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Maria I. New
Joe Leigh Simpson *Editors*

Hormonal and Genetic Basis of Sexual Differentiation Disorders and Hot Topics in Endocrinology

Proceedings of the 2nd World Conference

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Hormonal and Genetic Basis of Sexual Differentiation Disorders and Hot Topics in Endocrinology

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Preface

The 2nd World Conference entitled 'Hormonal and Genetic Basis of Sexual Differentiation' was held in Miami on January 15, 2010, and was directed by Maria I. New and Joe Leigh Simpson.

The success of the conference was helped in part by the endorsement of the Endocrine Society and was jointly sponsored by the University of Miami Miller School of Medicine.

The history of this meeting dates back to 2004 when Dr. Maria New organized the 1st world conference in Arizona. The conference brought together world experts in the field of disorders of sexual differentiation and allowed them to share their vast amounts of knowledge and cutting-edge research with each other. It was also a way to bring these scientists to a single room where ideas and theories could be discussed as well as new collaborations. The conference was a great success and could be seen in the literature by the surge of peer-reviewed papers and collaborations around the world. Even though disorders of sexual differentiation have been noted throughout history, dating over 2,000 years, little was known and discoveries were waiting to happen. Dr. New would have loved to have the meeting on an annual basis but was unable to until now.

Thanks in part to the 2004 meeting, significant advances have been made in the biological, genetic, and psychological differences between a man and a woman. Also, in recent years, great controversy has risen in the mainstream media dealing with elite athletes and their desires to engage in sports. Who should be playing on the 'boys' team versus who should be playing on the girls team? This question has stimulated interest in the definition of sexual identity in the elite athlete and has gone as far as to question gender. This includes questions such as can an XY female compete as a female? Guidelines put out by different athletic boards vary and the lack of a general consensus of what should be done has sparked great anger and suspicion for some athletes. Accusations follow which can be very embarrassing on a local and in some cases an international level.

The 2nd World Conference sought out the best scientist in the field of sexual differentiation both academic and clinical. It opened a new platform where surgeons, obstetricians, psychiatrists, endocrinologists, and pediatricians were able to have creative discussions and learn about the influences they have on each other. The platform that was created allowed for a purposefully interactive environment in which

new discoveries and outstanding science over the past 6 years were discussed. The conference specifically addressed the controversy concerning biological (gonadal and anatomical) differences between a man and a woman. Those presenting at the conference were world class scientists who achieved high recognition for their work over the years on the biological, genetic, and psychological differences between the sexes. They covered recent advances which could be used to clarify confusions and to address controversies among athletes like the South African track star at the International Amateur Athletic Federation meet in Berlin in August 2009. Her eligibility to compete as a female athlete brought her international media attention and embarrassment as to what gender is she. The conference presented an extraordinary amount of data that can help avoid such international attention. The conference taught ways to evaluate, diagnose, and treat those with disorders of sexual differentiation to clinicians who normally do not see these types of patients, may have them in their practice unknowingly, or see them on a regular basis without knowing what to do next. Also, knowing the great importance of modalities such as hormonal assays and psychological tests used along with DNA analysis.

The large scale of the meeting became greatly apparent when the International Olympic Committee (IOC) not only expressed that they would like to be present at the meeting but also desired to sit with the experts at the meeting and discuss future testing regulations that would help avoid attention such as that seen by the South African runner. A roundtable discussion was further organized by Dr. New so that the experts, with their vast knowledge of gender issues, could advise the IOC as well as the IAFF (International Association of Athletics Federation) on how to determine an athlete's eligibility by using better testing modalities as well as clearer definitions of what it means to be a male as well as a female. It was also important to express that there are times where a situation will shift from being a sporting issue to a medical issue. The IOC, 10 years ago, had abolished mandatory gender testing but with recent attacks on athletes questioning their gender in a very public manner, it has become apparent that a review of current guidelines and the need for the development of new guidelines are needed. The roundtable discussion encompassed both the science and the ethics of this dilemma and helped clarify the medical aspect of these issues.

The scope of the meeting went well beyond just the science behind many of these disorders; it also dealt with many issues that are beneficial to the practitioner. Sex determination in newborns born with genital ambiguity can be found dating back more than 2,000 years (ANCIENT HIST OF CAH). It can be found in the art and writings throughout time and across many cultures. But, even though it has been dealt with in all societies for over a millennium, an accurate and effective way to quickly diagnose and assign gender to these newborns is still under investigation. The meeting took great care in this issue and we hope that with the open discussions during the meeting as well as that which was presented will help the medical practitioners worldwide to not only help diagnose and treat newborns but also help those who were misdiagnosed or left undiagnosed at birth and present later in life with questions or confusion about changes in their bodies (i.e., a 46,XX male presenting because he is menstruating from his penis).

We hope that the success of this meeting has not only answered questions but stimulated new questions. We also hope that in sharing the contents of the conference with those who were unable to attend will spark new interest and new questions in this great field driving it forward with the great momentum initiated by these great scientists. We hope that you enjoy the proceedings of the 2nd World Conference of Hormonal and Genetic Basis of Sexual Differentiation and hope to see you at a future meeting.

New York — Maria I. New
Miami, Florida — Joe Leigh Simpson

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Part X Posters

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Part I
Genetic Disorders of Steroids – Speakers

Chapter 1
Congenital Adrenal Hyperplasia Owing to 17α-Hydroxylase/17,20 Lyase and P450 Oxidoreductase Deficiencies

Christa E. Flück

Congenital adrenal hyperplasia (CAH) due to genetic mutations in the genes for *CYP17A1* (*MIM 609300*) and *CYP OR* (*MIM 124015*) are rare and may cause disordered sexual development (DSD).

The CYP17A1 enzyme catalyzes two distinct reactions in the steroid pathway; its 17α-hydroxylase (17OHase) activity is essential to produce 17-hydroxypregnenolone (17OHPreg) and 17-hydroxyprogesterone precursors of cortisol, and its 17,20 lyase activity is needed for the production of C19 precursors of sex steroids. As a consequence, lack of 17OHase activity causes glucocorticoid (GC) and sex steroid deficiency. However, compensatory overproduction of corticosterone and deoxycorticosterone with weak GC and significant mineralocorticoid action results in subclinical hypocortisolism but severe hypertension and hypokalemia. By contrast, lyase deficiency causes a lack of sex steroids leading to 46,XY DSD with severe undervirilization in 'male' newborns, and absent or incomplete pubertal development in both sexes. While the first clinical and biochemical description of patients with 17OHase deficiency is dated 1966 [1] and 1970 [2], it was not until 1988 that the underlying genetic defect was found [3]. Over the last two decades numerous point mutations, deletions/insertions, and splicing mutations have been reported for the CYP17A1 gene. Generally, mutations affecting the steroid-binding domain of CYP17A1 or disturbing the interaction with P450 oxidoreductase (OR) for electron transfer cause combined 17OHase and lyase deficiency. Although there seems no 'hot spot' in the gene for mutations, CYP17A1 mutations W406R and R362C predominate in a larger cohort of Brazilians originating from Spain or Portugal suggesting a founder effect [4]. By contrast, only four CP17A1 point mutations (E305G, R347C/H, and R358Q) are reported to cause isolated lyase deficiency [5–7], first described in 1997 [5]. These mutations are thought to interfere with either cofactor cytochrome b5 in the specific electron transfer from

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OR to CYP17A1 during the lyase reaction [8] or to alter substrate binding for 17OHPreg specifically [6].

OR is the obligate electron donor to the steroidogenic enzymes CYP17A1, CYP21A2, and CYP19A1. Therefore, patients with OR deficiency have a complex pattern of disordered steroidogenesis that was initially described as apparent combined 21OHase and 17OHase deficiency in 1985 [9]. Usually mineralocorticoid production remains normal while cortisol and sex steroid production are impaired to variable degrees with different OR mutations. Because of affected CYP17A1 and CYP19A1 activities leading to impaired testosterone and estrogen production, *OR* mutations may cause severe sexual ambiguity in both sexes (46,XY DSD or 46,XX DSD). Affected girls may present with different degrees of virilization at birth suggesting prenatal androgen exposure (Prader III–V). Affected boys present with varying degrees of undervirilization ranging from micropenis to severe hypospadias. In addition, patients with 'severe' OR mutations may present with skeletal malformations previously described as Antley Bixler craniosynostosis syndrome (*MIM 207410*) with genital anomalies [10]. This is explained by the fact that OR interacts with all microsomal type II P450s including enzymes involved not only in steroidogenesis but also in bone development, cholesterol biosynthesis, drug metabolism, and more [11]. Since the first description in 2004 [12, 13], about 40 inactivating *OR* mutations have been described (http://www.cypalleles.ki.se/por.htm) in patients presenting with an extremely broad range in phenotype [11]. Several patients previously misdiagnosed for having 21OHase or 17OHase deficiency were identified. Genotype–phenotype correlation is currently being studied by several groups [14, 15]. Overall, OR A287P mutation appears to predominate in European patients while R457H is mostly found in Japanese patients [11]. Mutations destroying the FAD binding to OR such as R457H seem to inactivate all interacting P450s [13–16]. By contrast, mutations not directly involved in the electron transfer but involved in the direct interaction with partners or cofactors (e.g., A287P) seem to affect activity of partner proteins to different degrees [16, 17]. In addition, a large number of sequence variations have been identified in 842 healthy individuals of 4 ethnic groups and the missense mutation A503V was found on 27% of all alleles [18]. Further, (ongoing) studies focus on the impact of OR mutations on drug-metabolizing P450s [19, 20] to elucidate the impact of OR variants for pharmacogenomic aspects.

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Chapter 2
Congenital Adrenal Hyperplasia Owing to 11β-Hydroxylase Deficiency

Perrin C. White

Humans have two isozymes with 11β-hydroxylase activity that are, respectively, required for cortisol and aldosterone synthesis. CYP11B1 (11β-hydroxylase) converts 11-deoxycortisol to cortisol and 11-deoxycorticosterone to corticosterone, is expressed at high levels, and is regulated by ACTH. CYP11B2 (aldosterone synthase) is normally expressed at low levels and is regulated mainly by angiotensin II and potassium levels. In addition to 11β-hydroxylase activity, the latter enzyme has 18-hydroxylase and 18-oxidase activities and thus can synthesize aldosterone from deoxycorticosterone. Mutations in the CYP11B1 gene cause steroid 11β-hydroxylase deficiency, a form of congenital adrenal hyperplasia. Mutations in CYP11B2 result in aldosterone synthase deficiency, which can cause hyponatremia, hyperkalemia, and hypovolemic shock in infancy. These are both recessive disorders. Unequal crossing over between the CYP11B genes can generate a duplicated chimeric gene with the transcriptional regulatory region of CYP11B1 but sufficient coding sequences from CYP11B2 so that the encoded enzyme has aldosterone synthase (i.e., 18-oxidase) activity. This results in glucocorticoid-suppressible hyperaldosteronism, a form of hypertension inherited in an autosomal dominant manner. This review concentrates on steroid 11β-hydroxylase deficiency.

Except in particular populations such as Moroccan Jews, this disorder comprises only a few percent of cases of congenital adrenal hyperplasia. Because 11-deoxycortisol can be converted to androstenedione, signs of androgen excess are prominent including virilization of affected females and rapid somatic growth, premature epiphyseal closure, and short adult stature in untreated males and females. These features are similar to those seen in patients with 21-hydroxylase deficiency. Additionally, 11β-hydroxylase deficiency patients have high levels of deoxycorticosterone and metabolites that are mineralocorticoids, causing sodium retention and hypertension in most patients. This contrasts with the salt wasting seen in many patients with 21-hydroxylase deficiency. Severely virilized females with

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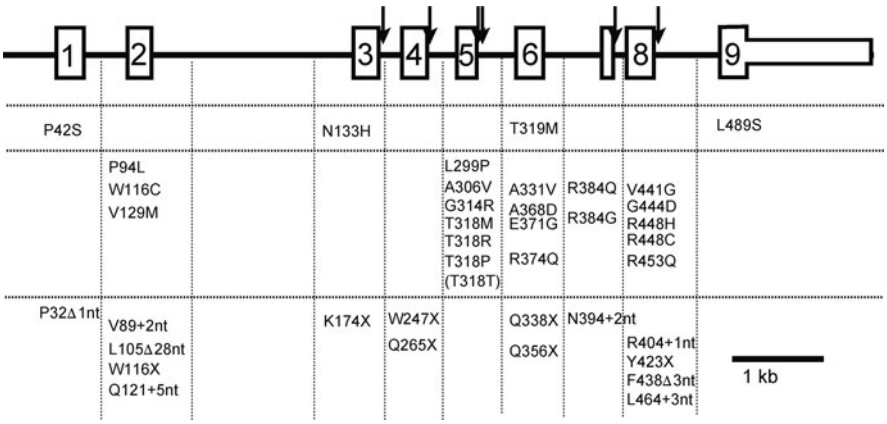

Fig. 2.1 Mutations causing 11β-hydroxylase deficiency. Exons are numbered and a 1 kb scale is shown. *Arrows* denote splice site mutations. The top line of mutations are those that yield partial enzymatic activity and are associated with nonclassic disease. The next group are more severe missense mutations, and the bottom group are nonsense and frameshift mutations that destroy enzymatic activity. For clarity, *vertical lines* group mutations by their locations within particular exons

11β-hydroxylase deficiency can thus sometimes survive for years without diagnosis and be raised as males. This previously occurred in up to half of affected females in the Moroccan Jewish population, but most females have been reared as girls in recent years.

Whereas many Moroccan Jewish patients carry a characteristic mutation, R448H, many other causative mutations have been identified (Fig. 2.1). Whereas most 21-hydroxylase deficiency mutations are caused by recombinations between the normally active gene and an adjacent pseudogene, there is no corresponding 11β-hydroxylase pseudogene to act as a donor of deleterious mutations. Thus most CYP11B1 deficiency alleles carry sporadic missense, nonsense, or splice site mutations. Gene deletions due to unequal crossing-over between CYP11B1 and CYP11B2 have been reported rarely. Mild, nonclassic 11β-hydroxylase deficiency is apparently unusual. Approximately two-thirds of classic patients are hypertensive regardless of genotype.

Chapter 3
46,XY DSD due to 17β-HSD3 Deficiency and 5α-Reductase Type 2 Deficiency

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Introduction

The 46,XY disorders of sex development (46,XY DSD) are characterized by ambiguous or female external genitalia, caused by incomplete intrauterine masculinization. Complete absence of virilization results in normal female external genitalia and these patients generally seek medical attention at pubertal age, due to the absence of breast development and/or primary amenorrhea. 46,XY DSD can result either from decreased synthesis of testosterone or from the impairment of androgen metabolism or action [1, 2].

46,XY DSD due to 17β-HSD3 Deficiency

This disorder consists in a defect in the last phase of steroidogenesis, when androstenedione is converted into testosterone and estrone into estradiol [3].

There are five steroid 17β-HSD enzymes which catalyze this reaction [4] and 46,XY DSD results from mutations in the gene encoding the 17β-HSD3 isoenzyme [4, 5]. The *HSD17B3* gene contains 11 exons and is located on chromosome 9q22.

Phenotype: Patients present female-like or ambiguous genitalia at birth, with the presence of a blind vaginal pouch, intra-abdominal or inguinal testes and epididymides, vasa deferentia, seminal vesicles, and ejaculatory ducts. Most affected males are raised as females [6, 7], but some have less severe defects in virilization and are raised as males [4]. Virilization in subjects with 17β-HSD3 deficiency occurs at puberty. This late virilization is usually a consequence of the presence of testosterone in the circulation as a result of the conversion of androstenedione

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to testosterone by some other 17β-HSD isoenzyme (presumably 17β-HSD5) in extra-gonadal tissue and, occasionally, of the secretion of testosterone by the testes when LH levels are elevated in subjects with some residual 17β-HSD3 function [4]. Bilateral orchiectomy resulted in a clear reduction of androstenedione levels indicating that the principal origin of this androgen is the testis [4, 7]. 46,XY DSD phenotype is sufficiently variable in 17β-HSD3 deficiency to cause problems in accurate diagnosis, particularly in distinguishing it from partial androgen insensitivity syndrome [6, 8].

Most 46,XY patients are raised as girls during childhood and change to male gender role behavior at puberty has been described in subjects who were reared as females [7, 9–11] including members of a large consanguineous family in the Gaza strip [12]. 46,XX subjects homozygous for *HSD17B3* mutations presented normal phenotype [13].

We report here clinical, psychological, and therapeutic studies of eleven 46,XY DSD subjects from eight Brazilian families with 17β-HSD3 deficiency. The 11 subjects were evaluated and followed at the Hospital das Clinicas, University of Sao Paulo, and were assessed by the same psychologist. Sexual ambiguity was noted at birth in nine subjects, but all were registered and raised as females because of incomplete virilization of the external genitalia. Two were diagnosed prepubertally, and the diagnosis was made in the others between 12.5 and 34 years. At the time of diagnosis, the external genitalia were characterized by proximal hypospadias, a small phallus (<2 SDS below the age-matched normal range), a bifid scrotum, and a blind-ending vaginal pouch. Nine subjects had separate urethral and vaginal openings and two had a single perineal opening with a short urogenital sinus.

All subjects had bilateral inguinal testes except one whose right testis was intra-abdominal. The testes in the postpubertal subjects were normal or near normal in size. All subjects of postpubertal age had male *habitus* and phallic enlargement but penile length remains below <2 SDS in the three male patients. All subjects had normal heights for age, and bone age was compatible with the chronologic age. In three subjects gender identity changed from female to male at puberty; in these subjects, penile length ranged from 5.0 to 6.8 cm. The remaining eight affected subjects (including two who were castrated prepubertally) have maintained a female social sex; in these subjects penile length ranged from 4.5 to 7.0 cm. In one family, the two siblings carrying the same mutation had a different gender role [14]. Gynecomastia was present in only one case (Tanner III stage) in contrast to the report of frequent gynecomastia in earlier studies [3].

Biochemical diagnosis: Laboratory diagnosis is based on elevated serum levels of androstenedione and estrone and low levels of testosterone and estradiol in basal conditions and after hCG stimulation resulting in elevated androstenedione/testosterone and estrone/estradiol ratios indicating impairment in the conversion of 17-keto into 17-hydroxysteroids. At puberty, serum LH and testosterone levels rise in all affected 46,XY subjects and testosterone levels may be in the normal adult male range [7].

Molecular defect: The disorder is due to homozygous or compound heterozygous mutations in the *HSD17B3* gene which encodes the 17β-HSD3 isoenzyme and several mutations have been reported [4, 15].

The diagnosis of 17β-HSD3 deficiency was confirmed in all eight families by analysis of the mutations, demonstrating compound heterozygous mutations in one family and homozygous defects in seven. Identical mutations recurred in apparently unrelated families, namely, R80Q in two families, 326-1, G→C in three families, and A203V in two families. The R80Q mutation was originally described in a Palestinian family from the Gaza strip but we were unable to document Palestinian ancestry in the Brazilian families. The A203V, S209P, E215D, and 326-1, G→C mutations appear to be unique to Brazil. Whether the existence of the same mutation in different families is due to recurring new mutations or to a common ancestor is not known. Family 2 is Black, while 17β-HSD3 deficiency has rarely been described in Blacks (13).

Treatment: Gonadectomy and estrogen replacement at puberty are indicated for patients reared in the female social sex. In male patients, androgen replacement is necessary when they present low levels of testosterone. In patients with mild defects testosterone replacement is not usually necessary.

5α-Reductase Type 2 Deficiency

This disorder is the only DSD condition in which the brain of affected subjects is prenatally exposed to normal testosterone levels. There are two steroid 5α-reductase enzymes that catalyze 5α-reductase reaction [16–18]. 46,XY DSD results from mutations in *SRD5A2* gene which encodes the steroid 5α-RD2 isoenzyme [19–21]. The *5α-RD2* gene contains five exons and four introns and is located at chromosome 2 p23. The 5α-RD2 isoenzyme promotes the conversion of testosterone to its 5α-reduced metabolite dihydrotestosterone (DHT).

Phenotype: Affected patients present with ambiguous external genitalia, micropenis, normal internal male genitalia, prostate hypoplasia, and testes with normal differentiation with normal or reduced spermatogenesis. The testes are usually located in the inguinal region, suggesting that DHT influences testis migration to the scrotum [21]. Virilization and deep voice appear at puberty, along with penile enlargement and muscle mass development without gynecomastia. These patients present scarce facial and body hair and absence of temporal male baldness, acne, and prostate enlargement, since these features depend on DHT action. Most of the patients are reared in the female social sex due to female-like external genitalia at birth but many patients who have not been submitted to orchiectomy in childhood undergo male social sex change at puberty [14, 21–25]. In our experience with 30 cases of 46,XY DSD due to 5α-RD2 deficiency from 18 families, all subjects were registered in the female social sex except for 2 cases – one who has an affected uncle and the other who was diagnosed before being registered [14, 26]. Fourteen patients changed to male gender role. No correlation was observed between *SRD5A2*

mutation, T/DHT ratio, and gender role change in these patients. In one family, the two siblings carry the same mutation but presented a different gender role [14]. Ten cases are adults now and nine of them are married. Three cases adopted children and in two cases in vitro fertilization using the patient's sperm cells resulted in twin siblings in one family and in a singleton pregnancy in the other 14, 26]. Fourteen patients maintained the female sexual identification. Three of them were castrated in childhood and the others, despite the virilization signs developed at puberty, kept the female social sex and sought medical treatment to correct the absence of breast development and primary amenorrhea. None of the 10 adult female patients, now aged 24–52 years are married but 8 of them have satisfactory sexual activity.

Inheritance: The mode of inheritance for 5α-RD2 deficiency is autosomal recessive. A different mode of transmission of 5α-RD2 deficiency due to uniparental disomy was described in two unrelated patients [27].

