

# Nonclassic Congenital Lipoid Adrenal Hyperplasia: A New Disorder of the Steroidogenic Acute Regulatory Protein with Very Late Presentation and Normal Male Genitalia

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**Context:** Lipoid congenital adrenal hyperplasia is a severe disorder of adrenal and gonadal steroidogenesis caused by mutations in the steroidogenic acute regulatory protein (StAR). Affected children typically present with life-threatening adrenal insufficiency in early infancy due to a failure of glucocorticoid (cortisol) and mineralocorticoid (aldosterone) biosynthesis, and 46,XY genetic males have complete lack of androgenization and appear phenotypically female due to impaired testicular androgen secretion *in utero*.

**Objective:** The objective of this study was to investigate whether nonclassic forms of this condition exist.

**Patients and Methods:** Sequence analysis of the gene encoding StAR was undertaken in three children from two families who pre-

sented with primary adrenal insufficiency at 2–4 yr of age; the males had normal genital development. Identified mutants were tested in a series of biochemical assays.

**Results:** DNA sequencing identified homozygous StAR mutations Val187Met and Arg188Cys in these two families. Functional studies of StAR activity in cells and *in vitro* and cholesterol-binding assays showed these mutants retained ~20% of wild-type activity.

**Conclusions:** These patients define a new disorder, nonclassic lipoid congenital adrenal hyperplasia, and represent a new cause of non-autoimmune Addison disease (primary adrenal failure). (*J Clin Endocrinol Metab* 91: 4781–4785, 2006)

L IPOID CONGENITAL ADRENAL hyperplasia (CAH) (OMIM no. 201710), the most severe form of adrenal hyperplasia, significantly impairs adrenal and gonadal steroidogenesis by a defect in the conversion of cholesterol to pregnenolone (1). Affected infants experience salt loss from impaired mineralocorticoid and glucocorticoid synthesis, but hormonal replacement therapy permits long-term survival (2). Because the fetal testis is affected, 46,XY genetic males fail to produce testosterone and are born with female external genitalia. The defect in lipoid CAH is primarily in the steroidogenic acute regulatory protein (StAR) (3, 4), which facilitates the entry of cholesterol into mitochondria, where it becomes the substrate for the cholesterol side-chain cleavage enzyme, P450<sub>scc</sub> (1, 5). Unlike abnormalities in StAR, which is not expressed in placenta, defects in P450<sub>scc</sub> will also disrupt placental progesterone synthesis, presum-

ably interrupting pregnancy, although rare mutations in P450<sub>scc</sub> have been reported in children with adrenal failure (6–9).

In most steroidogenic disorders, such as steroid 21-hydroxylase deficiency, a spectrum of clinical findings results from different missense mutations. However, in lipoid CAH, the clinical findings are remarkably similar; all individuals reported to date have had female external genitalia irrespective of chromosomal sex and have had evidence of salt loss in the first year of life, usually within the first 2 months (3, 4, 10–13).

We report three children from two families who have a novel, mild form of lipoid CAH with their initial clinical manifestations at 2–4 yr and normal development of the male genitalia in 46,XY individuals. This new disorder, termed nonclassic lipoid CAH, illustrates that the clinical presentations of steroidogenic disorders can vary substantially from their classic descriptions and identifies a new form of non-autoimmune Addison disease (primary adrenal failure).

## Patients and Methods

### Case histories

Patient 1, a Pakistani (Gujakhan) 46,XX phenotypic female, had an uneventful infancy, experienced fever and vomiting at 2 yr of age, and was hypoglycemic during a viral illness at 4 yr. Progressive hyperpig-

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Abbreviations: CAH, Congenital adrenal hyperplasia; CT, computed tomography; IMM, inner mitochondrial membrane; NBD, nitrobenzoxadiazol; OMM, outer mitochondrial membrane; PRA, plasma renin activity; 22R-OH, 22R-hydroxycholesterol; StAR, steroidogenic acute regulatory protein.

