated sequencer and analysis by Lasergene sequence analysis software (DNASTAR, Madison, WI, USA). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was carried out for mutation confirmation using specific primers listed in **Supplemental Table 1**.

### *mRNA EXPRESSION ANALYSIS*

Tissues were homogenized mechanically in TRI Reagent® Solution (Ambion) until completely dissolved prior to isolation of total cellular RNA by chloroform precipitation. Reverse transcription of 1µg RNA was performed using Multiscribe™ Reverse Transcriptase and random hexamer primers (Applied Biosystems, Foster City, CA, USA). cDNA amplification was carried out employing specific primers for *SULT2A1*, *PAPSS1*, and *PAPSS2* listed in **Supplemental Table 1**. Quantitative mRNA expression analysis was carried out using an ABI 7500 system (Perkin Elmer Biosystems, Warrington,UK). Specific human real-time primers and probes were purchased as predesigned expression assays (Applied Biosystems). All reactions were correlated to expression of ribosomal 18S as internal reference;  $\Delta\text{Ct}$  values were calculated as the difference between Ct of the target gene minus Ct of 18S, where Ct is the cycle number at which logarithmic PCR plots cross a calculated threshold line.

***FUNCTIONAL ANALYSIS OF PAPSS2 MUTANTS***

Mutant *PAPSS2* cDNAs were generated *in vitro* by site-directed mutagenesis employing the QuikChangeII site-directed mutagenesis kit (Stratagene) and specific primers (**Supplemental Table 1**) with the bacterial expression vector pGEX6 containing the wild-type coding sequence of *PAPSS2a* as a template; all resultant inserts were sequenced in their entirety to confirm the mutations. Subsequently, the pGEX6 vectors were transformed into *Escherichia coli* strain BL21 (DE3) obtained from Stratagene. Transformed cells were grown at 30°C to an OD600 of 0.4 in Luria Broth supplemented with 50 µg/l of carbenicillin (Sigma) and then induced with 50 nM IPTG for 16 hours. Cells were harvested, resuspended into lysis buffer (PBS) with 1% Triton X-100), and lysed by 3 rounds of rapid freeze/thaw followed by sonication. Cytosolic fractions were prepared by centrifugation at 30,000g for 15 min at 4°C; protein content was measured using a Bradford assay (Bio-Rad).

DHEA sulfation assays co-incubating bacterially expressed human PAPSS2a and SULT2A1 protein were performed in a total volume of 200 µl in assay buffer (150 mM Tris HCl (pH 7.0), 50 mM KCl, 15 mM MgCl<sub>2</sub>, 3 mM EDTA, 45 mM dithiothreitol), supplemented with 5 mM ATP and 10 mM sodium sulfate. Reactions were carried out using 5 µM <sup>3</sup>H DHEA (Amersham) and a 1:1 mix of PAPSS2a (wild-type or mutant) and wild-type SULT2A1 protein incubated for 60 min at 37°C, determined as the linear range of the enzymatic reaction by preceding experiments. Steroids were extracted with 5 ml dichloromethane, concentrated by evaporation at 55°C, separated by thin layer chromatography on PE SIL G/UV silica gel plates (Whatman, Maidstone, Kent, UK), and developed using a solvent system consisting of chloroform/methanol/acetone/acetic acid/water (8:2:4:2:1). Steroids were quantified using a Bioscan 2000 image analyzer (Lablogic, Sheffield, UK).

**AUTHOR CONTRIBUTIONS**

W.A. designed the study; C.N. and V.D. contributed to the design. C.N. identified the patient and gathered clinical and biochemical data, with the help of H.C.v.d.G., J.A.S., and F.C.G.S. Genetic and *in vitro* data were gathered and analyzed by V.D., J.C.M., F.S., N.H., N.K., and R.S. Data analysis was overseen by W.A. and C.N, who both vouch for the data and the analysis and decided to publish the paper. The first draft of the paper was written by C.N., V.D., and W.A.; all authors contributed to the writing of the manuscript, and W.A. wrote the final draft.

**SUPPLEMENTAL TABLE 1.**Specific primer pairs for genetic and mRNA expression analysis of *SULT2A1*, *PAPSS1*, and *PAPSS2*.