Biochemical diagnosis: After hCG stimulation, affected children show lower DHT levels and elevated T/DHT ratio [5, 28]. Postpubertal affected patients present normal or elevated testosterone levels, low DHT levels, and elevated T/DHT ratio in basal conditions. Low DHT production after exogenous testosterone administration is also capable of identifying 5α-RD2 deficiency [14]. Elevated 5β/5α urinary metabolites ratio is also an accurate method to diagnose 5α-reductase 2 even at prepubertal age and in orchiectomized adult patients [14, 29].

Molecular defects: There are more than 50 families with this disorder described in several parts of the world [21–24]. In a few cases of 46,XY DSD due to 5α-RD2 deficiency diagnosed by clinical and hormonal findings, no mutations were identified in *SRD5A2* gene [19, 21–24].

Treatment: In male patients with 5α-RD2 deficiency, higher doses of testosterone esters (250–500 mg twice a week) are used to increase DHT levels and consequently penis size and male secondary characteristics. Maximum penis enlargement is obtained after 6 months of high doses and after that the normal dosage is reinstituted [14, 21]. The use of topic DHT gel is also useful to increase penis size with the advantage of not causing gynaecomastia and promoting a faster increase of penis size as it is 50 times more active than testosterone. DHT is not aromatized, allowing the use of higher doses than testosterone during prepubertal age.

At diagnosis, the comparison of mean penile length among 46,XY groups showed that 5α-RD2 deficiency group had the smallest penile length. At final evaluation, after surgical and hormonal treatment, mean penile length was also smaller in the 5α-RD2 deficiency group (–5.4 ± 1 SDS) compared to the groups with testosterone production deficiency ($p < 0.05$). There was no statistical difference of the mean penile length before and after treatment in each of the two etiological groups ($p > 0.05$) indicating that current treatment do not result in penis size enlargement. Two variables were significantly associated with the change of male social sex in patients 46,XY female which were registered and non-castrated in childhood: male kids games and self-perceived physical appearance as male or ambiguous in childhood.

Overall, most of our patients reported satisfaction with the treatment, although specific complaints about small penile length, sexual activity, and urinary symptoms

were frequent. A recent review analyzing articles which reported on mental or physical health outcomes of DSD patients concludes that it is still not clear how sexual function contributes to quality of life of these patients [30].

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Chapter 4
Studies of a Cohort of 46,XY with DSD Including Steroid Biosynthesis Deficiencies

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Mutations of genes involved in sex development have been largely reported in the literature, but few data are available to evaluate the proportion of each gene defect in 46,XY DSD patients. We report molecular studies of a cohort of 644 families.

Clinical and Biological Classification

Before sequencing, we have classified our cohort according to hormonal data and presence or absence of a uterus.

Our best criterion for this classification was the evolution of AMH concentrations, especially during the first months of the life. Recently, we have determined the normative values of AMH in a cohort of 110 male neonates without abnormalities of external genitalia three times, around 15th day and 3rd and 9th months of life (Plotton et al., Horm Res, 2009, *72*, suppl 3, 365). These values were expressed in pmol/L as follows:

	Mean ± 1 SD	Value range
13–20 days	882 ± 335	286–2116
2.8–5.1 months	1816 ± 577	704–3250
8.5–9.8 months	1736 ± 616	639–4364

Basal LH, FSH, and testosterone concentrations were determined during the minipuberty. If these data were not available, testosterone and its precursors after stimulation by hCG test (6 × 1500 IU each 2 days) have been determined. Two groups and some subgroups have been determined.

All pediatric endocrinologists, geneticians, and urologists of French DSD Network have contributed significantly to this work.

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– Group 1: Disorders of testicular development attested by the low AMH level (172 families):
  – With complete female phenotype
  – With undermasculinization
– Group 2: Disorders in androgen synthesis or action because normal AMH was in favor of a normal testicular formation (472 families). Two subgroups have been determined based on the testosterone level:
  – 2a: Low testosterone has suggested a disorder of testosterone biosynthesis (StAR, CYP11A, CYP17, HSD3B2 or HSD17B3, and POR)
  – 2b: Normal or high testosterone has suggested an abnormality of androgen receptor gene and rarely an SDR5A2 defect

Strategies for Molecular Studies

Our sequencing strategy was dependent on the clinical and biological data of each 46,XY patients.

In group 1, first *SRY* gene was first sequenced if the phenotype was a complete female form; in other cases, *SF1* gene. Second, rearrangements (deletion or duplication) of several genes (*DAX1*, *WNT4*, *SOX9*, *WT1*, *SRY*, etc.) were researched by MLPA. Afterward, *SF1*, *WT1*, *DHH*, and *FGF9* genes have been sequenced. When syndromic features or mental retardation is associated with the testicular defect, a CGH array study was performed.

In group 2a, the gene corresponding to the suspected defect has been sequenced. In subgroup 2b with normal or high testosterone, AR gene mutations have been sequenced. If basal T/DHT ratio performed when an activation of hypothalamic–pituitary–gonadal (HPG) axis occurs (24 h after birth or minipuberty) was superior to 7, the *SRD5A2* gene has been sequenced.

Results: Incidence of Each Defect

The nature of these genetic lesions of group 1 was reported in the table below. All patients with an identified genetic lesion were heterozygous or hemizygous.

Families (number)	57	115
Phenotype of external genitalia	Complete female	Undermasculinized
With genetic lesion	52%	28%
SRY	9	0
SF1	8	28
DAX1 (duplication/triplication)	2	1
SOX9	7	3
WT1	1	2
With no detected lesion	48%	72%

In our cohort of 46,XY with hormonal data suggesting a disorder in biosynthesis of testosterone (about 98 families, group 2a), these defects have been confirmed by sequencing: *HSD17B3* (35), *HSD3B2* (28), *StAR* (15), *CYP11A* (5), *CYP17* (10), and *POR* (5); no mutation has been found in only three (two lipoid adrenal hyperplasia and one HSD17B3 deficiency).

Finally, in our group 2b (483 families) with normal testicular function (normal AMH and testosterone >10 nmol/L after hCG), the determination of genetic lesions was dependent on phenotype.

Families	160 (208 patients)	323
Phenotype of external genitalia	Complete female	Undermasculinized
With genetic lesion	95.6%	16.4%
Androgen receptor mutation	146	51
SRD5A2	7	2
With no detected lesion	4.4%	83.6%
No androgen receptor mutation	7	270

It is interesting to underline that the genetic lesion was often identified in 46,XY patients with complete female phenotype: in almost all cases (96%, 160 families, 208 patients) with normal testicular formation (group 2) and less in cases (48%, 57 families) with gonadal dysgenesies (group 1). In contrast, few genetic lesions were detected in other 46,XY patients with incomplete virilization: about 28% (group 1) and about 16% (group 2b) of genetic lesions.

In summary, our screening was successful for patients with complete female phenotype, but less efficient in patients with incomplete virilization. Rapid sequencing of *AR* gene at birth helps for the gender assignment. A genetic counseling should be done if mutations of *SF1*, *AR*, and steroïdogenic genes have been identified.

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Chapter 5
Aromatase Deficiency and Its Consequences

Melvin M. Grumbach

Origin of scientific creativity: To know when to be astonished.
–Louis Pasteur

New developments have challenged long-held concepts of the role of estrogen in the human male and prenatally in the conceptus, have uncovered widespread effects of estrogen in diverse tissues in the male and female, and have emphasized the role of extraglandular estrogen synthesis and paracrine and intracrine actions. Three illuminating developments are mainly responsible for the challenge to conventional wisdom: (1) description of a man with a homozygous null mutation in the ERα (estrogen receptor α) gene that led to estrogen resistance (ERαR) and the detection of 7 men and an infant boy, and 11 prepubertal or pubertal age females with severe estrogen deficiency as a consequence of a variety of homozygous and compound heterozygous mutations in the CYP19 gene, the gene that encodes aromatase (P450 aromatase), the enzyme responsible for the last and irreversible step in estrogen synthesis from androgens by the gonads and extragonadal tissues, and which has a wide tissue distribution in the human; (2) the concurrent development of mice that lack the gene encoding ERα (αERKO mice) and the gene encoding aromatase (Arko mice); and (3) the discovery of a second widely distributed estrogen receptor ERβ and the development of ERβ knockout mice (βERKO), and later of mice in which the gene encoding each receptor has been disrupted (αβERKO). Estrogen deficiency or resistance in the men led to tall stature without a pubertal growth spurt, eunuchoid proportions, delayed skeletal maturation, and severe osteopenia despite high testosterone levels. In the aromatase-deficient men but not the ERαR man, low-dose E replacement induced within 6–7 months rapid epiphyseal fusion and cessation of growth and by 3 years of E Rx striking improvement in bone mass and repair of the osteoporosis without inducing gynecomastia. These observations highlight the critical role of estrogen but not androgen in the male as well as female on skeletal

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growth (including skeletal proportions) and maturation – an effect on growth plate chondrocytes on the one hand, and the role of estrogen sufficiency in the accrual and maintenance of bone mass and density which is regulated by osteoblasts and osteoclasts. In both sexes, estrogen deficiency leads to a dissociation between (1) skeletal growth (it continues at a steady rate without a pubertal growth spurt) and (2) skeletal maturation and accrual of bone density and mass. Prepubertal estrogen concentrations in the normal female are apparently important in the well-defined sex difference in the rate of skeletal maturation and possibly the age of pubertal onset.

P450 aromatase deficiency in the male leads to hypergonadotropism, macroorchidism, and increased serum testosterone concentration. It has an important effect on carbohydrate and lipid metabolism including the development of persistent insulin resistance and can be associated with premature onset of coronary atherosclerosis. Apparently, it does not have a critical effect in the male on psychosexual differentiation or behavior.

The clinical consequences of mutations in CYP19 on the fetal–placental unit have elucidated the critical role of placental aromatase in the protection of the female fetus from androgen excess, and the prevention of androgen-induced XX DSD (female pseudohermaphrodism) and of virilization of the mother (Table 5.1).

Table 5.1 46,XX DSD (female pseudohermaphrodism[a])

A. Androgen induced
  1. Fetal source
    a. Congenital virilizing adrenal hyperplasia (defective 21-hydroxylation, 11β-hydroxylation, 3β-hydroxysteroid dehydrogenase-2, P450 oxidoreductase (POR)
    b. Glucocorticoid receptor mutation
  2. **Feto-placental source**
    a. ***P450 aromatase deficiency***
    b. P450 oxidoreductase deficiency (affecting aromatase)
  3. Maternal source
    a. Iatrogenic
      (i) Testosterone and related steroids
      (ii) Certain synthetic oral progestagens
    b. Virilizing ovarian or adrenal tumor
    c. Virilizing luteoma of pregnancy
    d. Congenital virilizing adrenal hyperplasia in mother[b]

B. Non-androgen induced
  1. Disturbances in differentiation of urogenital structures associated with malformations of intestine and lower urinary tract (non-androgen-induced XX, DSD) (e.g. cloacal anomalies, müllerian agenesis (MURCS), vaginal atreasia, and labial adhesions)

[a]This term is previous terminology and is no longer recommended and should be abandoned. It is listed in parentheses during this transitional period

[b]In pregnant patient with CAH whose disorder is poorly controlled or who is non-compliant especially during the first trimester

Table 5.2 Aromatase deficiency and severe estrogen deficiency

In the mother
Virilization during pregnancy by an affected fetus, common
In the female
46,XX DSD (female pseudohermaphrodism)
Polycystic ovary syndrome
Virilization at puberty
In the male
Normal sex differentiation
Macroorchidism
In both sexes
Delayed sexual maturation
Tall stature
Osteopenia
Increased bone turnover
Insulin resistance
Abnormal plasma lipids
Psychosexual orientation appropriate for phenotypic sex
Role of placental aromatase
In the affected male and female fetus and pregnant mother
In the physiology of pregnancy

Virilization of the mother of an affected fetus due to increased placental androgen is a common feature of the disorder and a clue to the diagnosis. Fetal estrogen synthesis (or responsiveness through fetal ERα) is not essential for implantation, survival of the conceptus, or the timing of parturition. Nor is estrogen a mediator of fetal sex differentiation of the female genital tract (Table 5.2).

Table 5.3 Manifestations of aromatase deficiency in the female

Prenatal
Fetal: masculinization of urogenital sinus external genitalia: androgen-induced female pseudohermaphrodism; low plasma estrogen and very elevated androgen levels
Mother: Virilization, low plasma estrogen and elevated androgen levels
Infancy
Elevated plasma levels; undetectable plasma E_2 (±multicystic ovaries)
Puberty
No female secondary sex characteristics: severe estrogen deficiency
Tall stature
No pubertal growth spurt despite increased serum androgens
Delayed skeletal maturation
Virilization with progressive enlargement of the clitoris
Hypergonadotropic hypogonadism
Increased levels of plasma androgens
Polycystic ovaries
Osteopenia
Female psychosexual orientation

Aromatase deficiency syndrome in the female has distinct features in the fetus, during childhood, and at the age of puberty including androgen-induced XX DSD (female pseudohermaphrodism) virilization at puberty and without or incomplete feminization, polycystic ovaries, and hypergonadotropic hypogonadism (the phenotype may vary depending on the magnitude of the aromatase deficiency). The striking polycystic ovaries that occur in aromatase-deficient females during infancy, childhood, and adolescence appear to be a consequence of the increased intraovarian concentration of androgen and a high concentration of circulating FSH and do not require estrogen. Estrogen replacement therapy at puberty in both sexes arrests and corrects the functional changes and induces feminizing puberty in the female (Table 5.3).

The aromatase deficiency syndrome is most florid in nonsense mutations of the CYP19 gene. Aromatase deficiency varies clinically from florid to subtle depending on the magnitude of the aromatase deficiency.

Chapter 6
Gonadotropin-Regulated Testicular Helicase (GRTH/DDX25): A Master Post-transcriptional Regulator of Spermatogenesis

Maria L. Dufau, Hisashi Sato, Ravi Gutti, and Chon-Hwa Tsai-Morris

Male germ cell maturation is orchestrated by a cascade of temporally regulated factors. Gene expression during spermatogenesis requires temporal uncoupling of transcription and translation [1, 2]. GRTH, a testis-specific member of the DEAD-box family of RNA helicases discovered in our laboratory, is a target for gonadotropin-induced androgen action and a post-transcriptional regulator of key spermatogenic genes [3–8]. This helicase, which is expressed in germ cells (spermatocytes and round spermatids) and Leydig cells, has ATPase and bidirectional RNA-unwindase activities and increases the translation of messages. GRTH contains 483 amino acids and shared all conserved motifs of members of the DEAD family of RNA helicases with otherwise low amino acid sequence similarity with other members of the family [6]. The GRTH/DDX25 gene is TATA-less and contains 12 coding exons. Its basal transcriptional activity is driven by transcription factors Sp1/Sp3 that bind rich GC-rich sequences at the promoter [9]. The GRTH gene is transcribed as a single mRNA species of 1.6 kb [6].

GRTH Expression and Regulation

Three species of GRTH protein resulting from alternative utilization of translation initiation codons were observed in the rat testis. In the rat, germ cells (round spermatid and spermatocytes) utilized predominantly the first codons and contained major protein species of 61 (phosphorylated) and 56 kDa (non-phosphorylated), with minor use of the second ATG. Leydig cells utilized the second ATG codon with expression of 48/43 kDa, which lacks the nuclear localization signal and is expressed only in the cytoplasm. In the mouse testis, the first ATG codon is

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exclusively utilized [4]. The cell-specific 61/56 kDa species is essential for spermatogenesis. In germ cells (pachytene spermatocytes and round spermatids) GRTH is present in the nucleus (56 kDa non-phosphorylated) and at cytoplasmic sites (61 kDa phophorylated species) of germ cells including the chromatoid body of round spermatids and polyribosomes [5, 10]. GRTH is the only helicase known to be regulated by hormones. It is developmentally regulated and its expression is observed at puberty and adult life. Its expression is transcriptionally regulated by gonadotropin via cAMP and androgen, directly in Leydig cells and indirectly in spermatids presumably through genes induced by androgen/androgen receptors in Sertoli cells [6]. The 1 kb fragment 5′ to the ATG codon of GRTH gene contains sequences for androgen regulation of its expression in Leydig cells [7].

The Chromatoid Body in Germ Cells

The chromatoid body (CB) is a non-membranous filamentous cytoplasmic mRNA storage organelle of *nuage* appearance, located adjacent to the nucleus [11, 12]. It contains members of the small interfering RNA pathway (MIWI/argonaute proteins/RNA-induced silencer complex (Risc)/Dicer endonuclease/and decapping enzyme Dcp1a, mi/si/pi RNAs) that might participate in the RNA-mediated silencing/degradation/pathway in haploid germ cell [13] and is functionally related to somatic P-bodies [14]. In germ cells this complex also contains MVH (mouse vasa homologue) a germ cell RNA helicase that resides in the CB and cytoplasm and does not bind RNA. No interaction of GRTH with MVH or RISC members was observed in both protein and RNA [15].

Export and Transport Function of GRTH: Its Central Role in the Structure/Function of the Chromatoid Body

GRTH null male mice generated in our laboratory are sterile due to spermatogenic arrest at step 8 of round spermatids and their failure to elongate [10]. Their blood testosterone is normal, excluding androgen reduction as the cause of the arrest. The CB in spermatids is markedly reduced in size in GRTH null mice. Treatment of round spermatids (wild type) with nuclear export or RNA synthesis inhibitors caused nuclear retention of GRTH and its absence in the cytoplasm and CB [15]. The nuclear level of GRTH bound to RNA messages was significantly enhanced and a major reduction was noted in the cytoplasm, indicating GRTH main transport function of mRNAs to the cytoplasm and CB. The MVH and MIWI proteins present in the CB were excluded from this organelle and accumulated in the cytoplasm upon treatment with the nuclear export inhibitor. This was also found to occur in spermatids of GRTH-KO mice. A change from lobular filamentous to a small condensed structure was observed in spermatids after treatment, resembling the CB of

the GRTH-KO. Therefore, GRTH acting through its export/transport function as a component of mRNP is required to maintain the CB structure in spermatids and to preserve its function (i.e., storage and processing of mRNAs) during spermatogenesis. The fact that no direct interactions were noted between GRTH with any of the factors involved in the micro-RNA pathways residing in the CB indicates that GTRH delivers essential RNAs to the CB, presumably for silencing/storage and/or degradation of genes via the small mRNA pathway during germ cell development [15] (Fig. 6.1).

GRTH is a binding protein and, as an integral component of messenger ribonucleoprotein particles (mRNP) in germ cells, associates with relevant messages whose

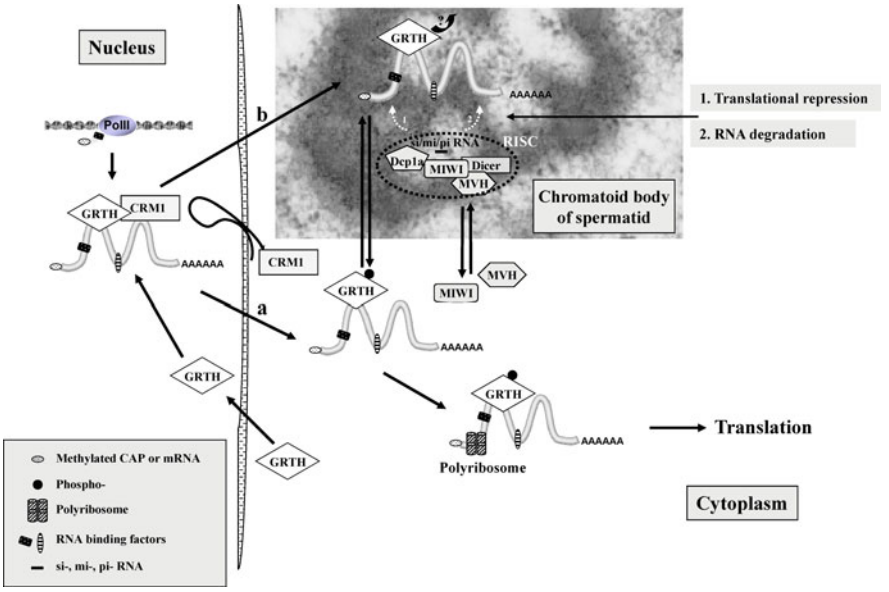

Fig. 6.1 Model of GRTH action in male germ cell: GRTH protein enters to the nucleus, where it binds messages of relevant spermatogenic genes as well as its own message and associates with CRM1. As an integral component of ribonuclear protein particles (RNPs) it exports these messages through nuclear pores via the CRM1 pathway to the cytoplasm *a* and the chromatoid body (CB) *b*, either directly, via nuclear pores adjacent to or associated with the CB *b* or indirectly, via the cytoplasmic route *a* to silence/store/degrade transcripts in the CB through the RISC complex and si/mi/pi pathway (see below). GRTH undergoes phosphorylation in the cytoplasm 61 kDa species (nuclear GRTH is non-phosphorylated—56 kDa species). Phospho-GRTH associates with polyribosomes where it regulates translation of associated mRNAs. GRTH–RNP complexes are required to maintain the CB filamentous-lobulated, *nuage* structure. GRTH delivers mRNAs to the CB, but does not directly interact with protein members or factors of the si/mi/pi small RNA pathway which reside in the CB (RISC:RNA-induced silencing complex: decapping enzyme Dcp1a, Argonaute proteins, MIWI, MVH, Dicer) for either storage *1* or degradation *2* of messages. GRTH probably dissociates from mRNAs at the CB site during this process. At specific times during the progress of spermatogenesis, messages are transported from the CB by GRTH to polyribosomes for translation. Some messages bypass the CB and proceed by the *a* route transported by GRTH directly for translation in polyribosomes [3, 8, 15]

proteins are expressed at different steps of spermatogenesis (i.e., TP1 and TP2, Prm1, Prm2, tACE, and others). The 56 kDa GRTH nuclear non-phosphorylated species, in association with and via the chromosome region maintenance-1 protein (CRM1)-dependent pathway, exports target mRNAs from the nucleus to cytoplasmic sites for storage in the CBs of spermatids, to be released for translation during spermatogenesis [5] or translated in polyribosomes. Besides participating in the transport of relevant spermatogenic genes, GRTH was found to transport its own message to cytoplasmic sites [15]. A 61 kDa phosphorylated GRTH species associates with polyribosomes to regulate the translation of mRNAs encoding spermatogenic factors. GRTH selectively regulates the translation of specific genes, including histone 4 and HMG2 in germ cells, and participates in the nuclear export of RNA messages in a gene-specific manner including PGK2, tACE, and TP2, TP1. Some GRTH-associated messages bypass the CB route and are directed for translation (Fig. 6.1).