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**TABLE 1.** Clinical and biochemical characteristics of patients with nonclassic lipoid CAH

	Val187Met	Arg188Cys		Normal range
	Patient 1	Patient 2	Patient 3	
Age at investigation (yr)	4.5	2.2	2.8	
Presentation	Hypoglycemia, pigmentation	Pigmentation	Pigmentation	
Adrenal imaging	Normal US	Normal CT	Normal CT	
ACTH (pg/ml)	>1250	>1250	>1250	10–50
Cortisol				
Basal ( $\mu\text{g/dl}$ )	<1.0	6.2	11.3	5–15
Peak ( $\mu\text{g/dl}$ )	<1.0	5.6	10.5	>20
PRA (ng/ml/h)	5.3	5.4	20.2	0.3–3.9
Aldosterone (ng/dl)	15.5	6.7	12.4	4–31
Treatment	Hydrocortisone	Hydrocortisone, fludrocortisone	Hydrocortisone, fludrocortisone	

Conversion to Systeme International units: ACTH, picograms per milliliter  $\times 0.22$  for picomoles per liter; cortisol, micrograms per deciliter  $\times 27.6$  for nanomoles per liter; PRA, nanograms per milliliter per hour  $\times 0.77$  for picomoles per milliliter per hour; aldosterone, nanograms per deciliter  $\times 27.7$  for picomoles per liter. US, Ultrasound scan.

mentation prompted referral at 4.5 yr. Basal cortisol was undetectable ( $<1.0 \mu\text{g/dl}$ ), ACTH was elevated ( $>1250 \text{ pg/ml}$ ), and cortisol did not respond to stimulation with cosyntropin (Table 1). Plasma renin activity (PRA) was mildly elevated ( $5.3 \text{ ng/ml/h}$ ; normal range,  $0.3\text{--}3.9$ ), but sodium ( $138 \text{ mEq/liter}$ ), potassium ( $4.2 \text{ mEq/liter}$ ), and aldosterone ( $15.5 \text{ ng/dl}$ , normal range  $4\text{--}31$ ) were normal. She has remained well on hydrocortisone replacement therapy ( $11\text{--}13 \text{ mg/m}^2/\text{d}$ ), with normal electrolytes and PRA ( $1.1\text{--}2.9 \text{ ng/ml/h}$ ).

Patients 2 and 3, Pakistani (Memon) 46,XY brothers from a consanguineous family, were born after uneventful pregnancies and had normal male genitalia with descended testes. The older boy developed hyperpigmentation at 1.5 yr; investigation at 2.2 yr of age revealed low basal cortisol ( $6.2 \mu\text{g/dl}$ ), elevated ACTH ( $>1250 \text{ pg/ml}$ ), and no cortisol response to cosyntropin stimulation, indicating compensated primary adrenal failure (Table 1). PRA was mildly elevated ( $5.4 \text{ ng/ml/h}$ ), and aldosterone was at the lower range of normal ( $6.7 \text{ ng/dl}$ ). The younger brother also became progressively pigmented. At 2.8 yr of age, his cortisol ( $11.3 \mu\text{g/dl}$ ), aldosterone ( $12.4 \text{ ng/dl}$ ), and electrolytes were normal, but his ACTH ( $>1250 \text{ pg/ml}$ ) and PRA ( $20.2 \text{ ng/dl}$ ) were elevated, and cortisol did not respond to cosyntropin. Computed tomography (CT) scans did not show adrenal enlargement. Both brothers responded to replacement therapy with  $9\alpha$ -fludrocortisone and hydrocortisone.

#### Sequence analysis, mutagenesis, transfection, and steroid measurement

DNA was extracted from blood leukocytes with institutional review board approval. The seven exons of the human StAR gene were amplified by PCR and sequenced as described (11, 14). PCR-based site-directed mutagenesis was used to recreate the mutations found in the patients' DNA in a full-length StAR cDNA expression vector (15), and the resulting plasmids were sequenced to ensure accuracy. Using Lipofectamine (Invitrogen, Carlsbad, CA), nonsteroidogenic monkey kidney COS-1 cells were cotransfected at 50–80% confluence with a StAR expression vector and the F2 plasmid expressing a fusion protein of the cholesterol side-chain cleavage system ( $\text{H}_2\text{N-P450scc}$ -adrenodoxin reductase-adrenodoxin-COOH) (16). Culture media were immunoassayed for pregnenolone 48 h later.