		<i>SULT2A1</i>	<i>PAPSS1</i>	<i>PAPSS2</i>
<b>Primers for mRNA expression analysis *</b>				
	Fwd	caggaagaacctagagaagatctg	ctgctggcatgcctcatc	cactcccctcaaaggttc
	Rev	gtcttacacaatgaccccagtc	gtggccccctctgttactag	cagcgtctcgtaatagtc
<b>Sequencing primers **</b>				
Exon 1	Fwd	ggtggctacagttgaaacce	gccctcctcttgctac	aagggagtgcgacgtgtc
	Rev	tgactgaatggacaggaac	gttctcccacgaacg	cactcttactctcctc
Exon 2	Fwd	gcgtgagccacatgctc	gtggaaagcagtaaacac	catgtatcagtttcgaattaaag
	Rev	ggcatatcagtgttgaaaggc	gttatatgctgtgatgctc	catctcccagcctccttctaatg
Exon 3	Fwd	tcaaaaagagtgaggattgactg	gcagagcaagactcttg	gtcatcttaaatatccaggccg
	Rev	gggtgtcaaaagaggctcgg	aacaagagatggtagctg	cttgactgtgtgtgggaatcg
Exon 4	Fwd	tagactggatgcctgctctc	cactaaattggatgagaag	ggctattgaaaaccaaagtacacag
	Rev	taaggatgggtgagaggg	tcaagtcactccaatc	caaggaagatttctgaggacag
Exon 5	Fwd	gccagccttgtctttctc	gcgagataactaacttc	gtcaaggatggctgtttgacc
	Rev	catgcatgccgtgtattctg	cacaacacctcacacac	gaaatgaacagcattgtaaaaac
Exon 6	Fwd	ggtggagacaggtaaggag	tagcactcacagccttc	ggtaggtgaaccggttgc
	Rev	caaggacaggagaatcaatgctc	gctaataccaccctg	ggagaagagggttaaaaataactgg
Exon 7	Fwd		ctcatccttactgtttg	catagaaggttctgccctcatc
	Rev		agtcttataatgctacctc	acactgtaaatgatccaaacag
Exon 7B	Fwd			tgctgtaagattcgtttggt
	Rev			gaactaatagcgattccaactg
Exon 8	Fwd		gtcactggatctacagc	cttgatttgggtcttaatgcttc
	Rev		ccaatgaaaagtcaatacac	cattctccacctaateccag
Exon 9	Fwd		agagtagcttacaacgac	ctgaaggcagttcttaactgtaac
	Rev		tattfacctgtactcatcg	gttcagtgagctgagatcg
Exon 10	Fwd		tgcgtatcctttgaaag	gccagtgataatgaatgcacag
	Rev		cagaaaagcaatcggatg	cagaaaggatcccagagactca

Exon 11	Fwd	agatttctaccgataatg	gttgactcacattgctgaattac
	Rev	gtcatcagtaaagattagac	gagagttttcaagggcc
Exon 12	Fwd	gaacagaaggaacaaatgc	ccattatttcccttcttctg
	Rev	ccaacagagactatgtg	agcacttcagaaagaactc
<b><i>Primers for PCR-RFLP analysis***</i></b>			
c.143C>G mutation	Fwd		catgtatcagtttcgcaattaaag
	Rev		ctttcacagttgacttgatc
c.985C wild-type	Fwd		agggtagctatcttac
	Rev		cattctccacctaatcccag
c.985C>T mutation	Fwd		agggtagctatcttat
	Rev		cattctccacctaatcccag

\* Amplification of specific products by polymerase chain reaction was carried out using the following conditions: initial denaturation at 94°C (5min) followed by 32 cycles of 30 seconds at 94°C, 30 seconds at 55°C (*PAPSS1/PAPSS2*) or at 50°C (*SULT2A1*) and 30 seconds at 72°C with a final extension of 7 minutes at 72°. Gel electrophoresis analysis for *SULT2A1* and *PAPSS1* was performed on 1% agarose gels, whereas *PAPSS2a* and *PAPSS2b*, with a size difference of only 15bp, were separated on a 3% MetaPhor® (Lonza, Rockland, ME, USA) gel. The gels were stained with ethidium bromide and PCR products visualized under UV light.

\*\* Polymerase chain reactions were carried out using the following conditions: initial denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 95°C for 30 s, annealing (starting at 60°C, with stepwise reduction by 2°C every four cycles down to 50°C for the last 16 cycles) for 30 s, and extension at 72°C for 30 s. Final extension was done for 7 min at 72°C.

\*\*\* The point mutation c.143C>G was confirmed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) employing the specified primers for amplification, followed by enzymatic digestion employing the restriction enzyme *DdeI* for 1 hour at 37°C and subsequent fragment separation on a 1.5% agarose gel. The point mutation c.985C>T was confirmed by amplification with primer pairs designed to only amplify the wild-type and the mutant allele, respectively.