GRTH in Leydig Cells

Leydig cells of GRTH-KO mice have reduced lipid droplets and swollen mitochondria lacking normal central cristae, indicative of hyperstimulation. In these mice, basal circulating levels of testosterone are normal and the Leydig cells in vitro show no changes in the basal levels of androgen production [10]. However, upon in vivo hCG stimulation significant increases in circulating levels of testosterone production are observed over those of the wild type. Similarly marked increases in testosterone production were observed upon stimulation of Leydig cells from KO with hCG compared to wild-type mice. This indicated that GRTH has a negative influence in steroidogenesis induced by the gonadotropin stimulus [16].

GRTH Anti-apoptotic Function in Spermatocytes

Also, as a component of mRNP particles GRTH is a negative regulator of tumor necrosis factor receptor 1 and caspase pathways and promotes NF-κB function to control apoptosis in spermatocytes of adult mice [17]. Pro- and anti-apoptotic factors were found to be under GRTH regulation and many of their messages associated with GRTH protein. Pro-apoptotic factors associated with GRTH might be either down-regulated or silenced through the small RNA degradation pathway. It could also favor the export of nuclear anti-apoptotic messages and subsequent translational events during spermatogenesis. GRTH-KO mice displaying marked apoptosis in spermatocytes entering the metaphase of meiosis had decreased levels of Bcl2 and Bcl-xL (anti-apoptotic factors); an increase in Bid, Bak, and Bad (pro-apoptotic); and marked reduction of phospho-Bad (anti-apoptotic) known to be associated with the cell survival mechanism that raises the threshold of cytochrome C release from

the mitochondria into the cytosol. These changes, which caused increased cleavage of caspase 9, 3 and PARP and caspase activation, are known to induce DNA fragmentation and could contribute to induction of the apoptotic cascade in the spermatocytes of KO mice. The half-life of caspase 3 transcripts was markedly increased in KO mice, indicating that GRTH has a negative role in its message stability. IκBα, which sequesters NF-κB at cytoplasmic sites preventing its transcriptional activation of pro-apoptotic genes (Bcl2 and Bcl-xL), was highly elevated in KO, and its phospho-form that promotes its dissociation was reduced. In addition, the increase of HDAC1 and abolition of p300 expression in KO indicated a nuclear action of GRTH on NF-κB-mediated transcription of anti-apoptotic genes. GRTH also influences the death domain pathway by acting as a regulator of downstream death receptor-associated adaptor protein TRADD in the TNFα-associated pathway. In the KO mice TRADD is markedly increased. TRADD in turn could activate the caspase 8 cascade by recruiting FADD to trigger the release of mitochondrial factors and activation of caspase 3, resulting in cell death. Association of GRTH with TRADD mRNA as an mRNP complex could render it unstable or inhibit its translation, excluding its participation in the death domain signaling of apoptosis in the wild-type mice. These studies have provided evidence for a central role of GRTH in the prevention of germ cell apoptosis in the adult male gonad.

Polymorphism of the GRTH Gene

A unique heterozygous SNP Arg^{242} His (exon 8) was found in azoospermic Japanese men (5.8%) [18]. This mutation impacts on the post-transcriptional modification of the expressed GRTH, since expression of the cytosolic 61 kDa species was absent and only the 56 kDa nuclear species was present. The SNPs of exons 8 (missense) and 11 (silent) found in our studies were not apparent in infertile patients from west China [19]. Also, the exon 10 silent mutation reported in the infertile Chinese patients, although present in the Japanese infertile patients, was not significantly different in the allele change from controls. These differences suggest that SNPs of the GRTH gene might be associated with an ethnic background of male infertility among Asian men. The absence of the phospho-GRTH species of the mutant protein could be relevant to functional aspects of the protein (translational events and cytoplasmic functions) that impact on germ cell development and/or function.

Conclusions

These studies have demonstrated that GRTH is a multifunctional RNA helicase that is essential for the completion of spermatogenesis. Also, its association with some cases of male infertility underlines its importance as a central, post-transcriptional

regulator of spermatogenesis. Moreover, GRTH links androgen action and spermatogenesis. These studies on GRTH function and regulation provide insights into the intrinsic requirements of spermiogenesis and open new avenues for studies on the regulation of spermatogenesis, infertility, and male contraception.

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Part II
Prenatal Diagnosis of Congenital Adrenal Hyperplasia – Speakers

Chapter 7
Long Range Outcome of Prenatal Treatment

Maria I. New and Alan A. Parsa

Preliminary medical results are presented in Figs. 7.1, 7.2, and 7.3. Reported fractures in those prenatally treated were less frequent than those who reported fractures. The data do not show any apparent difference in the rate of fractures between those prenatally treated with dexamethasone and those not prenatally treated. With respect to obesity there is no evidence that those who were treated with dexamethasone prenatally had a higher BMI than those not treated. Hypertension was infrequent and the blood pressure in one patient who was treated was no different from the other patient who was not treated. There were no patients with diabetes observed in the long-term follow-up.

PRELIMINARY MEDICAL RESULTS

Bone fractures

	Reported fractures	No reported fractures	Total
Dex–treated	12 (37.5%)	20 (62.5%)	32
Not Dex–treated	13 (26.0%)	37 (74.0%)	50
Total	25 (30.5%)	57 (69.5%)	82

Fig. 7.1 The data do not show any apparent difference in the rate of fracture between those prenatally treated with dexamethasone and those not prenatally treated

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Obesity

	Dex treated ($n=24$)	No prenatal dex ($n=29$)
Normal (BMI 18–25)	17 (70.8%)	17 (59.0%)
Overweight (BMI 25–30)	4 (16.7%)	9 (31.0%)
Obese (BMI > 30)	2 (8.3%)	3 (10.3%)
Underweight (BMI < 18)	1 (4.2%)	0 (0.0%)
Total	24	29

Fig. 7.2 Prenatal Dex treatment does not increase the risk of overweight or obesity

Fig. 7.3 Prenatal dexamethasone does not increase the risk of hypertension

Hypertension

	Dex treated ($n=18$)	No prenatal Dex ($n=28$)
Hypotensive (<90/<60)	0	0
Normal (90–139/60–89)	17	27
Hypertensive (>140/>90)	1	1

Preliminary psychoendocrine outcome including cognition, gender, behavior, and performance revealed a marginal difference between dexamethasone exposed versus dexamethasone unexposed affected females. Feminine hobby preference and female gender behavior were increased in the dexamethasone exposed group. There was no psychoendocrine difference in dexamethasone exposed or unexposed males whether affected or unaffected with CAH (Table 7.1). Based on the Behavior Problem Scale no significant changes between dexamethasone exposed and dexamethasone unexposed affected males or females were seen except that the dexamethasone unexposed patients had significantly higher attention problems ($P < 0.02$) and were marginally more aggressive ($P < 0.095$). There were no differences in the education outcome in those treated or untreated.

Table 7.1 Study enrollment (USA and France)

	No dex				Partial dex				Full dex				
	M		F		M		F		M		F		
	Fr	A	Fr	A	Fr	A	Fr	A	Fr	A	Fr	A	Total
SW	10	8	17	7	4	3	0	1	1	0	6	3	60
	18		24		7		1		1		9		
SV	2	1	0	3	1	1	0	0	0	0	1	0	9
	3		3		2		0		0		1		
NC	0	1	0	1	0	1	0	0	0	0	0	0	3
	1		1		1		0		0		0		
No CAH	10	6	8	4	7	8	9	9	0	2	0	1	64
	16		12		15		18		2		1		
Total	38		40		25		19		3		11		136

FR - France; A - America.

Summary: These preliminary data show no obvious detrimental effects in patients treated with low-dose dexamethasone prenatally.

Chapter 8
Novel Non-invasive Prenatal Diagnosis as Related to Congenital Adrenal Hyperplasia

Joe Leigh Simpson and Farideh Bischoff

Prenatal treatment of female fetuses having congenital adrenal hyperplasia (CAH) requires diagnosis of gender for genotype prior to genital differentiation. This approach currently involves chorionic villus sampling (CVS). In experienced hands CVS carries minimal risk of pregnancy loss, comparable to that associated with amniocentesis. Although offering considerable advantage by first trimester diagnosis, CVS is typically performed no earlier than 9-week gestation (7-week embryonic age) and usually not until 10–12 weeks. The procedure can be performed as early as 6–7 weeks, but it is technically difficult at that gestational age and associated with limb reduction defects. A preferable approach is definitive *non-invasive* diagnosis of fetal disorders. This could be achievable by analysis of either intact fetal cells or cell-free fetal DNA in maternal blood.

Intact Fetal Cells

Intact embryonic or fetal cells exist but are rare in maternal blood (1 fetal cell per cc or $10^7/10^8$ maternal cells); thus, one must enrich a maternal blood sample for the efficient analysis of rare fetal cells. Recovery can be accompanied through the use of beads to which antibodies are attached or by cell capture devices (i.e., microfluid-based system). These devices capture cells by attachment chemistry and fluid dynamics. One such device is CEE^{TM} (Biocept, Inc.), a hollow device (75 μm $\times$ 12 mm $\times$ 30 mm) that contains randomly distributed vertical posts. Microfluid-based cell capture devices can be coated with capture antibody (i.e., glycophorin-A (Gly-A)) to enrich for the fetal cell population. FISH (21-specific; X- and Y-centromeric probes) can then be performed within the device using standard fluorescent microscopy.

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In pregnancies with a male fetus, fetal red blood cell detection was achieved in the majority of pregnancies, as validated by a Y interphase signal concomitant to independent confirmation by PCR of a Y sequence. Accuracy is essentially 100% in informative cases. Confirming fetal/embryonic cell origin in pregnancies with a female fetus requires an independent marker to distinguish fetal XX from maternal XX cells. However, difficulty arises in preserving optimal integrity for chromosome-specific FISH probes in the presence of independent markers, for which reason far fewer pregnancies with a female fetus are informative. Thus, intact fetal red blood cells in blood are considered to have limited value at present for definitive non-invasive prenatal diagnosis.

Cell-Free Fetal DNA

An alternative for definitive non-invasive prenatal diagnosis involves cell-free fetal DNA (cffDNA) in maternal blood. At least 5% of cell-free DNA in first trimester maternal blood is derived from the fetus; cffDNA can be recovered from plasma or maternal whole blood, through controlled lysis of cells (targeting weaker, apoptotic, fetal cells) and size fractionation. Cell-free fetal DNA for prenatal diagnosis is applicable earlier in pregnancy (6-week gestation) than CVS and is without risk of pregnancy loss. The strategy employed involves excluding a mutant paternal allele whose transmission is necessary for the fetus to be affected, even if substantial admixture exists with fetal and maternal blood. Cytogenetic indications include detecting fetal trisomy, being pursued by analyzing chromosome-specific fetal versus parental polymorphisms or by massive parallel genomic sequencing. Specific single gene indications include the following: (a) Determining fetal sex in couples at risk for X-linked recessive traits; (b) detecting a mutation transmitted from a father who has a DNA sequence that the mother lacks (e.g., Marfan syndrome or achondroplasia), as discussed above; (c) determining Rh(D) fetal status in an Rh-negative (dd) mother whose partner is heterozygous (Dd); and now (d) performing linkage analysis to detect 21-hydroxylase deficiency, linkage analysis required given existence of pseudogenes.

In conclusion, prenatal diagnosis has evolved from invasive diagnostic tests to risk-free definitive non-invasive diagnosis (cffDNA), while management can be initiated earlier in pregnancy.

Part III
Treatment of Congenital Adrenal Hyperplasia – Speakers

Chapter 9
Medical Treatment of Classic and Nonclassic Congenital Adrenal Hyperplasia

Phyllis W. Speiser

Medical Maintenance Therapy of CAH

The main goals of medical therapy for congenital adrenal hyperplasia (CAH) are (1) to replace deficient cortisol with a suitable glucocorticoid (GC), (2) to reduce ACTH oversecretion and thereby prevent excessive androgen secretion, and (3) to replace deficient aldosterone with suitable mineralocorticoid (MC) and sodium supplements. Appropriate steroid treatment prevents adrenal crisis and virilization, allowing normal growth and development. A secondary goal is to preserve reproductive potential. While simple in principle, good management of classic CAH represents a set of trade-offs to achieve a tenuous balance between endogenous hyperandrogenism and iatrogenic hypercortisolism. Undertreatment carries the risk of adrenal crisis and allows increased adrenal androgen production, with accelerated bone age and loss of growth potential. On the other hand, overtreatment may suppress growth, increase blood pressure, and cause Cushing syndrome.

Adrenal hormone levels are usually markedly elevated at the time of diagnosis in the newborn period. In order to suppress these hormones, one may temporarily exceed recommended GC doses. However, it is important to rapidly reduce the dose once target serum adrenocortical hormone levels are achieved. Frequent reevaluation is needed in infancy. Serum 17-OHP levels should not be completely suppressed, as this practice results in overtreatment. Moreover, ACTH levels should not be used to titrate dosing. During childhood, the preferred GC is hydrocortisone (HC) at doses of about 10–15 mg/m^2/day. HC's short half-life minimizes the adverse side effects of more potent longer acting GCs, such as prednisone and dexamethasone, especially growth suppression [1]. In one trial, the estimated growth-suppressive effect of prednisolone was about 15-fold more potent than HC [2]. Dexamethasone is still more potent and long acting, estimated at 70- to 80-fold the glucocorticoid equivalency of HC [3].

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HC suspension and HC tablets are not bioequivalent. HC oral suspension may be unevenly distributed and has been removed from the market in the USA due to inadequate control of CAH in children treated with these preparations [4]. Good control can be achieved in small children by orally administering crushed HC tablets mixed with a small volume of liquid immediately before administration. There are scant data to recommend administering a higher dose of HC in either morning or evening [5]. When HC equivalent doses exceed 20 mg/m^2/day in infants and 15–17 mg/m^2/day in adolescents, there is loss of height SD score (SDS) and shorter adult height SDS [6]. Thus, although prednisolone and dexamethasone treatments are effective in suppressing adrenal androgens in children with CAH, these more potent drugs are somewhat more difficult to titrate and, thus, are more likely to impede statural growth. The 2010 Endocrine Society Task Force recommended using hydrocortisone as the first line of GC treatment in children [7].

During puberty hormonal control may deteriorate as a result of more rapid cortisol clearance [8]. Whereas the hormone profile is difficult to control in adolescence, it is important to note that height correlates negatively with the dose of GC administered in puberty. Patients treated with less than 20 mg HC/m^2/day at start of puberty are significantly taller than those who were given higher HC doses [1].

At or near completion of statural growth, long-acting GCs may be used, although HC remains a treatment option. Prednisolone suspension or dexamethasone elixir can be used to titrate the dose more finely than with tablets. If prednisone is used, typical adult doses do not exceed 7.5–10 mg daily. An average adult dexamethasone dose would be about 0.25 mg nightly. As in children, steroid dosing must be individualized according to adrenal hormone levels, blood pressure, and weight gain.

Salt-wasting 21-hydroxylase deficiency, comprising 75% of all classic cases, requires MCs as well as supplemental sodium chloride in addition to GC treatment. The requirement for sodium is absolute in infancy and generally decreases with advancing age as more salty food is consumed. While aldosterone deficiency is clinically important only in the most severe salt-wasting forms of CAH, subclinical aldosterone deficiency is present in all forms of 21-hydroxylase deficiency. This can best be evaluated by the aldosterone to PRA ratio [9]. Thus, all patients with high PRA or aldosterone to PRA ratio for age benefit from fludrocortisone therapy and supplemental dietary sodium. Typical doses of oral fludrocortisone are 0.1–0.2 mg in one or two daily doses. Maintenance of sodium balance reduces vasopressin and ACTH levels, contributing to lower GC doses, leading to better auxological outcomes [10].

Sensitivity to MCs may vary over time, and recovery from salt wasting has been described in some patients, most probably secondary to extraadrenal 21-hydroxylation [11, 12]. Therefore, the need for continuing MCs should be reassessed periodically based on blood pressure, PRA, the aldosterone to PRA ratio, growth pattern, and adrenocortical hormone profile. Infants who are often initially treated with high doses of MC should have blood pressure measurements at each visit.

Stress Dosing with Glucocorticoids

In classic, severe forms of 21-hydroxylase deficiency, there is an insufficient cortisol response to stress, such as is required to cope with febrile illness, gastroenteritis with dehydration, surgery, or trauma. Therefore, such patients require boost doses of GC for these episodes. Exercise and psychological stresses (e.g., anxiety and examinations) do not require increased GC dosing [13]. When pharmacological doses of HC are given (as parenteral Solu-Cortef), MCs are not needed because high-dose parenteral HC has MC-like effects. Typical empiric stress doses are 25, 50, or 100 mg, respectively, for infants, school-age children, and adolescents or adults initially, and then three times the maintenance dose every 6–8 h thereafter. Maintenance GC doses should be resumed when the patient is clinically stable. Salt-wasting CAH patients should avoid fasting during acute illnesses. Glucose and electrolyte supplementation should be given to young children during illness and extreme physical exertion or stress. Fludrocortisone doses need not be increased during illness. There is no currently available parenteral form of fludrocortisone.

Experimental Forms of Glucocorticoid Treatment

New treatment approaches have been explored to enhance stature in children with CAH. Short stature in CAH patients may be caused by hypercortisolism, hyperandrogenism, inadequate mineralocorticoid treatment, late start of treatment, or perhaps most importantly lack of adherence to the prescribed medical regimen. Overtreatment during infancy [14, 15] or treatment with long-acting, high-potency GCs [1] may also result in shorter than expected stature. The pubertal growth spurt is often attenuated in CAH [1]. Despite these concerns, target height has been attained with strict adherence to standard GC and MC medication regimens, coupled with monitoring every 3 months (6). As we diagnose more patients at birth and avoid over-aggressive steroid treatment, it is expected that height outcomes will improve.

Evidence is lacking from carefully controlled trials in terms of growth outcomes in CAH. In a systematic review of the medical literature and meta-analysis of data meeting stringent criteria for inclusion, only 35 of 1,016 published reports met eligibility criteria for analysis [10]. All were observational studies with methodologic limitations, graded as very low quality evidence. Most patients were diagnosed before the era of newborn screening, fewer than half of these studies reported a mean age of diagnosis <1 year. Most did not give details of GC doses. The pooled data indicated a corrected adult height SDS of −1.05. Subgroup analysis revealed that the addition of MC treatment improved height outcome.

Patients with NCCAH can also have compromised adult height, but height deficit is less severe than with classic CAH. There are scant data to suggest that an early start of GC treatment before puberty can improve adult height [1, 16]. Similarly, there are limited studies to show that drugs which either enhance growth or delay puberty significantly improve height in children with classic CAH.

One such study involved a complex four-drug regimen of the antiandrogen flutamide, the aromatase inhibitor testolactone, reduced HC (8 mg/m^2/day), and fludrocortisone. This combination in a small cohort of children followed over 2 years resulted in a more age-appropriate growth rate, more normal weight velocity, and slower bone maturation as compared with conventional treatment with HC and fludrocortisone [17, 18]. Long-term safety data are unknown; blood chemistries and liver function must be carefully monitored during treatment with synthetic antiandrogens.

Another non-randomized clinical trial of children with CAH showed an improved growth rate and height *z* score for bone age after treating with growth hormone alone ($n = 12$) or in combination with gonadotropin-releasing hormone agonist (GnRHa) ($n = 8$, $p < 0.0001$) [19]. In a follow-up study, a combined regimen of growth hormone and GnRHa was administered to 14 patients selected for a predicted height of >1 SD below target height. Patients treated with this regimen plus conventional therapy for ~4 years had improved adult height (+1.1 SD) [20]. Adult height of the patients treated with this combined regimen for ~4 years was greater than the adult height of matched historical CAH controls treated with conventional therapy alone (SD −0.4 vs. −1.4, $p = 0.01$).

GnRHa treatment increases adult height in CAH children who develop central precocious puberty, the only FDA-approved indication for this class of drugs [21]. No randomized study has investigated the effect of GnRHa alone on adult height in children with CAH and normally timed puberty. Similarly, there are no data to suggest that aromatase inhibitors would benefit CAH children in terms of height outcomes, although data seem promising in trials of adolescent boys with other growth problems.

In summary, evidence is lacking to recommend the use of sex steroid blockade, growth hormone, GnRHa, or aromatase inhibitors in children with CAH outside of controlled IRB-approved trials. Normal adult height can be achieved in CAH with judicious use of standard GC and MC therapies, and height-enhancing drugs are not recommended for individuals whose height is, or is expected to be, > –2.25 SD.

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Chapter 10
Apparent Mineralocorticoid Excess – Update

Saroj Nimkarn

Apparent mineralocorticoid excess (AME) is a rare inherited form of hypertension caused by 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD) deficiency. The disorder was first described biochemically and hormonally in 1977 by New et al. in a Native American girl with severe hypertension [1]. The syndrome is caused by non-functional mutations in HSD11B2 on chromosome16q22. More than 40 causative mutations have been described. AME defined an important "pre-receptor" pathway in steroid hormone action and their specificities to the receptor. The exploration and elucidation of this disease opened a new area in receptor biology as a result of the demonstration that the specificity of the mineralocorticoid receptor (MR) function depends on a metabolic enzyme (11β-HSD2) rather than the receptor itself [2–6]. This enzyme functions to protect the MR by inactivating cortisol to its inactive metabolite cortisone, thereby enabling the mineralocorticoid aldosterone to occupy the MR in vivo [7, 8]. Aldosterone is not metabolized by 11β-HSD2 because it forms a C_{11}–C_{18} hemi-ketal group in aqueous solution. The MR is non-selective in vitro and cannot distinguish between the glucocorticoid cortisol and its natural ligand, aldosterone [9, 10]. Therefore, lack of protection of the receptor owing to the enzyme defect allows cortisol, which has higher circulating levels than aldosterone, to bind to the MR and to act as a mineralocorticoid. Clinical manifestations of AME mimic those of excessive mineralocorticoid activity, but no elevation of known mineralocorticoids is present in the AME patients. Three metabolite ratios are calculated, each reflecting a different aspect of enzyme function: (1) tetrahydrocortisol (THF) + allo-THF/tetrahydrocortisone (THE) (global function of HSD) [11]; (2) allo-THF/THF ratio (defect in 5β-reductase activity) [12, 13]; and (3) urinary-free cortisol (UFF)/urinary-free cortisone (UFE) (kidney HSD function) [14]. Originally AME was described through the plasma half-life of [11-^{3}H]cortisol (which when metabolized by 11β-HSD2 yields tritiated water and cortisone), which may more accurately reflect renal 11β-HSD2 activity [15].