#### Biochemical assays

The modifications of the pTWIN1 intein vector (New England Biolabs, Beverly, MA) for bacterial expression of wild-type and mutant N-62 StAR, the preparation and quantitation of proteins, the steroidogenic assays with isolated MA-10 cell mitochondria, and the assays of cholesterol binding were performed as described previously (17), except that nickel-nitrilotriacetic acid chromatography was added after the chitin-binding column for the Val187Met mutant.

#### Structural modeling of missense mutants

Our computational model of human wild-type N-62 StAR (17, 18) was modified by introducing missense mutations using the Swiss-Model program (<http://expasy.ch/spdbv/>). Energy minimization was performed with Amber 7 (<http://amber.scripps.edu>) (University of California, San Francisco, Computer Graphics Laboratory). The resulting models were checked with WHAT IF (<http://swift.cmbi.kun.nl/WIWWWI/>), and images were generated with Chimera (<http://www.cgl.ucsf.edu/chimera>).

## Results

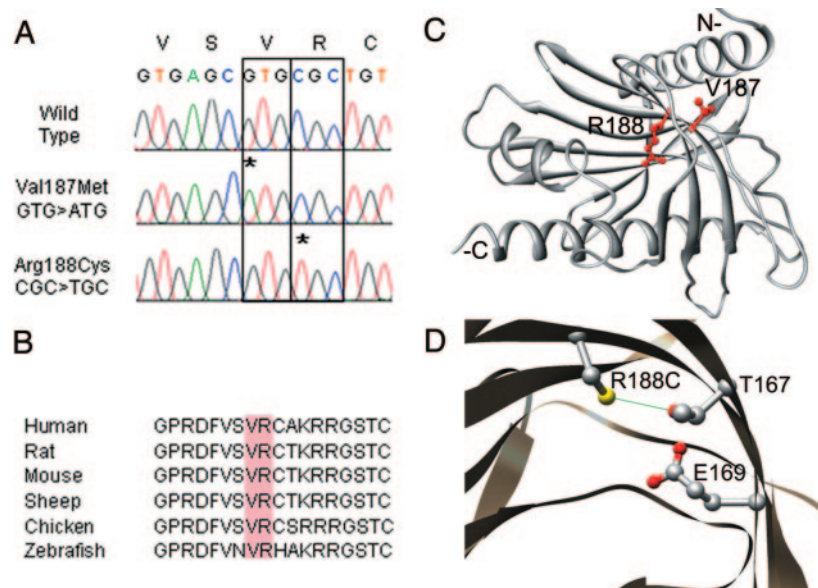
#### Mutation analysis

The clinical findings of compensated glucocorticoid and mineralocorticoid deficiency in the presence of apparently normal fetal testicular steroidogenesis suggested an abnormality in a gene affecting adrenal function, but sequencing of the ACTH receptor (MC2R), DAX1 (AHC, NR0B1), and SF1 (steroidogenic factor 1, NR5A1) was normal. However, the gene for StAR contained the homozygous mutation Val187Met in patient 1 and the homozygous mutation Arg188Cys in patients 2 and 3 (Fig. 1A). These residues are highly conserved among vertebrate species (Fig. 1B). The parents were heterozygous, and these mutations were not detected in 200 control Pakistani alleles, indicating these were functional mutations and not polymorphisms.

#### Structural modeling of missense mutations

To visualize the effects of these mutations, we used a validated computational model of human StAR, showing that Val187 and Arg188 lie in sheet  $\beta_6$ , which contributes to the sterol-binding pocket (17, 18) (Fig. 1C). Arg188 forms a salt bridge with Glu169, coordinating with the  $3\text{OH}$  group of cholesterol to facilitate its binding (Fig. 1D). Mutation of Glu169 to Gly or Lys causes lipoid CAH (4), confirming the essential role of this residue, but the folding of the Glu169Gly mutant is not as greatly disturbed as other severe mutants (19). The adjacent Val187 residue does not have an obvious function, but the modeling shows that its mutation to Met should inhibit cholesterol binding sterically.