## SUPPLEMENTAL FIGURE LEGENDS

### **SUPPLEMENTAL FIGURE 1. Skeletal x-rays taken in the patient at the age of 12 years.**

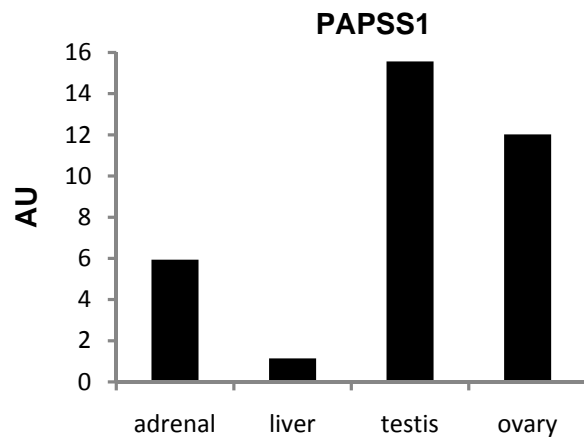
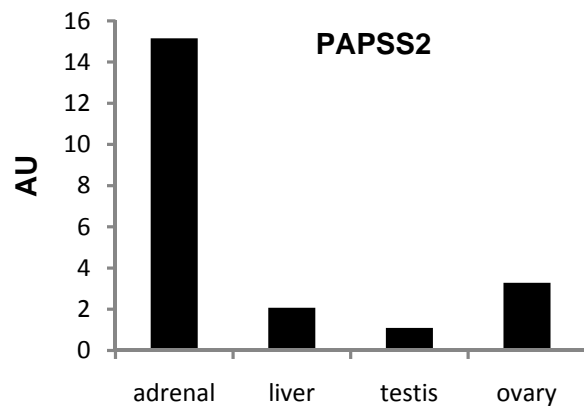
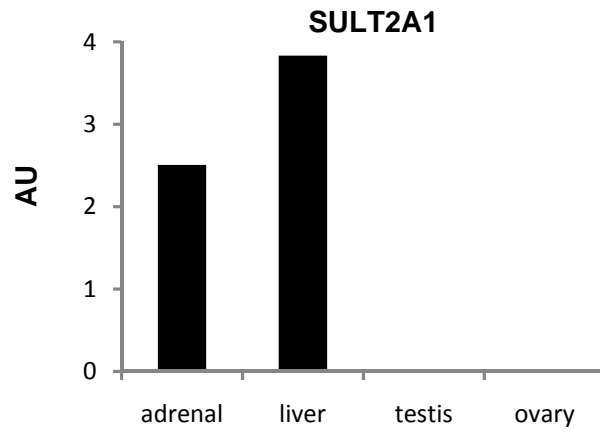
Spinal x-ray (**Panel A**) depicting flattened vertebrae (platyspondyly) with reduced intervertebral disk space and slightly irregular endplates within the thoraco-lumbar region. By contrast, x-ray analysis of the long bones (**Panels B+C**, left leg; **Panel D**, left am) did not reveal any evidence of epiphyseal or metaphyseal changes.

**SUPPLEMENTAL FIGURE 2. Results of real-time analysis of quantitative mRNA expression of *SULT2A1*, *PAPSS2* and *PAPSS1*** confirming adrenal and liver as major sites of DHEA sulfation co-expressing *SULT2A1* and *PAPSS2* at high levels. Conversely, ovary and testis express only relatively low *SULT2A1* and *PAPSS2* mRNA levels but contain high levels of *PAPSS1* mRNA that is found in much lower abundance in adrenal and liver.

Results are expressed in arbitrary units (AU) with the table providing  $\Delta$ CT values  $\pm$  standard deviation.



**Suppl.  
Fig. 1**



	<b>SULT2A1</b>	<b>PAPSS2</b>	<b>PAPSS1</b>
<b>Adrenal</b>	11.9 ± 0.1	12.7 ± 0.0	17.4 ± 0.0
<b>Liver</b>	11.3 ± 0.1	15.6 ± 0.1	19.7 ± 0.2
<b>Testis</b>	18.6 ± 0.0	16.5 ± 0.1	16.0 ± 0.0
<b>Ovary</b>	20.8 ± 0.1	14.9 ± 0.0	16.3 ± 0.0

**Suppl.  
Fig. 2**