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Longstanding research in this field allowed us to understand the natural history and outcome of AME patients. Although AME is very rare, mild or intermediate phenotypes of AME patients may be linked to common human disorders via alteration in cortisol–cortisone shuttle. These include several forms of hypertension, kidney failure, inflammatory processes (cirrhosis and cardiac fibrosis), low birth weight/fetal programming of adult diseases, and, lately, carcinogenesis.

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Chapter 11
Clitoroplasty in Congenital Adrenal Hyperplasia: Description of Technique

Dix P. Poppas

Female patients with congenital adrenal hyperplasia (CAH) may develop masculinized genitalia in utero upon exposure to excess androgen. This masculinization may be characterized by hyperpigmented or fused labioscrotal tissue, enlarged labia majora, a urogenital sinus, and clitoromegaly. Clitoromegaly may vary, ranging from an ordinary clitoris to a significantly enlarged clitoris similar to a near-normal penis, with any degree of intermediate variation. Surgical management of clitoromegaly may ensure adequate sexual function, promote consistent female gender identity development, and provide a typical clitoris aesthetic. Patients and families considering clitoral surgery should make every effort to understand the options, risks, and benefits of this surgery. If surgery is being considered, it should only be performed in centers with significant experience.

Reduction clitoroplasty is the most widely accepted and practiced surgical approach for clitoromegaly and maximizes clitoral aesthetic while preserving sexual function through neurovascular conservation. Specific surgical techniques vary with respect to the extent of glanular and corporal preservation as well as incision location. Consequently, outcomes are difficult to quantify objectively secondary to unsystematic record keeping and lack of large patient populations who have a consistent surgical procedure. Further, reservations about surgical intervention exist, centered on inconsistent or outdated surgical protocols, fears of nerve destruction or sexual dysfunction, a lack of histological data as a benchmark of surgical success, and little consistent clinical follow-up regarding sensation and function.

In an effort to maximize postoperative somatosensory function, the reduction clitoroplasty was modified in consideration of the dorsal nerves of the clitoris. The anatomic location of these important nerves has only recently been delineated in great anatomical detail. Briefly, the dorsal nerves course dorsally along the clitoral erectile bodies; they are most prominent at the 11 and 1 o'clock positions and send

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branches around the tunica until the 5 and 7 o'clock positions, between which nerves are absent.

The dorsal nerves serve several vital functions. They are the primary provider of somatosensory innervation to the clitoral body and glans. Dorsal nerve stimulation causes the cavernous nerve to initiate and maintain engorgement. Finally, the dorsal nerves carry nNOS-positive fibers, which may be efferents for clitoral engorgement. Both the somatosensory and erectile capacities are essential for normal female sexual function.

Beginning in 1996, our team has focused on providing the most specific surgical approach for performing reduction clitoroplasty. We have assessed the outcome of a modified ventral approach reduction clitoroplasty by analyzing hypertrophic erectile tissue obtained from patients undergoing this procedure. The erectile tissue has been examined for the presence and size of nerves from patients undergoing this procedure. Specific attention was paid to the circumferential nervous tissue, reflecting the course of the dorsal nerves. We have determined that no large nerve fibers remain with the excised erectile tissue indicating that they are left intact within the patient. Additional studies by our group have also confirmed that clitoral viability with respect to tissue perfusion and lack of ischemia is excellent. Furthermore, in a limited number of patients, clitoral sensation was evaluated and was determined to be intact to both touch and vibration.

Dorsal nerve preservation via a modified ventral approach reduction clitoroplasty may maximally preserve sexual function. Postoperative histologic and clinical data provide evidence that this modified ventral approach conserves dorsal nerve fibers and functions within these patients. The ventral approach reduction clitoroplasty described in this report leaves the dorsal neurovascular bundles of the corporeal bodies and the glans clitoridis intact. This is a safe and reliable approach for patients and families who are considering clitoral surgery. Sexual and social function of our patient cohort is difficult to assess until all patients reach sexual maturity. Continued, long-term follow-up is ongoing to document sexual function using this approach for patients following clitoroplasty.

Chapter 12
Genitoplasty/Vaginoplasty

Richard C. Rink

The surgical management of DSD/CAH is one of the most complex problems a pediatric urologist will encounter. Furthermore, this surgery remains quite controversial and has been featured by nearly every major television and print media network. The parents must be made aware of these controversies and to understand the pros and cons, not only of having surgery but also of not having surgery. They should also be made aware of the options for timing of surgery: neonatal versus pubertal, with the advantages and disadvantages of each. The parents should be made aware of and also have access to support groups such as the CARES Foundation and Accord Alliance.

There is a spectrum of severity of virilization in the child born with CAH. Nearly all girls will have some degree of clitoral hypertrophy. The labia majora may have mild to nearly complete fusion and the labia minora are usually absent. The labia majora are anteriorly placed in relation to the ultimate vaginal orifice. These virilized girls will also have a persistent communication of the vagina with the urinary tract that may occur at any point from the urethra to the bladder neck. The two structures join and exit on the perineum as a common "urogenital sinus" channel. Given these anatomical findings, surgery usually has three components: clitoroplasty, labioplasty, and vaginoplasty [1–4].

Prior to any surgical intervention, the genitourinary tract has historically been evaluated by genitography and endoscopy. These studies are used to identify the level of the confluence of the vagina with the urethra. Vaginal size is also noted. We have recently reported that the anatomy is accurately defined in only 72% of cases with genitography and that it did not provide any information that could not be obtained by endoscopy in the child with CAH. Furthermore, it did not influence the type or timing of surgery [5]. Endoscopy may be done at the time of reconstruction. This avoids a separate anesthetic for Endoscopy in the child with CAH.

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Clitoroplasty will not be discussed here as it is covered in Chapter 11 by Dr. Poppas. Again, it is important to stress the controversy surrounding this surgery and whether or not this surgery is needed. All aspects of surgery or no surgery, pros and cons, must be discussed with the family. They should have the opportunity to discuss and question this surgery with all care givers and they must have access to support groups.

Labioplasty consists of creation of labia minora and mobilization of the labia majora by Y-V plasty to move them inferiorly along either side of the vagina [6]. This improves the cosmetic outcome and avoids having the vagina appear as an isolated hole as was common in previous early repairs.

It is nearly universally accepted that *vaginoplasty* is necessary, making it much less controversial than clitoroplasty. However, the timing and type of procedure are still debated. The literature notes a 30–98% incidence of stenosis, as well as potential for painful intercourse or impaired sensation. There has been remarkable progress in vaginoplasty techniques over the past decades. "Cutback" vaginoplasties have often been reported in the old literature but with improvements in surgical techniques should no longer be used. This procedure nearly always results in stenosis, and only rarely provides opening into the normal caliber proximal vagina. Though many types of vaginoplasties have been described, most children with a low- to mid-level vaginal confluence UG sinus can currently be successfully treated by "flap" vaginoplasty [3, 4]. A posterior-based perineal flap is placed into the posteriorly opened vagina to allow it to exit onto the perineum. When done correctly it will achieve an adequate caliber vaginal introitus. It is important to note that a "flap" vaginoplasty serves only to enlarge the introitus and it does not change the level of the confluence of the urethra and vagina. With this procedure the posterior wall of the vagina will be made of skin and the urethra will be somewhat hypospadic.

For the rare high confluence, a "pull-through" vaginoplasty may be required. In this procedure the vagina is completely separated from the urinary tract and is then advanced toward the perineum. The urogenital sinus becomes the urethra. The vagina often cannot be moved to the perineum and therefore multiple skin flaps may be necessary to reach the vagina. This more complex procedure results in increased risks of stenosis, fistula, incontinence, and sensory loss [3, 4]. Furthermore, the skin flaps often turn out to be hair-bearing and lack lubrication. Fortunately, "pull-through" vaginoplasties are rarely needed in CAH and are reserved for those with a very high confluence.

"Total urogenital mobilization" (TUM), described by Alberto Pena, is a concept whereby the entire urogenital sinus, bladder, and vagina are circumferentially mobilized toward the perineum. This is not a vaginoplasty technique but a means to allow a more easily performed vaginoplasty. There is less blood loss and because the vagina is moved toward the perineum there is less need for the more complex "pull-through" vaginoplasty. TUM initially gained great popularity. However, there are no long-term results in CAH and there are concerns about the potential for stress incontinence or loss of sensation to the vagina and clitoris. Due to these concerns we reported "partial urogenital mobilization" (PUM) [6]. In our PUM technique, the dissection stops at the pubourethral ligament. We believe this reduces these

risks. Our PUM concept has been supported on a neuroanatomic basis by Dr. Larry Baskin's research. In the original description of the TUM the redundant mobilized sinus tissue was discarded. We have now shown that the use of the mobilized sinus tissue in the reconstruction eliminates the need for skin flaps which are often hair-bearing and tend to stenose [7]. Cosmesis is also improved. We have used this mobilized urogenital tissue to create a mucosal-lined vestibule and an anterior and posterior vaginal wall. Our urogenital mobilization techniques are well illustrated and described in British Journal of Urology International [8].

We do not believe that any prepubertal child should undergo vaginal dilation. Though we support early surgery in infancy, parents should be aware that there has historically been a high incidence of patients needing further surgery after puberty, primarily for vaginal stenosis. These secondary repairs have generally been minor outpatient repairs. Some have recommended delaying the vaginoplasty portion of the surgery until puberty, but this results in a two-stage repair. Others have called for no surgery until the patient is at the age where they can decide when and what type of surgery is to be performed. Future research should be directed to define which path results in the best outcomes.

In conclusion, there have been major advances in genital reconstruction for CAH. However, there is still much to be achieved. The parents must be supported during this difficult time and be allowed to participate in all decision making regarding surgery versus observation. Some suggest waiting until the patient can make an informed decision about surgery and this option should always be presented to the family. We need long-term studies that focus not only on cosmetics, vaginal size, and urinary continence but also on pain-free intercourse with normal sensation and orgasm. Excellent hormonal control is imperative to achieve the best surgical outcomes. Unfortunately, the level of optimal hormonal control is never reported in the literature. Furthermore the initial severity of masculinization and the level of the confluence are seldom reported, making it difficult to accurately compare results. The optimal timing of surgery is yet to be determined. The rights of the child versus the parents must be determined. The effect of raising a child with genital ambiguity who has not undergone reconstruction remains unknown.

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Chapter 13
Insights into the Aetiology of Ovotesticular DSD from Studies of Mouse Ovotestes

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In mammals, *Sry* (sex-determining region Y gene) is the master regulator of male sex determination. It induces a cascade of gene expression that regulates the differentiation of the bipotential genital ridges into testicular tissue. In the absence of *Sry*, or if SRY function is impaired, the genital ridges develop into ovaries. Subsequently, hormones produced by the testes and ovaries direct the differentiation of all secondary sexual characteristics (for review, see Wilhelm and Koopman [1]).

Largely as a consequence of the location of *Sry* on the Y chromosome, this gene is subjected to mutations that cannot be repaired by mechanisms that rely on pairing with a duplicate chromosome. These mutations degrade the structure of the encoded protein and also potentially compromise the regulatory sequences required for correct and robust gene expression. This vulnerability of *Sry* to mutations means that the mechanisms controlling male sexual development are fragile, a situation that has profound biological and medical implications.

Sry encodes an HMG domain transcription factor whose primary role is to activate expression of a related *Sox* gene, *Sox9*. Like *Sry*, the activity of *Sox9* is both necessary and sufficient to induce testis development in the genital ridges. Once *Sox9* is activated, it upregulates a suite of genes required for maintaining the Sertoli cell phenotype and directing the development of other testicular cell lineages. *Sox9* also activates signalling pathways that stimulate *Sox9* expression in Sertoli cell precursors that do not express sufficient levels of *Sry* to cell-autonomously upregulate *Sox9*. We used XX–XY gonadal cell mixing experiments to identify prostaglandin D_2 (PGD_2) as a molecule secreted by Sertoli cells that stimulate *Sox9* expression even in XX gonadal cells lacking *Sry* [2]. We also found that SOX9 protein can directly activate transcription of the *Ptgds* gene encoding prostaglandin D_2 synthase, the enzyme producing PGD_2 [3]. Thus, *Sox9* and PGD_2 contribute to a positive reinforcement loop that recruits cells into the Sertoli lineage and operates to ensure testis development even when *Sry* activity is compromised.

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Cases exist of XY sex reversal even in the presence of a normal *Sry* gene. A classic example in mice is B6-Y^{DOM} sex reversal, characterized by the combination of a Y chromosome from some *Mus domesticus* subspecies, such as *Mus domesticus poschiavinus* (Y^{POS}), with the inbred C57BL/6 (B6) genetic background [4]. The severity of the phenotype varies and can include two ovaries, two ovotestes, or one ovary and one ovotestis. Each ovotestis consists of ovarian tissue at one or both poles of the gonad and testicular tissue in the centre. Studies of this mouse model of ovotestis development provide a rare opportunity to study the interaction of the testis- and ovary-determining pathways in the same tissue.

We studied expression of several markers of mouse foetal testis (SRY and SOX9) or ovary (FOXL2 and *Rspo1*) development in B6-XY^{POS} ovotestes by immunofluorescence and in situ hybridization, using normal testes and ovaries as controls [5]. In ovotestes, SOX9 was expressed only in the central region where SRY is expressed earliest, resulting in testis cord formation. Surprisingly, FOXL2-expressing cells also were found in this region, but individual cells expressed either FOXL2 or SOX9, not both. At the poles, even though SOX9 was not upregulated, SRY expression was downregulated normally as in XY testes, and FOXL2 was expressed from an early stage, demonstrating ovarian differentiation in these areas.

Our data show that SRY must (1) act within a specific developmental window to activate *Sox9*; (2) challenge the established view that SOX9 is responsible for downregulating *Sry* expression; (3) disprove the concept that testicular and ovarian cells occupy discrete domains in ovotestes; and (4) suggest that FOXL2 is actively suppressed in Sertoli cell precursors by the action of SOX9. In these respects our findings provide important new insights into the molecular regulation of testis and ovary development.

Our data indicate that compromise of the testis-determining pathway can tip the balance in favour of ovary or ovotestis development. Therefore, regulatory mutations affecting *Sry* expression levels or timing may explain some idiopathic XY DSD cases.

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Part IV
Receptor Defects – Speakers

Chapter 14
46,XY Disorders of Sex Development (46,XY DSD) due to Androgen Receptor Defects: Androgen Insensitivity Syndrome

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Androgens have a fundamental role in male sexual development and act by binding to the androgen receptor (AR), which is encoded by a gene located at the X chromosome. Androgen insensitivity syndrome (AIS) is a rare X-linked disorder in which 46,XY subjects have complete or partial impairment of androgen action throughout life due to abnormalities of the AR. Subjects with the complete form of AIS (CAIS) have a female phenotype, including female breast development that begins at the age of expected puberty, primary amenorrhea, and a paucity or absence of axillary and pubic hair. Partial AIS (PAIS) causes a spectrum of phenotypes, ranging from women with clitoromegaly to men with minor degrees of undervirilization; gynecomastia is common at puberty. In both CAIS and PAIS, androgen production is in the normal male range [1].

The importance of estrogens for the pubertal growth spurt and bone mineralization, in both males and females, has been recently shown. However, the direct effects of androgens and Y chromosome-specific genes remain less clear. Patients with androgen insensitivity syndrome constitute a natural model to study the effects of Y genes, which are present, and androgens, whose action is absent.

We studied the *AR* gene in 32 subjects (20 families) with 46,XY DSD. Study criteria were 46,XY karyotype, normal male basal and hCG-stimulated levels of serum testosterone and steroid precursors, gynecomastia at puberty, and in prepubertal patients, a family history compatible with X-linked inheritance. Mutations in the *AR* were found in all 9 families with CAIS and in 8/11 (73%) of families with PAIS. We summarize here the main clinical, hormonal, bone densitometry, molecular, and behavioral features of the 25 Brazilian subjects with AIS confirmed by identification of mutations in the *AR* [2].

Nine mutations had been previously reported and six were first reported in this cohort: 87% mutations were located in androgen-binding domain; 53% mutations

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were located in exon 5 or 7 (hotspot) [2, 3]. Identification of mutations in the androgen receptor was essential to classify patients with 46,XY DSD as PAIS. The presence of a family history and/or development of gynecomastia at the time of expected puberty was useful to select patients for genetic studies and increase the likelihood of finding a mutation in the AR.

Estradiol levels were within the normal range in all patients with CAIS, and many with PAIS, suggesting that gynecomastia developed in response to normal estrogen concentrations unopposed by androgen action. Serum LH, as well as the LH × T product, was elevated in all pubertal patients indicating resistance to androgen in LH feedback. Testosterone levels were normal or elevated. Serum FSH levels were normal unless the patient had testicular damage due to cryptorchidism and/or orchidopexy.

In patients with CAIS, absence of axillary hair was more frequent than absence of pubic hair, which usually was present but sparse. In patients with female social sex, vaginal dilation was useful to obtain an adequate length for sexual intercourse. In patients with PAIS, phallic size and its response to high-dose testosterone therapy were usually subnormal, but variable among patients (adult penile length varied from 5.5 cm after 250–500 mg/week testosterone esters to 10.0 cm without treatment). All patients with PAIS raised as girls, as well as those raised as boys, maintained the gender assigned before puberty, despite an overlap in their phallic sizes at puberty. This compares with patients with 46,XY DSD due to 5α reductase 2 and 17-hydroxysteroid dehydrogenase 3 deficiencies who, in our experience, frequently change from female to male gender at puberty.

There is no consensus if height and bone density in patients with AIS should be compared to male or female standards. Patients raised as girls will be socially compared to other women; biologically they harbor Y-specific genes but lack the androgen effects of normal males.

In our cohort, patients with CAIS had an adult height of 165.7 ± 8.9 cm, corresponding to a mean SDS of –1.35 (median SDS of –1.01) for men and mean SDS of + 0.59 (median SDS of + 0.96) for women. Patients with PAIS had an adult height of 168.7 ± 9.6 cm, corresponding to a mean SDS of –0.88 (median SDS of –0.91) for men and mean SDS of +1.08 (median SDS of +1.07) for women. Therefore, adult height in patients with AIS was intermediate between that of normal males and females ($P < 0.05$) [4].

The shorter height in relation to males might have resulted from an impaired androgen action on normal male statural growth, whereas the taller stature in relation to females might reflect an androgen-independent participation of Y-linked genes in height determination.

Bone mineral apparent density (BMAD) in subjects with CAIS and PAIS submitted to gonadectomy and estrogen replacement was normal in the femoral neck but deficient in vertebral bone ($z = –1.56 \pm 1.04$, $P = 0.006$, compared to female standards; and $z = –0.75 \pm 0.89$, $P = 0.04$, compared to male standards [4]). Low spine bone mineral density before and after gonadectomy and in estrogen replacement-compliant CAIS may reflect androgen resistance at the bone level and support a direct role of androgens on bone, apart from its effects after aromatization into

estrogens. Careful follow-up of subjects with AIS and surveillance for the incidence of fractures are necessary to determine which results of bone densitometry, BMD or BMAD, and which normative references, male or female, are more informative and lead to criteria for intervention.

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Chapter 15
Androgen Receptor Mutations Associated with Androgen Insensitivity Syndrome: A High Content Analysis Approach Leading to Personalized Medicine

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Androgen insensitivity syndrome (AIS) is a rare disease associated with inactivating mutations of AR that disrupt male sexual differentiation and cause a spectrum of phenotypic abnormalities having as a common denominator loss of reproductive viability. No established treatment exists for this condition; however, there are sporadic reports of patients (or recapitulated mutations in cell lines) that respond to administration of supraphysiologic doses (or pulses) of testosterone or synthetic ligands. The common denominator of these mutations is that they are located in the ligand-binding domain (LBD) and are associated with qualitative abnormal ^{3}H-DHT binding consisting of increased ligand–receptor dissociation rate.

We have utilized a novel high content analysis (HCA) approach to study AR function at the single cell level in genital skin fibroblasts (GSF) from patients and in HeLa cells stably transfected with plasmids containing wtAR or the mutation of interest fused to a green fluorescent protein (GFP) [1]. We have completed AR HCA analysis in three patients with AIS. While patients with mutations F764L and P766S were affected by complete androgen insensitivity syndrome (CAIS), mutation R840C was found in a patient affected by partial androgen insensitivity (PAIS). The biochemical phenotype of receptor mutants F764L and P766S consisted in normal K_d and B_{max} and increased ligand dissociation rate, while that of mutant R840C consisted in normal K_d, B_{max}, and ligand dissociation rate, but presence of thermolability (that is, the binding ability of this receptor decreased to more than 50% when the temperature was supraphysiologic). All three mutant receptors were greatly

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impaired in assays of transcriptional activity. AR HCA provides an opportunity to simultaneously quantify a myriad of cellular conditions associated with AR functions, is fully amendable to high throughput rates of data collection and automated analyses, and permits us to quantify AR activities in response to ligands, mutations, and various perturbations of the intracellular environment of the host cell and to identify anti-androgens with novel mechanism of action. With data based upon well-defined selection criteria from hundreds of cells per condition generated by the software PLP (Pipeline Pilot, Acelrys, San Diego, CA, USA) and CyteSeer (Vala Sciences, San Diego, CA, USA) we are able to define the ligand dose necessary to induce or inhibit (EC_{50}/IC_{50}) five sequential features that contribute to AR activation: (1) total AR protein level; (2) percent localization of signal in the nucleus (e.g., the nuclear:cytoplasmic ratio); (3) formation of AR-rich subnuclear "speckles" that correlate with AR transcriptional activity; (4) transcriptional reporter gene activity; and (5) AR nuclear export.