FIG. 1. Mutation analysis. A, DNA sequencing: *top*, wild-type control; *middle*, patient 1 with the Val187Met mutation; *bottom*, patient 2 with the Arg188Cys mutation. B, Alignment of the sequences of residues 180–197 of the human, rat, mouse, sheep, chicken, and zebrafish StAR proteins; residues 187 and 188 are *highlighted*. C, Ribbon diagram of N-62 StAR, positioned to permit both Val187 and Arg188 to be seen; the amino (N) and carboxy (C) termini are indicated. D, Arg188 normally forms a hydrogen bond with Glu169; changing Arg188 to Cys eliminates hydrogen bonding with Glu169 and creates a weak hydrogen bond with Thr167.



### Functional analysis

StAR increases the flow of cholesterol from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone to initiate steroidogenesis. The activity of StAR mutants is typically assayed by transfecting nonsteroidogenic cells with vectors expressing the cholesterol side-chain cleavage enzyme and StAR and measuring the amount of pregnenolone produced. Use of a null StAR vector provides a negative control, whereas addition of 22R-hydroxycholesterol (22R-OH), which bypasses the action of StAR, provides a positive control (3, 4, 13, 15). We cotransfected COS-1 cells with a vector expressing the F2 fusion of the cholesterol side-chain cleavage enzyme system and with a series of StAR expression vectors and assayed pregnenolone (Fig. 2A). The lipofectamine transfection protocol used here is more efficient than the calcium phosphate procedure used in a previous report (13), resulting in greater production of pregnenolone. In the absence of StAR, the F2 enzyme exhibits a low level of StAR-independent steroidogenesis (4, 16) at  $10.7 \pm 1.7$  ng pregnenolone/ml culture medium. In the presence of 22R-OH, cells expressing F2 made  $321.7 \pm 57.4$  ng/ml, indicating the maximal level of steroidogenesis achievable under these conditions. Using their endogenous cellular cholesterol as substrate, cells cotransfected with F2, and wild-type StAR generated  $105.2 \pm 11.7$  ng/ml. In contrast, the Val187Met mutant generated  $31.1 \pm 6.9$  ng/ml, and the Arg188Cys mutant generated  $23.5 \pm 5.0$  ng/ml. Thus, when the background of StAR-independent steroidogenesis is subtracted, the Val187Met and Arg188Cys mutants have 21.6 and 13.6% of wild-type activity, respectively; the activities of the two mutants were significantly different ( $P = 0.03$ ) by a paired two-tailed Student's *t* test.

### Biochemical analyses

To explore the mechanism by which these mutants retain partial activity, we expressed each StAR protein in bacteria using a self-splicing bacterial intein system. We purified

these proteins to homogeneity and tested their activities in two cell-free systems. First, as an independent assay of the capacity to induce steroidogenesis, we added each StAR protein to mitochondria isolated from steroidogenic mouse Leydig MA-10 cells and measured pregnenolone produced from endogenous mitochondrial cholesterol (Fig. 2B). Wild-type N-62 StAR elicited five times more steroidogenesis than did buffer or the wholly inactive StAR mutant Met144Arg (13, 17). After subtracting the buffer control, the Val187Met and Arg188Cys mutants elicited 28.6 and 17.7% of wild-type activity, respectively; this difference between the two mutants was statistically significant ( $P = 0.005$ ) by a paired two-tailed Student's *t* test. Second, we found that the reduced activity of these mutants was associated with reduced cholesterol-binding capacity (Fig. 2C). Wild-type N-62 StAR could bind either [ $^{14}$ C]cholesterol (not shown) or fluorescent nitrobenzoxadiazol (NBD) cholesterol, indicating that the NBD group did not affect binding (17). The cholesterol-binding capacity of the Val187Met mutant was 25.5% of control, and the capacity of the Arg188Cys mutant was 21.0% of the capacity of the wild-type N-62 StAR. Thus, both mutants retained partial cholesterol-binding capacity and partial activity to induce steroidogenesis.