For our studies we used HeLa cells transfected with the GFP-fused plasmid of interest and treated with logarithmic concentrations of DHT, mibolerone, or R1881. GSF from each patient and six normal controls received the same experimental treatment, while their endogenous AR was visualized by antibody staining. AR HCA was able to provide for HeLa cells data on the percent localization of the signal in the nucleus, the formation of AR-rich subnuclear speckles, and the transcriptional reporter gene activity. In contrast to GSF we were able to obtain data only on the percent localization of the signal in the nucleus and on the formation of AR-rich subnuclear speckles. Reporter gene transcriptional activity was not obtained because no AR-responsive promoter could be found that works in GSF.

The measured functions of P766S activity could be rescued at EC_{50} 10-fold higher compared to wild-type AR in both HeLa and GSF using each of the three ligands. F764L functions were rescued, but only in a mibolerone-dependent way and at EC_{50} concentrations 10- to 30-fold higher than wtAR, while R840C could not be rescued under any experimental condition. We hypothesized that the rescue of AR functions was associated with an increased level of ligand–receptor complex stability. To prove this we performed a two-hybrid analysis determining the AR NH_2–COOH-terminal domain interaction, an assay measuring the tightness of receptor–ligand binding. In agreement with the HCA data, these experiments showed that the degree of AR NH_2–COOH interaction increased as a function of ligand concentration for all three agonists using P766S. For F764L, AR NH_2–COOH interaction increased only as a function of mibolerone concentrations, while for R840C it did not change at all under any experimental conditions.

The conclusions of this study are that certain LBD AIS mutations associated with normal K_d and B_{max} but abnormal ligand dissociation rate can be rescued by AR agonists given at supraphysiologic concentrations and that higher concentration of agonist works by increasing the ligand–receptor complex stability. In addition, these experiments support the notion that HCA can be used for personalized treatment of patients affected by AIS.

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Chapter 16
Phenotypic Variation of SF1 Gene Mutations

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Knockout of the steroidogenic factor 1 (Sf1) gene in 46,XY mice causes complete adrenal failure associated with gonadal dysgenesis, undervirilization, and persistence of mullerian ducts [1]. SF1 (NR5A1, SF1/Ad4BP/FTZF1) is a member of the nuclear receptor superfamily, and it plays a crucial role in the fetal development of the adrenal gland [2] and testes [3]. Sf1 is considered to be the master regulator of the reproductive system because it regulates the expression of a wide array of genes required for testis and adrenal development and hormone synthesis [4]. Sf1 is involved in most of the enzymatic steps leading to testosterone synthesis and regulates AMH and INSL3 gene expression. In human, the *SF1/NR5A1* mutation has shown a broad range of phenotypes in the disorders of sex development (DSD), in addition to adrenal dysfunction [5]. *SF1* was first described in adrenal insufficiency (AI) combined with XY sex reversal [6], then in XY DSD patients without AI [7–10], and very recently as a cause of hypospadias [11] and premature ovarian failure [12]. Moreover, *SF1* polymorphisms have been associated with cryptorchidism [13] and micropenis [14].

We describe here the 20 *SF1* mutations (18 new and 2 previously published mutations) that we identified as part of an international collaborative program (Fig. 16.1). The first group of 16 patients displayed phenotypic features already reported in the literature: 3 patients had been referred for 46,XY sex reversal, 6 for XY DSD, and 7 for XY primary amenorrhea. In addition, explorations of other patient cohorts revealed new mutations in non-classical cases. We identified three previously unreported *SF1* mutations in patients with 46,XY sex reversal [9] and seven unreported mutations in patients with 46,XY amenorrhea and low plasma testosterone level [15].

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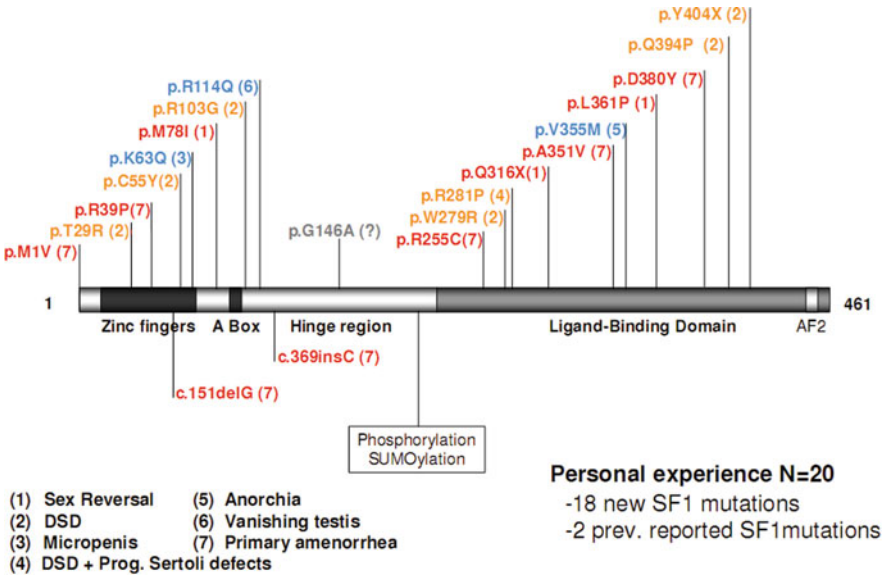

Fig. 16.1 Summary and localization of the 20 mutations identified in Montpellier

The first non-classical finding resulted from the screening for the *SF1* mutation in an anorchia cohort [16]. Of the 24 DNAs studied, we were able to identify one *SF1* gene abnormality in a patient with bilateral anorchia and micropenis. Mutational analysis revealed a heterozygous p.V355M substitution of the putative ligand-binding domain of SF1. This mutation affects a highly conserved amino acid in helix 7. Study of the parents and the other twin showed that the mother and twin brother carried the same heterozygous mutation. Sixty control DNAs were analyzed and this amino acid substitution was not found. Functional analysis of this mutant concluded to a mild partial loss of SF1 function (Fig. 16.2).

Screening in a cohort of patients with isolated micropenis ($n = 26$) and vanishing testis ($n = 1$) revealed an unreported p.K63Q mutation in young boys with subnormal penile length (6 cm at 12.6 years) and an unreported p.R114Q mutation in an adolescent boy with late vanishing testis. In vitro studies demonstrated a slight decrease in SF1 transactivation capability (Fig. 16.3).

More recently, analysis of a patient with XY DSD with a progressive Sertoli cell defect and posterior hypospadias led to the identification of a p.R281P mutation within the hinge domain of the SF1 protein. The in vitro functional analysis of this mutation revealed that it had drastically altered the transactivation of AMH transcription by 90% (Fig. 16.4), as confirmed in the patient by a high FSH level contrasting with decreased inhibin B and subnormal response to the HCG stimulation test (2 ng/ml). Interestingly, this mutation was found at low copy number in the father's DNA isolated from peripheral blood leukocytes, suggesting mosaicism.

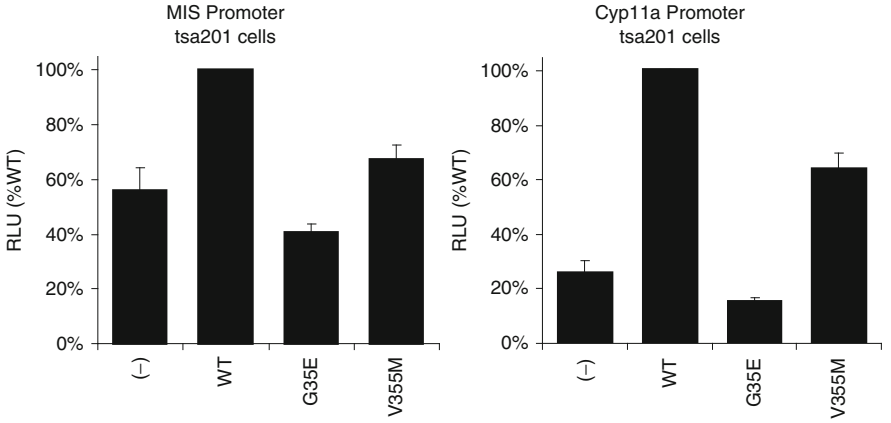

Fig. 16.2 Transcriptional activation of SF1 target gene promoters by WT and mutant p.V355M SF1. The activity of the p.G35E DNA-binding domain mutant associated with a more severe phenotype is shown for comparison. Data for the Mis and Cyp11a (scc) promoters in tsa201 cells expressed as percentage of WT activity. Data represent the mean + SEM of three independent experiments, each performed in triplicate

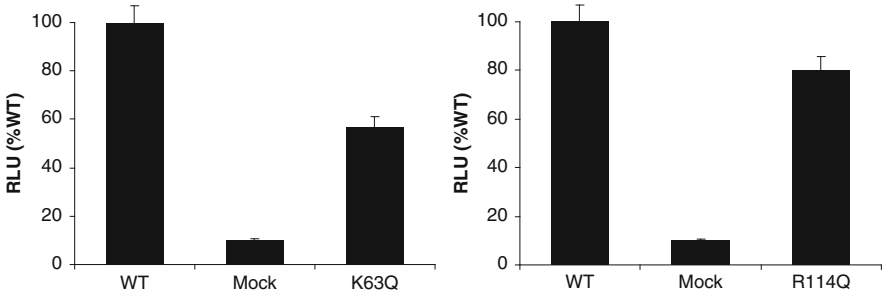

Fig. 16.3 Transcriptional activation of SF1 target gene promoter by WT and the p.K63Q and p.R114Q SF1 mutants. Data for minimal transactivation promoter in Cos-7 cells expressed as percentage of WT activity. Data represent the mean + SEM of three independent experiments, each performed in triplicate

Last, we regularly identified the SF1 variant p.G146A, which we consider to be a mutation with slight impact, in patients with testicular dysgenesis syndrome. The experiments on the transcriptional capability of the G146A variant suggested that this abnormality was responsible for the cryptorchidism and micropenis observed in these patients, either alone or in association with a particular genetic background.

In our experience, *SF1* mutations are associated with a wide range of phenotypes. Moreover, some important issues regarding these mutations in humans remain unsolved. First, it is not known whether patients with 'milder forms' of 46,XY DSD and seemingly normal adrenal function will eventually develop late-onset adrenal

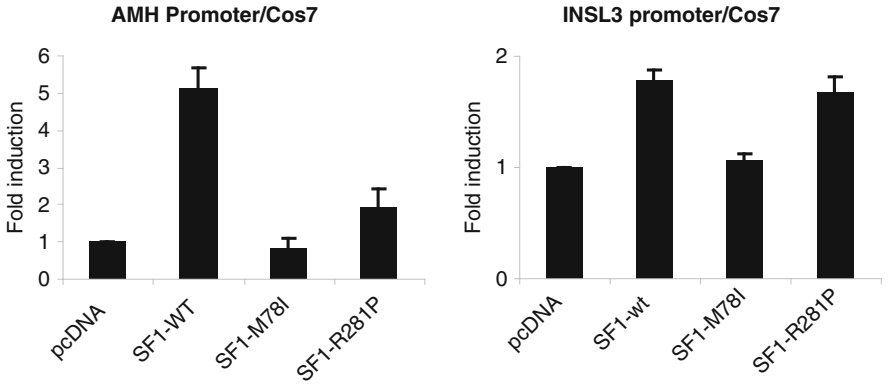

Fig. 16.4 Transcriptional activation of SF1 target gene promoters by WT and mutant p.R281P SF1. The activity of the p.M78I DNA-binding domain mutant associated with a more severe phenotype is shown for comparison. Data for the Mis and Insl3 promoters in HEK 293 cells expressed as fold inductions over Mock. Data represent the mean + SEM of three independent experiments, each performed in triplicate

insufficiency. Longitudinal follow-up of these patients is thus mandatory. Second, if patients with these milder forms of 46,XY DSD continue to show normal adrenal function even on long-term follow-up, why would the *SF1* mutation cause testicular dysgenesis or impaired androgen production, but not adrenal insufficiency? Third, the full phenotypic spectrum of 46,XY *SF1* mutation has not yet been documented. Previous publications have shown that most of the patients have 46,XY DSD without adrenal insufficiency. Thus, 46,XY *SF1* mutation may result in a wide spectrum of male reproductive phenotypes and could be involved in male or female infertility. Fourth, the molecular mechanism for the development of obesity in *SF1* deficiency needs further clarification, although mice studies have suggested the loss of VMH function.

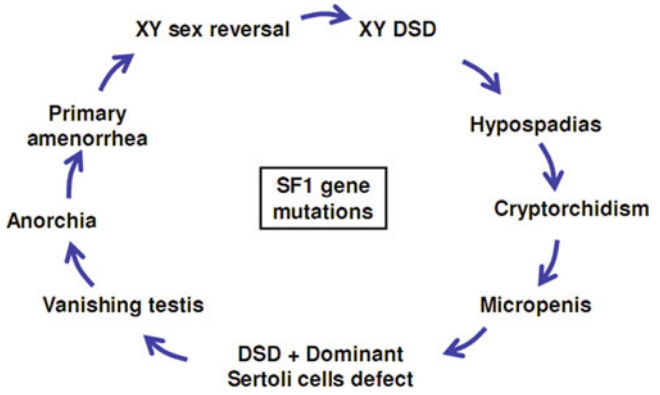

Fig. 16.5 Representation of the wide phenotypic spectrum of *SF1* mutations ranging from 46,XY complete gonadal dysgenesis to isolated micropenis and anorchia

To conclude, these data confirm the important role of SF1 in testis determination and steroidogenesis. The wide phenotypic variation in *SF1* mutations we report here (Fig. 16.5) suggests that SF1 is a relatively common cause of DSD in 46,XY patients.

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Part V
Psychological Aspects of Intersex – Speakers

Chapter 17
Transsexualism ("Gender Identity Disorder") – A CNS-Limited Form of Intersexuality?

Heino F.L. Meyer-Bahlburg

Major scientific societies (including the Endocrine Society [16]) as well as public health authorities of various countries are reexamining transsexualism – or, more broadly, gender identity disorder (GID; [1]) – regarding diagnostic categorization, etiology, clinical management, and human rights considerations. In this context, many transgender communities demand the "depathologization" of the condition, i.e., replacing its categorization as a "mental disorder" with the classification as a "neurological," "intersex," or "medical" condition, or even removing it entirely from both DSM-V and ICD-11 [34]. The purpose of this chapter is to examine the evidence supporting the interpretation of GID as an intersex condition.

In terms of behavioral or psychological presentation, persons with early-onset GID have much in common with individuals with somatic intersexuality – now subsumed under the category disorder of sex development (DSD; [18]) – who want to change their gender. From early on, they perceive an incongruence between their experienced/expressed gender and their assigned gender and frequently also their sexual anatomy, which may become strong enough to warrant a gender reassignment. However, by definition, individuals with non-DSD GID show no symptoms of somatic intersexuality, and their sex hormone levels appear normal for their assigned gender and age, except for about one-third of natal females with non-DSD GID who have moderately increased androgen levels in the range of hirsute women without genital ambiguity [7, 29]. Moreover, findings from studies of peripheral sex steroid-related nucleotide polymorphisms of steroid receptor or steroid enzyme genes in persons with non-DSD GID [3, 4, 15, 17, 33] have been inconsistent or negative.

The interpretation of GID as a CNS-limited form of intersexuality would need corroboration by one or more of several lines of evidence, among these (1) genetically based systemic prenatal sex hormone abnormalities that do not markedly affect the reproductive anatomy, but nevertheless influence brain and behavior; (2)

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abnormal sex hormone levels in the developing brain or respective CNS-limited hormone receptor defects; (3) abnormalities in the genes that interact with sex hormones to bring about the sexual differentiation of the brain; and (4) abnormalities of the neuroanatomic structures that underlie normative sex differences in gendered behavior.

While normative sex differences in the neuroanatomy of the human brain are pervasive on diverse structural levels [9, 21], the mechanisms of masculinization, feminization, demasculinization, and defeminization of the human brain are not yet well understood. It is clear from extensive research on non-human mammals that both genetic and hormonal factors play a significant role in the sexual differentiation of the brain. For instance, 4,508 genes are actively transcribed in the mouse brain; among these, 257 genes are more highly expressed in males and 355 in females [36]. Most sex-related genes are steroid dependent, but some are independent [10, 30]. Both androgens and estrogens play a role, although the role of estrogens appears to be relatively reduced in primates [2, 35]. In human gender development, social and cognitive factors such as differential reinforcement, observational learning, and self-socialization are expected to play strong additional roles [23, 31].

The prenatal and postnatal sex hormone situation in most syndromes of DSD is well known [14], and an association of gender-atypical prenatal androgen milieus with later gender-related behavior (including homosexuality) in a dose–response fashion has been emerging in recent studies [26, 27]. Yet, neuroanatomic and functional studies of the human intersex brain are just beginning [8, 22, 24] and not yet conclusive, providing, therefore, little guidance for what to expect from studies of non-DSD GID. A few postmortem studies of the brains of persons with non-DSD GID have yielded promising findings on the central portion of the bed nucleus of the stria terminalis [19, 37] and the uncinate nucleus [12] that are in line with expectations based on some of the known neuroanatomic sex differences. Brain function studies of persons with non-DSD GID by fMRI, when smelling odorous steroids [5], viewing erotic films [13], or performing a spatial cognition task [32], are also suggestive, although less consistent. However, the findings from such studies are not yet conclusive due to lack of replication, small sample sizes, and other methodological problems [28]. Moreover, most of the findings come from individuals with late-onset, heterosexual (relative to natal sex) GID, in whom GID development appears to be associated with a history of fetishistic transvestism (autogynephilia [6, 20]), which is more difficult to reconcile with an intersex interpretation. Also, we have previously shown in DSD children that their gender behavior shifts show a (group-level) correlation with prenatal hormone "dose," but not their (dimensionally measured) gender identity [25].

A straightforward neuroendocrine mechanism appears even less likely for documented other forms of identity-related psychiatric conditions, such as persons with body integrity identity disorder, 20% of whom have a history of GID [11]. Thus, we need a better understanding of the processes involved in identity formation in general, if we want to delineate the specific contribution of neuroendocrinological factors.

In summary, the data available permit the following overall conclusions:

1. Persons with non-intersex GID resemble intersex patients who request gender change in their current gender behavior.
2. However, there is currently no known intersex analog for non-intersex GID of late onset.
3. Neuroanatomy studies of intersex are very few and do not provide guidance to what brain effects to expect as the basis for GID in a putative CNS-limited form of intersexuality.
4. Findings in GID patients of atypical androgen levels apply only to about one-third of female-to-male transsexuals, and not at all to male-to-female transsexuals.
5. Sex steroid-related findings of genetic polymorphisms are weak, inconsistent, and largely unreplicated.
6. Neuroanatomic findings are few and unreplicated; distributions for GID patients overlap with those of controls and are therefore of questionable utility for GID diagnosis.
7. Given the complexity of gendered behavior and identity, the neuroanatomic basis of GID is likely to be networks rather than individual brain nuclei or regions.
8. Neurofunctioning findings are few and unreplicated.
9. In summary, the emerging data are interesting, but at this time insufficient to constitute a firm basis for a theory of GID as a form of CNS-limited intersexuality.

Note

A new brain-imaging study by MRI compared 24 gynephilic male-to-female transsexuals (MtF-TR) to heterosexual control women and men on various aspects of brain anatomy. The brains of MtF-TR were more similar to heterosexual men than to women, but also showed some differences from both control groups. The authors concluded that the data do not support the notion that the brains of MtF-TR are feminized (Savic I, Arver S: Sex dimorphism of the brain in male-to-female transsexuals. Cerebral Cortex 2011;doi:10.1093/cercor/bhr032).

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Part VI
Embryology of Sex Differentiation – Speakers

Chapter 18
Biology and Genetics of Anti-Müllerian Hormone

Nathalie Josso

AMH, A Member of the TGF-β Family "Specializing" in Sex

Anti-Müllerian hormone (AMH) also known as Müllerian inhibiting substance (MIS) is a homodimeric glycoprotein selectively involved in reproduction and development. As shown by Cate et al. [1], it belongs to the TGF-β family, with homology essentially to the BMP proteins. Like all members of the family, it is composed of a long N-terminus, which must be cleaved from the short C-terminus for biological activity. The N-terminus is inactive on its own but plays a role in folding and helps the C-terminus cross the cell membrane and get secreted. A 3D putative model of the AMH C-terminus has been constructed by analogy with crystallized membersof the BMP family [2].

AMH and BMPs also share receptors. Like all members of the TGF family, AMH has two kinds of receptors: AMH receptor type II (AMHR-II) is specific; a 3D model built by analogy with other crystallized members of the family is now available [3]. The type I receptors, ALKs 2, 3, and 6, are shared with the BMP proteins (reviewed in [4]). ALK3 is the most important, as demonstrated by targeted inactivation experiments [5].

Three-dimensional models are useful to explore the impact of mutations on protein function. Mutations of AMH or of its type II receptor lead to the so-called persistent Müllerian duct syndrome, PMDS, characterized by the persistence of uterus and tubes in a genetic and otherwise normally virilized male with cryptorchidism and/or inguinal hernia. The condition is autosomal recessive, in keeping with the autosomal location of the AMH (chromosome 19) and AMHR-II (chromosome 12) genes. Our group has studied more than 100 PMDS families to date, in 15% no mutation was identified, in the others, AMH and AMHR-II mutations are equally frequent. A 27-base deletion is found in approximately half the families with a receptor mutation [6].

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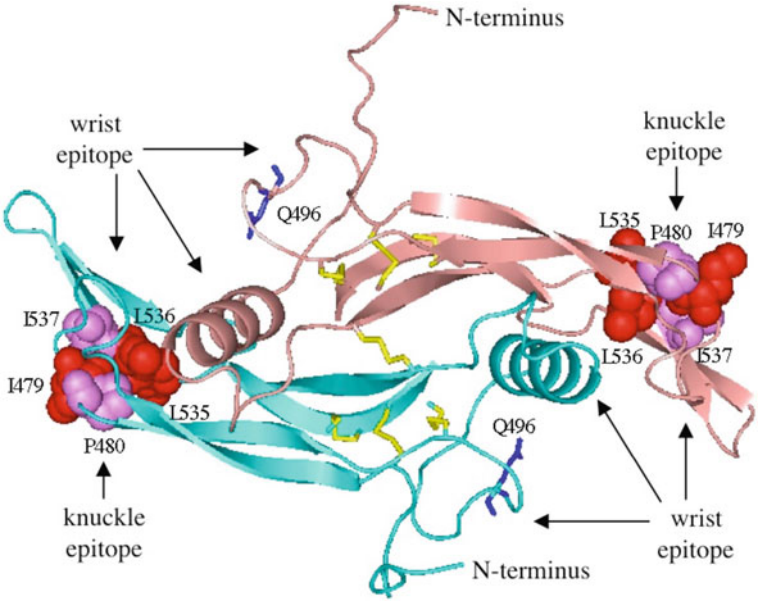
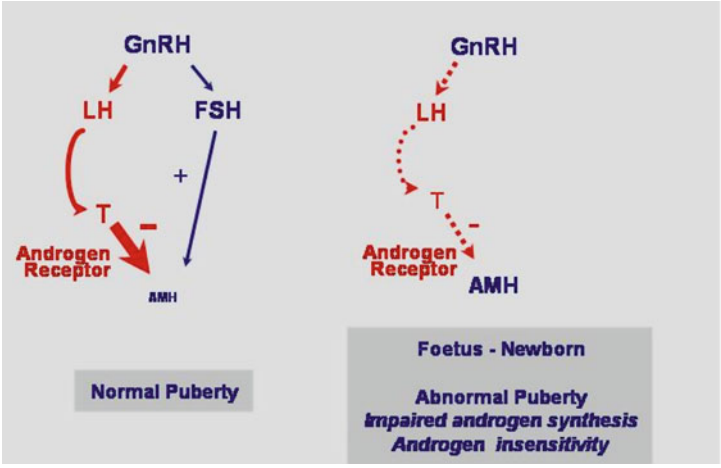


A 3D model for the dimeric AMHC_terminus: One monomer is shown in *blue* and the other in *pink*, disulfide bonds are in *yellow*. The type II receptor is thought to bind at the "knuckle" and the type I at the "wrist." Courtesy of Dr. Richard Cate

Biology of AMH in the Male

AMH is produced in high amounts by Sertoli cells from the time of their differentiation to puberty. In the adult, AMH expression is repressed by testosterone, provided Sertoli cells express the androgen receptor [7]. Androgen-insensitive patients or



Regulation of AMH by testosterone and FSH. Courtesy of Dr. Rodolfo Rey

those with defective androgen production express abnormally high amounts of AMH at birth and after puberty, due to uninhibited stimulation by FSH [8].

In the tammar wallaby, Sertoli cell androgen receptor is expressed earlier and AMH production is curtailed already at the time of virilization of urogenital sinus and external genitalia [9], which occurs in the pouch and not *in utero* as in eutherians.

Biology of AMH in the Female

AMH is produced by ovarian granulosa cells [10] from the end of fetal life to menopause and serves as a marker of follicular reserve [11]. AMH serum assay is now routinely included in the assessment of patients with infertility and PCOS and of those having undergone therapeutic procedures potentially damaging to germ cells. AMH opposes the recruitment of primordial follicles into the growing pool [12]; thus, inactivation of AMH in transgenic mice leads to premature loss of germ cells. Presently, it is not known whether this also occurs in women. Females with homozygous AMH or AMHR-II mutations are normal and fertile in early reproductive life.

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Chapter 19
Genetic Mechanisms Underlying 46,XY DSD with Gonadal Dysgenesis

Louisa Ludbrook and Vincent R. Harley

Genetic changes in key sex-determining genes lead to 46,XY disorders of sex development (DSD) in humans. Loss-of-function mutation in the *sex-determining region on Y* (*SRY*), *steroidogenic factor 1* (*SF1*), and *SRY-Box gene 9* (*SOX9*) genes causes varying phenotypic changes ranging from complete syndromic gonadal dysgenesis to isolated hypospadias. Similarly, gain of function of *dosage-sensitive sex reversal adrenal hypoplasia congenita on X gene 1* (*DAX1*) through duplications within the Xp21 region also affects typical testicular determination, together demonstrating the critical roles these genes play in determining sex. The molecular mechanisms through which these transcription factors act during sex determination are poorly understood and our current efforts aim at delineating the complex interactions that fail due to mutation or deletion in human DSD.

The expression of SRY in the developing XY gonad initiates a testis-specific developmental cascade leading to male-specific somatic cell differentiation, and the formation of testicular cords allowing sex hormone production and later for germ cell maturation. We determined that the biochemical action of SRY requires its import into the nucleus of pre-Sertoli cells, a process which is active and facilitated, in part, by calcium-dependent protein calmodulin (CaM) which interacts with the N-terminal nuclear localization signal (NLS) within human SRY protein [1]. We have now shown that when the SRY–CaM interaction is blocked by a CaM antagonist in embryonic XY gonads in culture, not only is the nuclear accumulation of SRY reduced but the cultured gonads show a sex reversal [2]. The nuclear import of SRY is therefore an early critical requirement for its sex-determining action.

Important evidence unmasking the mechanism of SRY action once inside the nucleus of pre-Sertoli cells came from transgenic mouse studies identifying a 1.3 kb core testis-specific enhancer element of SOX9 called *TESCO* [3]. This enhancer contains three SOX protein-binding sites and six SF1-binding sites. Activation of

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TESCO is proposed to occur in three steps: the initiation of *SOX9* expression involving SF1 binding to *TESCO*; the upregulation of SOX9 involving SRY and SF1 synergistic activation of *TESCO*; and the maintenance, in which SOX9 itself synergizes with SF1 to sustain its own expression in the Sertoli cells [4]. The importance of *TESCO* activation by key sex-determining genes in mouse is clear, and excitingly, in-depth comparative genomic analysis identified a evolutionarily conserved region (ECR) within *TESCO* that supports a conserved sex-determining role for this enhancer beyond mammals to vertebrate species that lack an XY sex-determining mechanism [5].

It is anticipated that characterization of the human testis-specific *SOX9* enhancer will lead to the identification of genetic changes within *TES* in some patients with 46,XY DSD of unknown genetic cause. So far, using in vitro cell transfection studies, we have revealed that the human SOX9 enhancer can be activated by SRY/SF1 and SOX9/SF1 but that this requires additional regulatory regions outside of the homologous *TESCO* sequence. In reporter assays, mutant SRY, SOX9, and SF1 encoded by missense mutations in 46,XY DSD patients show reduced ability to activate human *TES*, and increasing addition of DAX1 in transfection dose dependently represses human *TES* activation [6, 7]. This failure of sex-determining proteins from 46,XY DSD individuals to regulate the human testis enhancer of SOX9 in vitro could provide the molecular mechanisms underlying the atypical testis development.

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Part VII
Disorders of Sex Differentiation – Speakers

Chapter 20
Genetic Disorders of Sex Differentiation

Ken McElreavey and Anu Bashamboo

Introduction

Disorders of sex development (DSD) constitute a large array of disorders that affect the genito-urinary tract and often include the endocrine system [25]. 46,XY DSD includes 46,XY complete or partial gonadal dysgenesis and undervirilization or undermasculinization of an XY male due to defects in androgen synthesis or action. 46,XX DSD includes gonadal dysgenesis or more commonly overvirilization or masculinization of an XX female due to androgen excess. Ovotesticular DSD refers to an individual with both ovarian and testicular material present in the same or different gonads and 46,XX testicular DSD refers to an XX male with testes. Other forms of DSD include cloacal extrophy, severe hypospadias, vaginal atresia, and as part of other conditions such as Mayer–Rokitansky–Kuster–Hauser syndrome, Smith–Lemli–Opitz syndrome, or genito-palato-cardiac syndrome [47, 58]. DSD-related phenotypes include cryptorchidism and hypospadias. Some of these disorders are rare such as 46,XY complete and partial gonadal dysgenesis which affects approximately 1:50,000 individuals, while other DSD-related phenotypes are more common such as hypospadias and/or cryptorchidism, which can affect around 1:50 newborn males. In recent years there has been a considerable effort to identify the underlying genetic anomaly in these cases. However, only 50% of 46,XY children with DSD will receive a definitive diagnosis [25]. A specific molecular diagnosis is identified in only ~20% of cases of DSD [25]. Understanding the genetic basis of these disorders is a high priority because failing to understand the cause can lead to irreversible clinical interventions. The lack of understanding can have major consequences on the patient and the families concerned and gender identity can also be a major concern.

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Genetic Pathways Controlling Gonad Development

Since the Y chromosome gene *SRY* was identified 20 years ago there has been considerable progress in identifying the genetic factors involved in human and mouse sexual determination and differentiation, although the precise molecular mechanisms remain unknown (Fig. 20.1). In mammals the bipotential genital ridge is specified by several genes including *NR5A1*, *PBX1*, *CBX2*, *LHX9*, and *WT1* [55, 65]. Rodent studies indicate that the Y chromosome-linked testis-determining gene *Sry* acts during a restricted window of time during embryonal development to activate the downstream effector *Sox9*. However, in the human, *SRY* is expressed from the moment of sex determination in the male and then in the gonad during embryonic development right through to the adult [51]. Sertoli cells are one of the first somatic cell lineages to differentiate during testis formation. The expression of *Sry* ensures the differentiation of sufficient Sertoli cells to initiate testicular development by positively regulating *SOX9* expression. Once formed, Sertoli cells coordinate the cellular and morphogenetic events leading to primary sex determination and subsequent sexual differentiation. A recent model of testis determination suggests that the synergistic interaction of SRY and NR5A1 proteins activates *SOX9* expression via a testis-specific 1.4 kb enhancer (TESCO; [54]; Fig. 20.2). *Nr5a1* binds to multiple sites within the TESCO element and enhances the transactivation of a *Sox9* reporter fivefold. The addition of *Sry* increases reporter activity tenfold. Once SOX9 levels reach a critical threshold, several positive regulatory loops are initiated, including

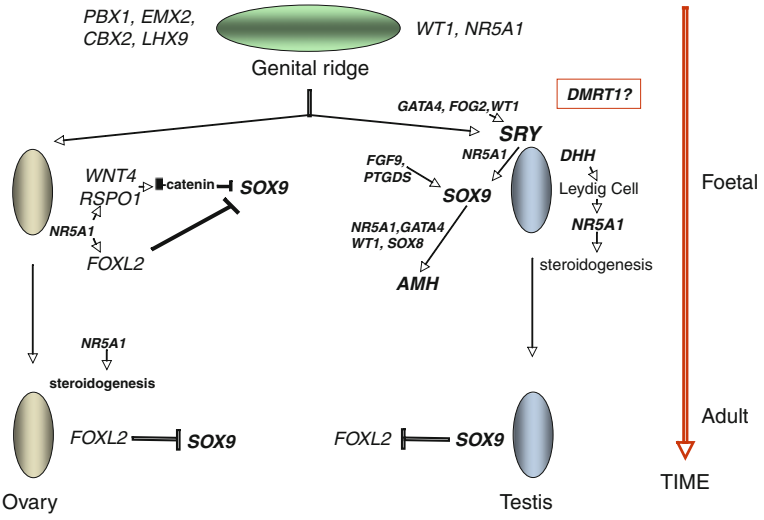

Fig. 20.1 The molecular and genetic events in mammalian sex determination and differentiation (see text for details). Much of this data has been generated from studies in mice

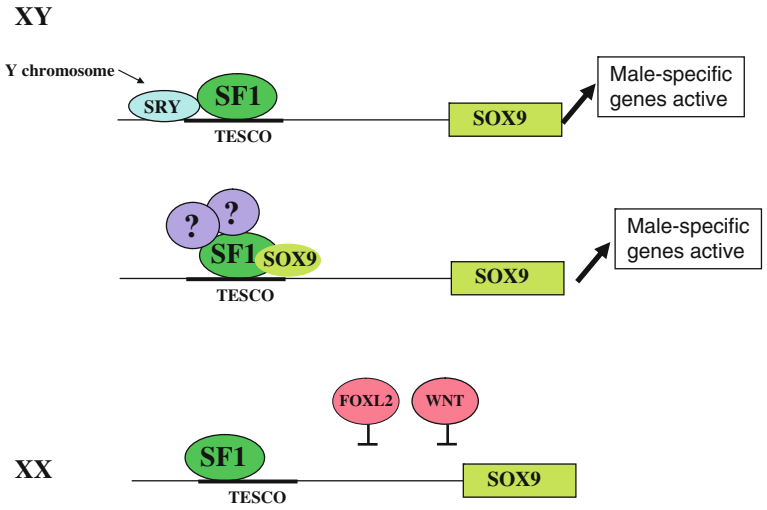

Fig. 20.2 A simplified model of mammalian sex determination. In this model *SOX9* expression is controlled in a synergistic manner through an interaction between SF1 (NR5A1) and SRY at the TESCO enhancer in the male. This leads to an upregulation of *SOX9* expression and the subsequent expression of male-specific genes. Following SRY expression, at least in the mouse, SOX9 can autoregulate its own expression. This process is likely to include a number of cofactors. In an XX individual, SRY is absent and the expression of SOX9 is repressed through probably independent FOXL2 and WNT pathways. The repression may involve direct protein/protein interaction with SF1 and/or protein binding to the TESCO element (modified from [55])

autoregulation of its own expression and formation of feed-forward loops via FGF9 or PGD2 signalling [55].

In the testis, SOX9 promotes the testis pathway, including Amh activation, and it also probably represses the ovarian genes WNT4 and FOXL2 [52, 55, 59]. Another key factor in this process may be *DMRT1*. Deletions of the distal chromosome 9p are associated with 46,XY gonadal dysgenesis [60, Veitia et al. 1997]. A gene containing a DNA-binding domain (DM domain), which exhibits sequence similarity to two genes involved in sex development in the *Drosophila* (*double sex*) and *Caenorhabditis elegans* (*mab3*), was identified within the 9p minimal region and termed *DMRT1* [48]. We cloned the other members of the DMRT gene family in the mouse and human, and we proposed that these genes play various roles in reproductive processes [42]. A *DMRT1* orthologue is sex determining in some species of fish and it is possible that *DMRT1* on the Z chromosome is the master determinant of sex in birds [57]. Our knowledge of the factors required for ovarian development is much less clear. In the XX gonad, the supporting cell precursors accumulate β-catenin in response to RSPO1/WNT4 signalling and repress SOX9 activity [52]. At later stages, FOXL2 may repress *SOX9* expression [59].

Genetic Mutations Associated with DSD

In approximately 80% of cases with 46,XX testicular DSD the *SRY* gene is present, usually on one of the X chromosomes [37]. Ten percent of cases of ovotesticular DSD can also be explained by the presence of *SRY*. In the remaining cases the aetiology is for the most part unknown. Some of these cases may be due to mutations in the *SOX9* locus that disrupt the repression of *SOX9* expression in an XX individual [29, 49] or due to duplications of the *SOX9* gene [24]. The remainder of these cases may be explained by the presence of Y chromosome mosaicism limited to the gonad or they may be caused by mutations that result in ectopic expression of other *SOX* genes in the XX developing gonad or to novel, and as yet unknown, sex-determining genes.

Loss-of-function mutations have been described in several of the genes known to be important for ovarian development (Fig. 20.1). The canonical Wnt signalling pathway acts by stabilizing β-catenin, which then acts as a cofactor for transcriptional activation. Heterozygote mutations in *WNT4* in the human are associated with Müllerian duct regression and virilization in an XX woman [8], whereas homozygote *WNT4* mutations are associated with SERKAL syndrome, a complex embryonic lethal phenotype that includes renal, adrenal, and lung dysgenesis as well as 46,XX testicular DSD [33]. Mutations in the R-SPONDIN1 (*RSPO1*) gene are also associated with a syndromic form of 46,XX DSD, and WNT4 and RSPO1 may act cooperatively to block the male testicular pathway in XX gonads [43]. Heterozygous *FOXL2* mutations cause the autosomal dominant blepharophimosis–ptosis–epicanthus inversus syndrome (BPES) that may be associated with premature ovarian failure [7]. Homozygous, loss-of-function mutations in *FOXL2* have not been described in the human to date but it is interesting to speculate that such mutations may lead to 46,XX testicular DSD.

46,XY gonadal dysgenesis is characterized by a failure of testis determination (reviewed by [6]). These cases can be subdivided into 46,XY complete gonadal dysgenesis and 46,XY partial gonadal dysgenesis. Since *SRY* is the primary sex determinant one would predict that individuals with anomalies of testis determination will harbour mutations in the *SRY* gene. Approximately 15% of 46,XY individuals with the complete form of gonadal dysgenesis carry mutations in the *SRY* gene, most of which are located in the evolutionarily conserved HMG domain [12]. Although the majority of mutations are de novo, a small minority are inherited from a normal fertile male [13, 53, 62]. The incomplete penetrance of these mutations may be caused by interaction between the variant SRY protein and another genetic determinant of testis determination that is independently segregating in the family. The importance of the genetic background in mammalian sex determination is well documented in the mouse, where *Sry* alleles from some mouse strains will cause sex reversal when placed in certain genetic backgrounds [15, 16]. A much smaller number of cases of 46,XY partial gonadal dysgenesis are associated with mutations involving the *SRY* gene [13, 36]. Other unusual phenotypes associated with *SRY* mutations include a de novo C to T transition resulting in a change from a glutamine to a stop codon at the second codon of the SRY open reading frame. This individual is a phenotypic female who had normal menarche, normal

secondary sexual characteristics, and at least partial ovarian function [11]. Other cases of ovotesticular DSD have been described associated with *SRY* mutations [10, 22].

Many autosomal genes are involved in testicular determination and differentiation and mutations have been described in many of these, and for the most part there are associated somatic anomalies. During mammalian development the Wilms' tumour gene, *WT1*, is expressed in the developing kidney, uterus, and testis. *WT1* transcripts are present in the bipotential genital ridge and subsequently in the Sertoli cells of the testis and the granulosa and eipthelial cells of the ovary [44]. A range of phenotypes are associated with mutations involving the *WT1* gene. In the human heterozygous *WT1* gene deletions are associated with mild genito-urinary anomalies and a predisposition to Wilms' tumour [18]. Heterozygous missense mutations give rise to Denys–Drash syndrome (early-onset renal disease with diffuse mesangial sclerosis, 46,XY complete or partial gonadal dysgenesis, and Wilms' tumour; [45]), and mutations in the donor splice site at the exon 9 boundary are responsible for Frasier syndrome (late-onset renal disease characterized by focal glomerular sclerosis, 46,XY complete gonadal dysgenesis, and the absence of Wilms' tumour; [3]). Since *WT1* is expressed in the same cell lineage as *SRY*, before, during, and after *SRY* expression, *WT1* may be acting upstream to SRY during the development of the genital ridge and perhaps controlling SRY expression.

DAX-1 (dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1) is orphan nuclear hormone receptor that plays critical roles in the development of the adrenal gland and reproductive systems [35]. Transcripts can be detected in specific endocrine tissues: the hypothalamus, anterior pituitary, adrenal glands, gonads, and placenta. The *DAX-1* gene is located at Xp21.3 [66]. In 46,XY males loss of *DAX-1* function is associated with congenital adrenal hypoplasia and hypogonadotrophic hypogonadism but testis determination is normal; however, cryptorchidism is frequent [39]. Duplications of a 160 kb region that includes *DAX-1* (the DSS locus) are associated with 46,XY gonadal dysgenesis and the consequent development of a female phenotype and are often, but not always, associated with multiple congenital anomalies [2, 4]. Likewise, loss-of-function mutations of *SOX9* are associated with campomelic dysplasia (a lethal skeletal malformation syndrome) and in 60% of 46,XY individuals gonadal dysgenesis [17, 63].

NR5A1, a member of the nuclear receptor superfamily, is a key transcriptional regulator of genes involved in the hypothalamic–pituitary–gonadal steroidogenic axis [32, 38]. The NR5A1 protein, also called steroidogenic factor-1, consists of a DNA-binding domain (DBD) including two zinc fingers, a flexible hinge region, a ligand-binding domain (LBD), and two activation function domains, AF-1 and AF-2 [23, 27, 50]. Newborn *NR5A1*$^{-/-}$ mice lack both gonads and adrenal glands and have impaired expression of pituitary gonadotropins [32]. *NR5A1* is expressed in foetal and adult Sertoli and Leydig cells of testis. *NR5A1* is also expressed in multiple cell types in the foetal, postnatal, prepubertal, and mature ovary [21, 26, 34]. In testis determination and differentiation NR5A1 is a positive regulator of SOX9 and AMH [30, 54]. NR5A1 also modulates the expression of many factors involved in cholesterol mobilization and steroid hormone biosynthesis including HMG-CoA

synthase, steroidogenic acute regulatory protein (StAR), 3β-hydroxysteroid dehydrogenase (3βHSD), and several cytochrome P450 steroid hydroxylase (CYP) enzymes [23, 30]. Until 2008, 18 *NR5A1* mutations were described in the human [30]. Three of these were originally identified in individuals with a phenotype similar to that seen in the mouse knockout. These three individuals had adrenal insufficiency, two associated with 46,XY DSD and the third a 2-year-old 46,XX girl with conserved ovarian function [30]. Other mutations in *NR5A1* are associated with a spectrum of 46,XY DSD phenotypes with no evidence of adrenal failure and this spectrum includes 46,XY partial and complete gonadal dysgenesis, penoscrotal hypospadias, and micropenis with anorchidia [19, 30, 46]. We have identified a further 19 *NR5A1* mutations including mutations in 4 familial cases [31]. These families had individuals with 46,XY DSD as well as 46,XX primary ovarian insufficiency (POI). The mode of inheritance of the phenotype in these families is consistent with either autosomal recessive or autosomal dominant transmission [31]. An analysis of a further 25 sporadic cases of POI revealed 2 additional mutations. One of the girls also had unexplained short stature and the other presented at 4 months of age with hypertrophy of the clitoris. We observed a reduction in transactivation of aromatase by each of these mutant proteins. These data reveal novel insights into the role of NR5A1 in ovarian development and function indicating that mutations of the *NR5A1* gene may be a significant cause of POI as well as 46,XY DSD [31].