### Discussion

StAR mediates the rapid actions of ACTH and angiotensin II on the adrenal and of LH on the gonad, permitting very rapid rises in the circulating concentrations of steroids in response to acute physiological stimuli. StAR elicits this action by facilitating rapid movement of cholesterol from the OMM to the IMM, where it is converted to pregnenolone. The mechanism by which StAR moves cholesterol from OMM to IMM is only partially understood. StAR acts exclusively on the OMM (15, 20), where it undergoes acid-induced conformational changes apparently induced by protonated OMM phospholipids (17, 21, 22). StAR has a sterol-binding pocket that accommodates one cholesterol molecule (17, 18), but each molecule of StAR can mobilize approximately 400 mol-



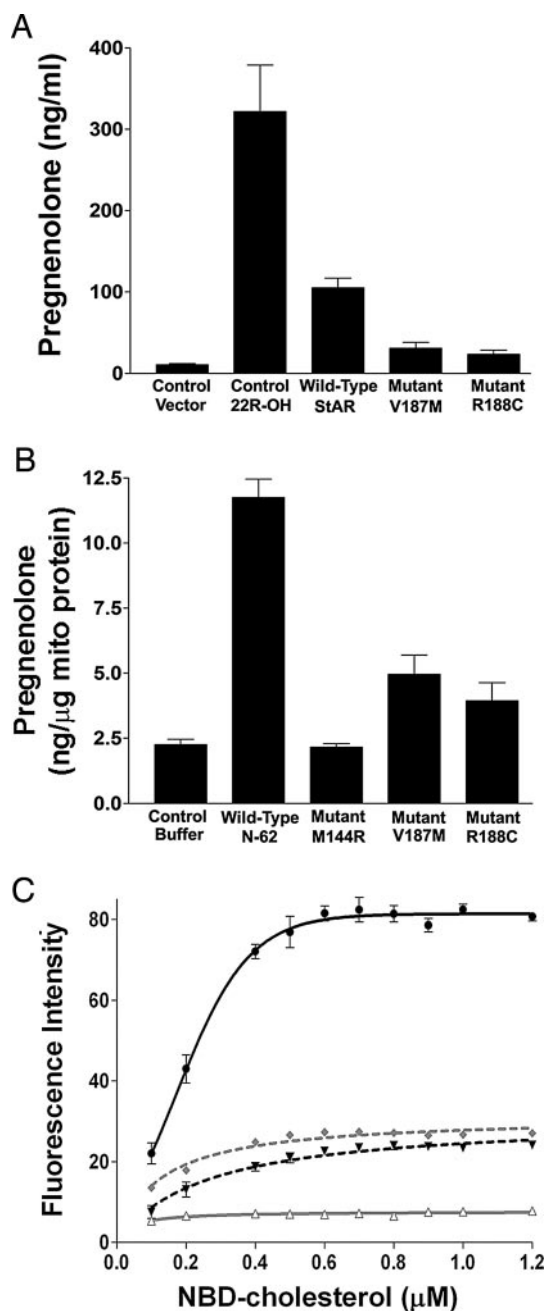


FIG. 2. Activity of StAR mutants. A, Activity in intact cells. COS-1 cells were cotransfected with expression vectors for the cholesterol side-chain cleavage system and the indicated StAR vector, and pregnenolone was measured 48 h later. Addition of the StAR-independent substrate 22R-OH in the absence of StAR indicates the maximum steroidogenic capacity of the cells. Data are the mean  $\pm$  SEM from six transfections, each performed in triplicate. B, Activity on mitochondria *in vitro*. Mitochondria were isolated from steroidogenic mouse Leydig (testicular) MA-10 cells, the indicated human N-62 StAR proteins were added, and pregnenolone synthesis from endogenous mitochondrial cholesterol stores was measured. Data are mean  $\pm$  SEM from three experiments, each performed in triplicate. C, Cholesterol-binding capacities. Binding of various concentrations of fluorescent NBD cholesterol by wild type (black solid line), Val187Met (gray dotted line), and Arg188Cys (black dotted line) was measured with a protein concentration of 1  $\mu$ M; control is buffer without protein (gray solid line). Data are mean  $\pm$  SEM for three experiments, each performed in triplicate.