Despite these advances, the genetic cause of most non-syndromic forms of DSD still remains unknown. Mutations in the androgen receptor (AR) gene are one of the most common genetic causes of 46,XY DSD. However, even in 46,XY underandrogenized subjects with testes who are suspected to have an AR defect, a pathogenic mutation is found only in less than half of the cases [1]. In cases of simple hypospadias or cryptorchidism a genetic cause is rarely detected despite epidemiological evidence indicating a major genetic contribution to these phenotypes [56]. New genes are likely to be involved since several pedigrees have been described where known candidate genes have been excluded [14, 28]. Also recent data suggest that errors in MAP kinase signalling may also lead to 46,XY DSD [9]. Our current understanding of the genetic causes of DSD is likely to dramatically change over the next few years with advances in sequencing technologies [5]. Recent studies show the power of next-generation sequencing approaches to detect pathogenic mutations causing disease without the need to have an a priori knowledge of the chromosomal location or the type of gene involved [40, 41]. This approach also offers the opportunity to identify genetic modifiers. Familial cases of both 46,XY and 46,XX DSD often show considerable variation in the expression of the phenotype, including families where the underlying genetic mutation has been identified [31]. The variation in the phenotype that is often seen in families may be explained by variations in other proteins that may interact with or influence the biological activity of the target gene. The generation of massive data sets of qualitative and quantitative information of DNA sequences in a patient sample at a relatively limited cost will transform the field of reproductive disorders by offering novel insights into the genetics and physiology of DSD.

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Chapter 21
The Clinical Spectrum and Treatment of Ovotesticular Disorder of Sexual Development

Rinus Wiersma

Introduction

In general practice worldwide, disorder of sexual development (DSD) is an uncommon condition. Although the proportions of individual conditions making up the spectrum of DSD vary from country to country, ovotesticular disorder of sexual development (OT-DSD) is regarded as a rare type, constituting between 3 and 10% of the total DSD [1].

In Southern Africa, for reasons that are still not understood, this condition is seen more frequently [2]. Forty-three percent of patients presenting with ambiguous genitalia and 51% of all patients with DSD are found to have OT-DSD [3].

Study

Over the past 25 years, 125 patients, with OT-DSD and age ranging from 0 to 13 years, have been managed and various aspects of their clinical features and management have been studied. All except two of these patients were black Africans, of whom 81% were referred from peripheral hospitals. There were no geographic areas and no family groups with a higher incidence of this condition.

Clinically, the external genitalia formed a permutation of penile size, labioscrotal folds, gonadal position, and perineal openings. Of the 125 patients, 61 fitted into the Prader classification 3 [4]. The overriding feature was that no patient looked like a totally normal male or female. Gonads were palpable in 59 patients, 17 of whom had bilateral and 42 had a single palpable gonad in an inguinal or scrotal position. The remaining 66 patients had bilateral gonads found within the pelvis and in 7 patients this is a single gonad only. The perineal orifices in 9 of our 125 patients

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showed a separate urethra and vaginal opening. Three patients had this in relation to a clitoris, and the remainder had a penile structure. Of the remaining patients, 91 had a urogenital sinus in varying positions, and 25 patients only had a urethra. Only two children, raised as females, presented with secondary sexual development of breast and pubic hair.

The non-clinical investigations showed that 46,XX was the commonest karyotype (87%) seen in our patients. Of the steroid assays, ACTH, cortisol, 17-OH progesterone, and 11-DO corticosterone levels were not helpful in gender determination, and testosterone production slowly decreased to below normal male levels in children >7 months old.

The internal genitalia were investigated using urethroscopy, laparoscopy, and gonadal biopsy in children from as young as a few days old. These investigations showed that only 73 patients had both a vagina and uterine structure. Due to the high incidence of OT-DSD all patients investigated for ambiguous genitalia had bilateral gonadal biopsies. On gross appearance the separate ovaries and testes looked normal. The ovotestes, however, were found to be of two main types, independent of the patient's karyotype. These were the globular looking mixed (89%) ovotestis and the bipolar ovotestis (11%). Histopathologically the ovotestes were divisible into three different histological patterns, two types of mixed and a bipolar ovotestis [5]. (a) The admixed ovotestis consisted of an outer mantle of ovarian tissue of variable thickness, surrounding a central core of stroma, containing scattered foci of ovarian and testicular tissue of different sizes. (b) The compartmentalized ovotestis consisted of ovarian tissue, which in the lower portion completely encapsulated a variable-sized core of testicular tissue. The tissue ratios ranged from 1:1 to 4:1 ovary:testis. (c) The bipolar ovotestis had a strict polar distribution of ovarian (craniad) and testicular (caudal) tissues, but had considerable interdigitation of the two tissues. All three ovotesticular patterns made any separation and excision of unwanted gonadal tissues impossible.

The full management protocol of these patients requires a long-term multidisciplinary approach, with input from psychologists, endocrinologists, etc. The surgical aspect of management deals with the investigation and assignment of gender and may be divided into several facets:

Assignment of gender: This is necessary where the child is seen de novo or the parents have not decided on a gender in the child who is under 6–8 years of age.

Management of the gonads: This is required as ovotestes consist of inseparable ovarian and testicular tissue. Although the risk of malignancy in ovotestes in patients with a 46,XY karyotype is low, these gonads are best removed as soon as practicable where the follow-up is poor or difficult. Testes lack spermatogenic potential and lose their hormonogenic ability during the first year of life. It is suggested that both these are removed after the first year [6]. Ovaries need only be removed if the child feels he is male at 6–8 years of age.

The surgical management: This may be subdivided into the following:

- Early interventional surgery: This allows the young child to fit into the assigned gender. As such correction of severe chordee can be undertaken without diminishing the chances of a change of gender later, should the child when old enough decide on this.
- Cosmetic surgery: This is for the older child who is able to determine the child's own gender. The gender establishing surgical procedures assist with giving gender confidence as either male or female.

Management of puberty: This is undertaken by the endocrinologist to control somatic growth.

Psychological management: This is of the child and the parent.

Conclusion

The overall care of patients with OT-DSD was based on the principles that

- all children with ambiguity of the genitalia required urgent investigations to define the diagnosis and most appropriate gender for that child;
- as these patients were mostly from a rural background, and follow-up was poor, all non-functional and potentially dangerous gonadal tissue, i.e., ovotestes and testicular tissue, should be excised;
- corrective genital surgery should only be fully implemented at a time when the child is old enough, 6–8 years of age, and can assist with the determination of the gender;
- these complex patients require a multidisciplinary long-term approach to their management.

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Chapter 22
The Genetics of Ovotesticular Disorders of Sex Development

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Disorders of sex development (DSD) encompass a very large spectrum of phenotypes, from minor malformations of the genitalia (hypospadias, cryptorchidism, and hypertrophy of the clitoris) to sexual ambiguity. Taken altogether, these anomalies have an estimated frequency of 0.5–1%. Moreover, sexual ambiguity has a major impact on quality of life. Recently, the debate about the management of patients with DSD has intensified over issues of gender assignment and the indication for early genital surgery. Yet the scientific data on patient outcome have remained poor. The main obstacles to the optimal management of patients with DSD have been a combination of the lack of controlled outcome data and the lack of understanding of their pathophysiology, which prevents precise diagnostic categorization of patients. Despite much progress in the past 15 years, the molecular mechanisms underlying mammalian sex determination are still far from understood, and the molecular basis of sex reversal in a large number of patients cannot yet be explained. Apart from XX testicular DSD, mainly caused by an X–Y translocation including SRY, the genetic etiology of XY gonadal dysgenesis and of ovotesticular DSD (OT-DSD) remains unknown for a majority of patients. The genetic causes of the latter, involving the presence of both testicular and ovarian tissues in the same patient, are particularly unappreciated.

Analysis of the chromosomal distribution of ovotesticular DSD shows that about 65% have an XX chromosomal constitution while only 10% are XY, and the remaining are complex mosaics containing a Y chromosome. The geographical distribution of OT-DSD shows an overrepresentation of Africa, with a number of published cases of 17 per 100 million inhabitants, followed by Europe at 15.3. Asia is underrepresented at 1.2 cases per 100 million. The gonadal distribution of OT-DSD is as follows. The most common form is unilateral with ovotestis/ovary at 34%, followed by bilateral (ovotestis/ovotestis) at 29%, and lateral (ovary/testis) at 25%. The least

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frequent is unilateral with ovotestis/testis (12%). A Y chromosome is more often observed when there is no ovotestis (e.g., lateral). Sex assignment of OT-DSD has been historically approximately divided equally between male and female. From a molecular perspective, the presence of SRY explains about 10% of XX OT-DSD, and in some patients, it is limited to gonadal mosaicism. A small subset of XY patients with OT-DSD carry point mutations in SRY. The cases of SRY-negative OT-DSD remain, for the most part, unexplained genetically. Mutations in the RSPO1 gene have been shown to be associated with a rare syndromic form of OT-DSD with palmoplantar keratosis and there are a few reports of partial 22q duplication (containing the SOX10 gene) with OT-DSD.

It seems clear, however, that SRY-negative OT-DSD and T-DSD are part of the same clinical spectrum. Additional genetic factors explaining OT-DSD are yet to be identified, and mouse models are invaluable to this process. C57BL/6J (B6) mice containing the *Mus domesticus poschiavinus* Y chromosome, YPOS, develop ovarian tissue, whereas testicular tissue develops in DBA/2J or 129S1/SvlmJ (129) mice containing the YPOS chromosome. C57BL/6J-YPOS fetuses develop gonads varying from ovary to ovotestis and constitute an excellent model of ovotesticular DSD. To identify novel genes involved in sex determination, we used a congenic strain approach to determine which chromosomal regions from 129SI/SvlmJ provide protection against sex reversal in XYPOS mice of the C57BL/6J.129-YPOS strain. Genome scans using microsatellite and SNP markers identified a chromosome 11 region of 129 origin in C57BL/6J.129-YPOS mice. To determine if this region influenced testis development in XYPOS mice, two strains of C57BL/6J-YPOS mice were produced and used in genetic experiments. XYPOS adults homozygous for the 129 region had a lower incidence of sex reversal than XYPOS adults homozygous for the B6 region. In addition, many homozygous 129 XYPOS fetuses developed normal appearing testis, an occurrence never observed in XYPOS mice of the C57BL/6J-YPOS strain. We conclude that a chromosome 11 locus derived from 129Sl/SvlmJ protects against sex reversal in XYPOS mice. Further backcrossing of C57BL/6J.120-YPOS with C57BL/6J mice combined with bioinformatics approaches based on sequence conservation allowed to refine the critical region of chromosome 11 protecting against XY sex reversal.

Candidate genes generated by this mouse model combined with other genetic approaches involving human cases of ovotesticular DSD will help elucidate the biology of OT-DSD.

Chapter 23
Growth Hormone Treatment in Children with Congenital Adrenal Hyperplasia

Madeleine D. Harbison, Karen Lin-Su, and Maria I. New

Final adult height is often compromised in children with congenital adrenal hyperplasia (CAH). This report examines the impact of growth hormone (GH) with or without LHRH analogue (LHRHa) on final adult height in patients with CAH due to 21-hydroxylase deficiency. Boys and girls with CAH who had a predicted final height of more than 2 SD below their mid-parental target height or more than 2 SD below the population mean were eligible for treatment with GH. Other inclusion criteria included open epiphyses (bone age <14 years for boys and bone age <13 years for girls) and bone age $\geq$1 standard deviation above the mean for chronological age. Subjects received GH (starting dose of 0.3 mg/kg/week) until final adult height was reached. Final adult height was defined as growth velocity $\leq$1.5 cm/year over a 6-month period and bone age $\geq$15 in girls or $\geq$17 in boys. Patients with early central puberty were also treated with an LHRHa. The primary outcome variable was final adult height. Secondary outcome variables were gain in height (final height minus initial predicted height) and height discrepancy (final height minus target height). We report the results of 31 GH-treated CAH patients who have reached final adult height. Mean duration of growth hormone treatment was 4.5 years for girls and 4.9 years for boys. Mean duration of LHRHa therapy was 4.2 years. Girls ($n = 15$) reached a mean final adult height of 162.1 cm, in contrast to a mean initial predicted height of 153.0 cm. Boys ($n = 16$) reached a mean final adult height of 172.4 cm, in contrast to a mean initial predicted height of 166.3 cm. The mean gain in height after GH treatment was 9.1 cm in girls and 6.3 cm in boys. Of the CAH patients treated with GH, 39.4% (12 out of 31) reached or exceeded their mid-parental target height, in contrast to only 19.7% (45 out of 229) of historical CAH patients who were never treated with GH or LHRHa. Our results indicate that GH treatment with or without LHRHa can improve final adult height in patients with CAH.

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Part VIII
Hormonal Hypertension – Speakers

Chapter 24
Genetic Modifications of Corticosteroid Receptors in Hypertension

John W. Funder

Gain-of-function mineralocorticoid receptor (MR) mutations and loss-of-function glucocorticoid receptor (GR) mutations are accompanied clinically by elevated blood pressure. This brief presentation explores how clinical studies have proven prismatic in terms of the evolution of MR and the implications for the pathogenesis of ACTH-induced hypertension. In 2000 Geller et al. published their findings on a point mutation in the gene encoding human MR, leading to a single amino acid substitution (S810L) and producing juvenile hypertension exacerbated by pregnancy (*Science*, 289 (5476):23–26). MR mutations in affected subjects are constitutive partial agonists, reflecting the proximity of and interaction between Ala 773 and the mutant Leu 810. Iterative Bayesian analyses of possible evolutionary trees do not distinguish between any of the four members of the steroid receptor subfamily (MR, GR, AR, and PR) as the first to branch off the putative ancestral protein. GR, AR, and PR have invariant Glu and Met at the positions equivalent to Ala 773 and Ser 810 in wild-type MR; in contrast, more distantly related receptors (Erα, ERβ, and RXRα), like the S810L mutant, have an invariant Ala and Ser at the equivalent site. Experiments of nature may thus illuminate not only structure, function, physiology, and pathophysiology but perhaps also the evolutionary process.

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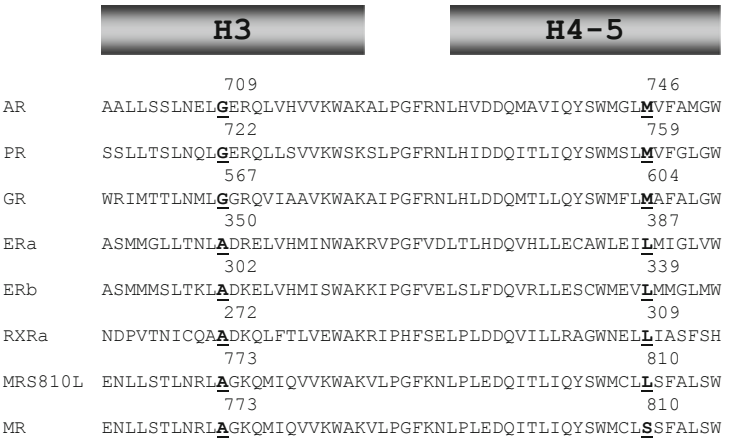
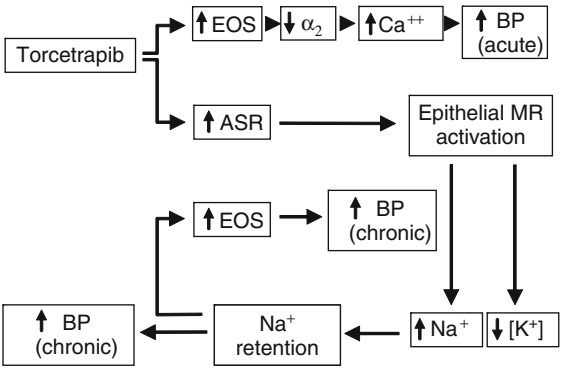
GR, PR, AR; S810L, ERa, RXRa - and the MR



In 2005 Lingrel's laboratory published a seminal paper (*PNAS*, 102:15845–15850) on the role of the cardiac glycoside (aka EO, endogenous ouabain) binding site of Na, K-ATPase in blood pressure regulation. Specifically, they showed that mutant α2 isoform, glycoside-resistant mice do not develop ACTH-induced hypertension and that ACTH-induced hypertension in wild-type mice is abolished by the GSK antibody Digibind. In the ILLUMINATE trial of the CETP inhibitor torcetrapib the excess mortality is commonly attributed to its off-target effects to raise plasma aldosterone, serum [Na^+] and [HCO_3^-], and BP and to lower serum [K^+]. Experimentally torcetrapib does not raise BP in adrenalectomized rats, but does in rats in which steroidogenesis is blocked by trilostane; in control studies torcetrapib raises BP in pithed rats and in animals under α- and β-blockade. EO secretion from the adrenal glomerulosa is stimulated by ACTH, by angiotensin via AT2R, and by *elevation* of sodium levels, consistent with a role for EO in the off-target effects of torcetrapib.

Response of aldosterone and (putatively) endogenous ouabain secretion to torcetrapib



A persisting enigma has been why aldosterone levels inappropriate for sodium status are so damaging in the cardiovascular system, whereas much higher levels in chronic sodium deficiency are not damaging but homeostatic. The answer may lie in the zona glomerulosa, in that EO secretion is stimulated by a sodium load and suppressed in sodium deficiency, squarely opposite to that of aldosterone. It thus may be that the enigma posed by the off-target effects of torcetrapib sheds light on a more basic enigma, that of how sodium status stochastically modifies tissue responses to mineralocorticoid receptor activation by aldosterone.

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Chapter 25
Angiotensin AT_2 Receptors: Control of Renal Sodium Excretion and Blood Pressure

Robert M. Carey

Angiotensin (Ang) II, the primary transducer peptide of the renin–Ang system (RAS), acts at two major receptors: type 1 (AT_1R) and type 2 (AT_2R). The majority of Ang II actions occur via AT_1Rs, including antinatriuresis. Renal cross-transplantation studies have demonstrated that renal AT_1Rs are both necessary and sufficient for the induction and sustainability of hypertension during Ang II infusion and that increased Na^+ reabsorption in the renal proximal tubule (RPT) is the major determinant of this response. In contrast, the role of AT_2Rs in the control of Na^+ excretion and hypertension has not been defined. AT_2Rs are expressed in the adult kidney predominantly in RPT cells.

Recent studies from our laboratory have provided evidence for a major role of RPT AT_2Rs in the inhibition of Na^+ reabsorption. In the normal rat, natriuretic responses to intrarenal AT_1R blockade with candesartan (CAND) were abolished with concurrent intrarenal AT_2R blockade with PD-123319 (PD). In the presence of systemic AT_1R blockade, intrarenal administration of Ang III engendered natriuresis that was abolished by simultaneous intrarenal PD, whereas Ang II at molar equivalent or higher doses did not alter Na^+ excretion. Our conclusion that Ang III is the preferred AT_2R agonist on natriuresis was significantly strengthened by the observation that intrarenal inhibition of aminopeptidase N (APN), the enzyme that metabolizes Ang III to Ang IV, markedly augmented the natriuretic response to Ang III, which again was abolished by PD. In addition, we showed that natriuretic responses to intrarenal Ang II could be unmasked by APN blockade but that the natriuresis was abolished by concurrent blockade of aminopeptidase A, the enzyme converting Ang II to Ang III. Taken altogether, this evidence indicates that, instead of Ang II, des-aspartyl^1-Ang II (Ang III) is the preferred AT_2R agonist inducing natriuresis. We also have evidence that intrarenal administration of dopamine D_1-like receptor (D_1R) agonist fenoldopam (FEN) induces natriuresis that is abolished with PD, indicating AT_2R dependency of D_1R-induced natriuresis. Furthermore, our

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studies have demonstrated in normal rats that natriuretic responses to both FEN and Ang III are accompanied by recruitment of AT_2Rs to the apical plasma membranes of RPT cells.

In contrast to Ang III-induced AT_2R translocation (to the apical plasma membranes of RPT cells) and natriuresis in 12-week-old Wistar Kyoto rats (WKY), we have demonstrated that hypertensive 12-week-old spontaneously hypertensive rats (SHR) both fail to recruit AT_2Rs and to mount natriuretic responses to Ang III. We also have shown that while 4- and 12-week-old WKY have natriuretic responses to intrarenal CAND, pre-hypertensive (4-week-old) and hypertensive (12-week-old) SHR do not, but that natriuretic responses can be restored in both pre-hypertensive and hypertensive SHR with concomitant intrarenal APN inhibition. Our results strongly suggest a defect in AT_2R-mediated natriuresis in SHR that predates the hypertension and is due, at least in part, to accelerated intrarenal Ang III metabolism.

Part IX
Skeletal Biology – Speakers

Chapter 26
The Pituitary-Bone Axis

Mone Zaidi, Li Sun, and Jameel Iqbal

Osteoporosis is a crippling disease, marked by skeletal fragility and resulting in a fracture in one out of every two individuals over the age of 50. What causes this increased bone fragility and resulting susceptibility fractures has, however, remained unclear. In the early 1700s the British surgeon John Hunter hypothesized that bone undergoes remodeling, that is, bone is forever changing itself by reorganizing into a newer, stronger configuration to resist ongoing stresses, such as physical activity [1]. Bone is lost when the cells degrading old bone, the osteoclasts, outpace the cells re-laying new collagen and mineral, the osteoblasts.