ecules of cholesterol (23). Although StAR is required for acute steroidogenic responses, low levels of StAR-independent steroidogenesis (e.g. in the placenta) proceed in its absence (3, 4). The presence of StAR-independent steroidogenesis explains the pathophysiology of lipoid CAH by a two-hit model (4). Mutations in StAR ablate the acute response (the first hit), resulting in low circulating steroid levels and compensatory increases in ACTH, angiotensin II, and LH. These increased tropic hormones stimulate cellular uptake of LDL cholesterol and increased *de novo* cholesterol biosynthesis, leading to accumulation of cholesterol and cholesterol esters in lipid droplets in steroidogenic cells, eventually destroying all steroidogenic capacity (the second hit). The two-hit model explains the unusual findings in lipoid CAH. Salt loss appears relatively late because fetal angiotensin II drive is minimal, so that the adrenal zona glomerulosa remains relatively undamaged until after birth; 46,XX patients spontaneously feminize in adolescence because the fetal ovary is steroidogenically quiescent and first experiences tropic stimulation at puberty. This model has been confirmed by clinical observations (14, 24) and by observations in StAR knockout mice (25).

Most disease-causing StAR mutations are devoid of measurable activity. Only three mutations causing lipoid CAH have had measurable activity in a transfected COS-1 cell assay equivalent to the one we employed. Ala218Val (6% activity) and Leu275Pro (10% activity) were both found in a 46,XY phenotypic female infant with salt loss at 2–4 months (4); hence, this very low level of StAR activity is insufficient to influence phenotype. Met225Thr (29% residual activity) was found in a minimally virilized (clitoromegaly, rugated labia, mild posterior labial fusion) 46,XY female who first experienced salt loss at 10 months of age (10). The coexistence of the severe Glu258Stop null mutant on this patient's other allele presumably accelerated the course of the second hit, so that the net effect was more severe than would be predicted for a Met225Thr homozygote (10). In contrast, the Val187Met and Arg188Cys mutations described in the present study, which retain approximately 20–25% of activity, were seen in homozygously affected individuals, permitting clear assessment of the phenotypic consequences of retaining this degree of StAR activity. The activities of the Val187Met mutant were slightly greater than those of the Arg188Cys mutant in all three assays, but it is not clear whether these differences were clinically significant. Although the partial activity *in vitro* correlates well with milder, later onset disease, the presentation of lipoid CAH can vary substantially among homozygotes within a single ethnic group; for example, among seven Saudi patients homozygous for Arg182His, the age of onset of symptoms ranged from 1–14 months, despite this mutant lacking detectable activity *in vitro* (13). The consequences of these nonclassical mutations on gonadal function cannot be assessed during childhood; long-term follow-up through puberty into adult life will be needed to assess the eventual impact of these mutations on ovarian and testicular function.

Lipoid CAH has been described in most ethnic groups and is common in Japan, Korea, and some isolated populations (1, 3, 4, 10, 11, 13, 24, 26–28). In contrast to previous reports of lipoid CAH, our patients presented late: patient 1, a 46,XX female homozygous for Val187Met, presented at 4 yr of age,

whereas patients 2 and 3, 46,XY phenotypic male siblings homozygous for Arg188Cys, presented at 2–3 yr. These unique clinical courses, which are consistent with the demonstrated partial activity of each mutation, establish a new entity, nonclassic lipoid CAH, showing that the clinical spectrum of StAR mutations is substantially broader than had been appreciated previously. Nonclassic lipoid CAH is thus a novel form of nonautoimmune Addison disease, which can present with or without salt loss. Other genetic bases of Addison disease probably remain to be identified.

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