What would cause the osteoclasts to degrade bone at a higher rate than the body's ability to re-build bone? During the mid-1900s, Fuller Albright at the Massachusetts General Hospital noted that the most dramatic bone loss occurred after menopause, either natural or surgically induced [2]. Thus, he hypothesized a link between the loss of sex steroids and osteoporosis, a *corpus* of observations that led to estrogen hormone replacement therapy becoming the first successful treatment for osteoporosis.

It is now clear that women have a precipitous drop in bone mass during the menopausal transition. Notably, bone loss is profoundly accelerated in the late perimenopause [3, 4]. In fact, nearly half of lifetime bone loss occurs within the first 5 years of menopause; this loss of bone mass is associated with microstructural degradation ultimately predisposing to fractures [5]. Surprisingly, however, during this phase of rapid, late perimenopausal bone loss, estrogen levels are normal. Thus, a conundrum: could the hypothesis – the loss of estrogen is the *sole* cause of osteoporosis – be wrong?

There is clear genetic and pharmacological evidence for a protective role of estrogen on the skeleton [6]. In vitro studies show that estrogen inhibits osteoclast formation through various mechanisms involving osteoclast precursors, osteoblasts, and T lymphocytes [7–12]. In addition to its inhibitory effect on osteoclast formation, estrogen has established anabolic actions mediated via osteoblasts [13].

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However, if estrogen protects the skeleton, why then is there striking bone loss occurring in the late perimenopause if estrogen levels during this period are unperturbed?

The answer to this question was ultimately inspired by our earlier studies on hyperthyroidism. In 2003, we discovered that the pituitary-derived thyroid-stimulating hormone (TSH) could bypass its primary endocrine target, the thyroid, and act directly on osteoclasts and osteoblasts to modulate bone turnover [14]. The results of those studies had profound implications for the bone loss associated with subclinical hyperthyroidism in which thyroid hormones are normal and TSH is low. For the first time, we could explain why bone loss occurred in these patients given their normal thyroid hormone levels – low TSH appeared directly to cause their bone loss [15].

Using a similar line of argument, we hypothesized in 2006 that changes in the circulating levels of the pituitary-derived follicle-stimulating hormone (FSH) may contribute to the late perimenopausal bone loss when estrogen levels are unperturbed. Several clinical studies on the menopausal transition, where bone loss was found to correlate with dramatic increases in FSH, bolstered this argument. The Study of Women's Health Across the Nations (SWAN), a longitudinal, cross-sectional study of 2375 perimenopausal women, found a strong correlation of FSH levels to markers of bone degradation [16] and demonstrated that changes in the levels of FSH over 4 years could predict decreases in bone mass [4]. Similarly, Xu et al. found a significant association between the incidence of osteoporosis and high serum FSH levels in a group of 689 native Chinese women [17]. Likewise, Sowers et al. show that spine and femoral neck bone loss accelerates in women between 47.6 and 51 years, i.e., during FSH stage 3 (34–54 mIU/mL), which corresponds to 2 years prior to the final menstrual period [18]. These strong correlations now help to clinically stratify women at a high risk of bone loss using serum FSH [19].

With the knowledge that FSH levels correlated strongly with bone loss during late perimenopause, we next investigated whether FSH could directly stimulate bone degradation by osteoclasts. We found that FSH augmented the formation, function, and survival of both human and mouse osteoclasts [20]. By activating the osteoclast FSH receptor, FSH triggered several of the signaling pathways used by RANK-L to transduce its pro-resorptive effects [20, 21]. Moreover, we and others found that FSH can indirectly stimulate osteoclast formation by enhancing the production of several pro-osteoclastogenic cytokines, TNFα, IL-6, and IL-1β [22, 23]. Recently, Pacifici and coworkers have shown that FSH increases CD40-ligand expression on T lymphocytes, thereby triggering increased TNFα production.

We also demonstrated a direct effect of FSH on the skeleton. Notably, mice lacking the β-subunit of FSH or the FSHR were protected from bone loss associated with estrogen deficiency, although these mice have a compensatory rise in serum androgens accounting for some of the skeletal phenotype [20]. Importantly, however, haploinsufficient FSHβ heterozygotes (animals having a 50% reduction in circulating FSH levels, but with normal estrogen levels) displayed increased bone mass [20]. This latter finding showed that, even in situations of normal estrogen,

FSH was acting independently to decrease skeletal mass [20]. We can thus extrapolate that the bone loss occurring in the perimenopausal period partly arises from elevated FSH levels [24].

Further studies take us toward establishing a cause–effect relationship between FSH and bone loss in vivo. First, amenorrheic women with similar estrogen levels having a mean serum FSH of 35 mIU/mL had greater bone loss than those with a level of 8 mIU/mL [25]. Second, exogenously administered FSH enhanced ovariectomy-induced alveolar bone loss in rats [26, 27]. The bone loss post-ovariectomy, as well as that induced by exogenous FSH, was significantly reduced by an FSH antagonist, providing unequivocal evidence for a direct effect of FSH on bone in vivo [26, 27].

Finally, and perhaps most importantly, Rendina et al. sought to tie the role of FSH in causing osteoporosis to the genetic variability that exists among the human population [28, 29]. To do so, they examined 289 postmenopausal women for FSHR polymorphisms at two sites, rs1394205 and rs6166, and then analyzed the influence these polymorphisms had on bone mass and bone turnover [29]. The results were impressive: the authors found that the SNP rs6166 of the FSH receptor gene significantly influenced bone mass in postmenopausal women [29]. Prior studies on AA rs6166 have associated this polymorphism with increased stimulation of the ovarian FSHR. Based on the knowledge that FSH acts to decrease bone mass, one would anticipate that women bearing an 'activating' FSHR polymorphism will have lower bone density. That was exactly the case: those women with AA rs6166 showed significantly lower bone density, higher bone resorption markers, and more than twice the fracture incidence compared to women with GG rs6166 [29]. The increased risk of osteoporosis in AA rs6166 women was independent of serum estrogen; this observation is clearly consistent with the estrogen-independent actions of FSH during the late perimenopausal period [29].

There is thus an ongoing paradigm shift in endocrine physiology whereby the classic pituitary hormones FSH and TSH act by design on a 'non-endocrine' tissue – bone [30]. It is possible that the discovery of polymorphisms in these non-classical pathways, such as the ones described by Rendina et al. or TSH receptor polymorphisms [31, 32], may define some of the complex, yet obscure, genetic variation in osteoporosis. As we bridge the gap in our understanding of what else causes osteoporosis, the future appears bright for targeting the pituitary-bone axis to a skeletal advantage.

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Chapter 27
Genetic Impact of Both Sex Hormones in Male-Typical Behaviors

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Androgens have a critical role in a wide range of biological processes. These include spermatogenesis, development of reproductive organs, and brain function. Most actions of androgens are mediated by the nuclear androgen receptor (AR), which acts as a ligand-inducible transcription factor. To elucidate physiological significance of AR in target tissues, we succeeded in disrupting the AR on the X chromosome using a Cre–loxP system. Male AR-null mutant (ARKO) mice exhibit abnormalities typical of testicular feminization mutants (Tfm), including female external genitalia with atrophic testis [1]. They also develop late-onset obesity [2] with glucocorticoid overproduction [3] and impaired bone growth coupled with high bone turnover [4]. Moreover, essential role of AR for normal folliculogenesis suggests that androgen/AR signaling is also physiologically important in females [5]. On the other hand, the physiological role of AR-mediated androgen signaling in brain masculinization has not been established. We describe here the cooperative role of sex hormones signaling in brain sex differences underlying the expression of male-typical behaviors.

Sex differences between male and female mammals in brain and behaviors develop under the influence of actions of both sex steroid hormones during the perinatal period. In male rodents, testosterone secreted from testes during this critical period results in the development of sexually dimorphic brain structures and is necessary for the expression of male-specific behaviors. However, these actions of testosterone appear to be exerted not through its androgenic activity, but rather through its conversion by brain aromatase [6, 7] into estrogen with the consequent activation of estrogen receptor-mediated signaling [8]. Thus, the role of AR in perinatal brain masculinization underlying the expression of male-typical behaviors had remained unclear owing to the conversion of testosterone into estrogen in the brain. To address this point, behavioral phenotype was tested by inactivation

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of AR gene in male mice. Male sexual behaviors were abolished in intact male AR knockout mice, whereas treatment with E2, but not non-aromatizable androgen (5α-dihydrotestosterone, DHT), rescued male sexual behaviors. When behavioral recovery did occur, estrogenic effect was only 50% of that observed in WT mice. Next, we attempted to verify the effects of DHT on male-typical behaviors in male ERαKO mice, as male-typical behaviors have been shown to be impaired in male ERαKO mice, but not in ERβKO mice [8]. In male ERαKO mice, DHT treatment restored impaired mount and intromission behaviors, but not ejaculation. These examinations of hormonal responses in ARKO and ERαKO mice suggested that androgen and estrogen receptor functions are convergent with regard to male sexual behaviors. We have therefore sought to evaluate the cooperative role of sex steroid signaling on physiology and behaviors in double steroid receptor knockout mice. Double AR and ERα mutant (DKO) male mice were born healthy but developed typical features of Tfm abnormality mimicking ARKO males. DKO males also showed complete loss of male-typical sexual and aggressive behaviors. Thus the dual requirement for androgen and estrogen signaling in male-typical behaviors suggests that these two pathways might interact genetically to control sexual dimorphic behaviors. It will be of interest in future experiments to determine the molecular basis of how these hormonal pathways intersect to control dimorphic behaviors by using triple-receptor (AR and ER α/β) knockout mice.

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Part X
Posters

Chapter 28
MAMLD1 (Mastermind-Like Domain Containing 1) Homozygous Gain-of-Function Missense Mutation Causing 46,XX Disorder of Sex Development in a Virilized Female

Maíra Pontual Brandão, Elaine Maria Frade Costa, Maki Fukami, Mariza Gerdulo, Natalia P. Pereira, Sorahia Domenice, Tsutomu Ogata, and Berenice B. Mendonca

Introduction

A number of reports indicate that premature ovarian failure (POF) may be inherited as an X-linked condition [1–3]. In particular, Xq deletions encompassing the region from q13 to q28 have been related to POF. A heterozygous female for a microdeletion involving Xq28 region has been described [4]. Recently, *MAMLD1* (mastermind-like domain 1) was identified as a candidate gene for 46,XY DSD. It is located at Xq28 and spans 70 kb in genomic sequence and comprises seven exons. Due to in-frame alternative splicing, exons 3–6 encode for two proteins of 701 and 660 amino acids, respectively, depending on whether the transcript includes or excludes exon 4. In situ hybridization analysis for the homologous mouse gene showed that the gene was barely detected until 2 weeks of age and was clearly identified in granulosa cells at the perifollicular regions of most Graafian follicles at 3 and 8 weeks of age [5]. To date, no *MAMLD1* mutations *have been* described in patients with 46,XX DSD due to gonadal dysgenesis. Therefore, the role of MAMLD1 in ovarian development and function remains unknown.

Objective

To screen *MAMLD1* mutation in patients with 46,XX DSD due to gonadal dysgenesis.

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Patients

We evaluated 14 Brazilian patients with 46,XX DSD due to gonadal dysgenesis. Mutations in the coding region of *FOXL2*, *BMP15*, *STRA8*, *Nanos1*, *Nanos2*, or *SF-1* were ruled out. One of these patients presented an unexpected clitoral enlargement and had a normal *WnT4* coding region. She was 44 years old, born from first cousin parents and had primary amenorrhea and failure of breast development. She had eunuchoid habitus and absence of hirsutism. Her height was 175 cm and Tanner IV pubic hair. *External genitalia*: c*litoromegaly* (4.2 cm), two perineal openings, and unpalpable gonads. *Basal hormonal levels*: elevated FSH levels and normal LH, progesterone, 17OH-progesterone, androstenedione, and testosterone levels (47 ng/dL) which did not increase after hCG stimulation (44 ng/dL). *Laparoscopy*: bilateral streak gonads, small uterus, and bilateral Fallopian tubes. *Anatomopathologic findings*: absence of left gonad, Fallopian tubes, and a dysgenetic right gonad with hilar cell hyperplasia and persistence of wolffian rests.

Methods

The entire coding region and the splicing site flanking regions of *MAMLD1* were sequenced. Transactivation function of *MAMLD1* was analyzed by COS-1 transiently transfected with the reporter vectors p-Hes3-luc and p-Hes7-luc, the expression vectors WT and mutant *MAMLD1*, and pRL-CMV vector as an internal control.

Results

The mutation p.V432A, previously described in heterozygous state in a 46,XY DSD patient [6], was identified in homozygous state in the patient with clitoromegaly. This mutation localizes at exon 3 into the glutamine-rich domain and was absent in 190 normal alleles. *Transactivation function of the V432A protein*: Significantly higher transactivation activity than WT for p-Hes3-luc (1.8-fold) and p-Hes7-luc (1.5-fold) was found ($p < 0.05$).

Discussion

Molecular function of MAMLD1 remains unknown although it transactivates the promoter of a noncanonical Notch target gene Hes3 without demonstrative DNA-binding capacity [7]. The virilization of this 46,XX DSD patient associated with

the presence of hilar cells suggests an underexpression of *WnT4* or an overexpression of *DHH* but the relationship of these pathways with *MAMLD1* remains to be determined.

Conclusion

We described the first homozygous gain-of-function mutation in MAMLD1 causing 46,XX DSD in a virilized female, indicating that this protein is involved in ovarian development.

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Chapter 29
Atypical Presentation and Novel StAR Protein Gene Mutation in a 46,XY Female with Lipoid Congenital Adrenal Hyperplasia

Oksana Lekarev, Yves Morel, and Maria I. New

Introduction

Congenital lipoid adrenal hyperplasia is a severe disorder of adrenal and gonadal steroidogenesis and is the most severe form of congenital adrenal hyperplasia. It is an autosomal recessive disorder caused by mutations in the steroidogenic acute regulatory protein (StAR) gene. The StAR protein facilitates the entry of cholesterol into the mitochondria. When this protein is defective, cholesterol cannot be converted to pregnenolone, and hence steroidogenesis cannot be initiated. Eventually all adrenal and gonadal steroidogenesis is destroyed. Patients usually present with adrenal salt-wasting crisis in the first 2 months of life. Those with a 46,XY karyotype have complete sex reversal; however, the phenotype can be variable. Adrenal insufficiency has been reported to occur later in life and patients can have partially virilized or even normal male genitalia. We report an atypical female patient with a 46,XY karyotype, who had mild genital virilization and presented with adrenal crisis at 6 months of age. She carries a previously unreported StAR protein gene mutation.

Patient Report

Our patient presented to us at 7 months of age. At birth she was noted to have mild clitoromegaly, mild posterior labial fusion, a single urogenital sinus, and bilateral inguinal hernias with gonads present. A pelvic sonogram revealed no uterus or ovaries. Of note, on prenatal testing the karyotype analysis of the amniocentesis fluid revealed a 46,XY karyotype, but the family chose not to know the results, and

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the delivery room staff was also not aware. The karyotype was confirmed postnatally after mild genital ambiguity was noted. No mutations in the androgen receptor were identified. At 3 months of age she underwent a bilateral orchiectomy, inguinal hernia repair, and vaginoplasty and tolerated the surgery well. Pathology revealed normal testes. At 6 months she had a prolonged respiratory illness and vomiting and presented with adrenal crisis. Her sodium level was 124 meq/mL and potassium was 7.7 meq/mL. She had low levels of all adrenal hormones, including testosterone, with no response to ACTH stimulation. Baseline cortisol was 10 mcg/dL, stimulating to only 12 mcg/dL 60 min after ACTH was administered. Plasma renin activity was significantly elevated at 14,345 ng/dL/h. Baseline ACTH level was also significantly elevated at 4781 pg/mL. Genetic analysis revealed c.178+1G>C intron 2 homozygous splicing mutation of the StAR gene, which is a novel mutation. The patient's father is Sephardic Jewish from Syria, Iraq, and Turkey and the mother is Sephardic from Syria and Ashkenazi (origin unknown). There is no reported consanguinity. Our patient was started on glucocorticoid (hydrocortisone) and mineralocorticoid (9α-fluorocortisol) supplementation, and since starting treatment she has been without major illness and growing and developing well.

Conclusion

Our patient had several clinical features consistent with non-classical or atypical StAR protein deficiency including unusually late presentation of adrenal insufficiency, tolerating the stress of surgery without developing adrenal crisis, a detectable cortisol level, and genital virilization. All of these findings suggest that the StAR gene was partially active. We postulate that the above-described novel mutation is associated with atypical congenital lipoid adrenal hyperplasia. In addition, it is important for the medical community to recognize that patients with StAR protein gene mutations are usually assigned to the female sex and female identity is established.

Chapter 30
Patient-Centered Care: Caring for Families Affected by Disorders of Sex Development

Anthony J. Asciutto, Emily Haddad, Janet Green, and David E. Sandberg

Disorders of sex development (DSD) are "congenital conditions in which development of chromosomal, gonadal, or anatomic sex is atypical" [1]. The conditions subsumed under this superordinate term are individually rare but, in the aggregate, have an estimated incidence of 0.5–1% [2]. Clinical management of DSD (formerly referred to as "intersex") had stood largely unchallenged from the mid-1950s until the early 1990s. At that time, criticism of various aspects of clinical practice in DSD emerged from several perspectives [3], perhaps most notably from affected adults who expressed dissatisfaction with their treatment [4, 5]. A confluence of advances in the diagnosis of DSD and appraisal of surgical and psychological outcomes has led to a reexamination of assumptions and clinical practice.

Clinical Management of DSD

Much of what is known about the psychological development of persons with DSD stems from research focused on elucidating the influence of atypical sex hormone exposure during steroid-sensitive periods of brain development [6–8]. In contrast to extensive research on gender-related phenomena, there has been relatively limited research addressing psychological adaptation and quality of life. An overemphasis on gender-related outcomes has also been associated with a relative scarcity of studies investigating endpoints that relate more directly to improving clinical care [9]. Where gaps exist in empirical evidence, health-care delivery in DSD is likely to be influenced by cultural ideologies of providers, patients, and families, with societal norms regarding gender and sexuality prompting additional ethical questions. Families and providers bring to the clinical interaction a set of personal "beliefs, values, goals, and expectations which are culturally constructed" by life experiences [10]. Addressing the interplay of varying perspectives involved in each

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individual case demands the involvement of a psychosocial specialist within the interdisciplinary team [10].

The historical stigmatization of DSD, the cultural questions raised during diagnosis and treatment decision making, and the highly personal experience for affected persons and their families demand a patient- and family-centered approach emphasizing effective information sharing and individualized communication. Religious beliefs and practices, marital customs, cultural ideologies of sex and gender, socioeconomic status, education level, the specific DSD diagnosis, and a myriad of additional demographic features unique to each patient/family necessitate an individualized care plan that structures treatment and long-term follow-up to achieve the quality of life outcomes important to the patient and the family.

Patient-centered care "requires that patient needs, preferences, and beliefs be respected at all times" with a focus on the patient's experience of "illness and healthcare" [11]. Patient- and family-centered care has developed along with the realization that psychosocial factors and health beliefs have an impact on well-being. The biopsychosocial model of well-being approaches health as a "process of stabilization" in which poor communication between patient/family and clinician acts as a destabilizing resistance [11]. If a patient/family-centered approach is adopted by interdisciplinary teams, then families will better understand the details of the child's DSD and the available treatment options. Moreover, families and affected persons will be able to articulate medical treatment preferences as they relate to personal definitions of quality of life.

Emerging evidence indicates that shared decision making and using patient-centered communication can be extremely beneficial to patients and their healthcare decision makers [12]. The process of shared decision making within a patient- and family-centered model involves detailed psychoeducational counseling of the family with a focus on cultural sensitivity, environmental influences, and the health beliefs of the family and the clinicians providing care. A strategic focus on quality of care elucidates efforts to define and improve processes of care and maximize desirable outcomes. Within a patient- and family-centered model of care, the important quality of life outcomes to be achieved and measured are those reflecting the perspective of the affected person and family.

Overview of Practice and Consensus on Model Approaches to Care

In response to advances in the molecular diagnosis of DSD, urogenital surgical strategies and techniques, inquiry regarding psychological outcomes of affected persons, and engagement with patient advocacy groups, an international consensus conference was convened in Chicago in 2005. The conference goals were to review clinical management practices, summarize findings from longer term psychosocial and medical outcome studies, and propose a research agenda to move inquiry forward. Sponsored by the *Lawson Wilkins Pediatric Endocrine Society* (as

of 2010 known as the *Pediatric Endocrine Society*) and the *European Society for Paediatric Endocrinology*, the conference comprised a diverse group of scientists and medical subspecialists as well as two patient advocacy organization representatives [1]. Among its many accomplishments, participants of the conference adopted a new diagnostic nomenclature. A salient modification to the existing classification scheme was the removal of terms perceived as imprecise and potentially stigmatizing such as "hermaphrodite" and "pseudohermaphrodite," as well as the politically charged and vague term "intersex." In their place, *disorders of sex development* was introduced. The proceedings of this conference represent important advancements in thinking about appropriate care for patients and families affected by DSD and are summarized in the Consensus Statement [1].

As noted in the Consensus Statement, DSD are now grouped into three major categories: sex chromosome DSD; 46,XX DSD; and 46,XY DSD. It is noteworthy that Klinefelter syndrome and Turner syndrome (and their variants) are categorized as sex chromosome DSD. Inclusion of syndromes in which genital appearance is typical, and gender identity is not in question, underscores that the newly established DSD nomenclature does away with the notion that "ambiguous genitalia" are the sine qua non of DSD [13].

Accompanying recommendations for changes in nomenclature, the Consensus Statement proposed a standard of care focused on improved quality of life through patient-centered care with an emphasis on interdisciplinary team model for health-care delivery [1]. It is recommended that children with DSD are cared for by an interdisciplinary team of pediatric subspecialists with expertise including endocrinology, surgery and/or urology, psychology/psychiatry, gynecology, genetics, neonatology, nursing, and social work. The optimal clinical management is characterized by the following features: gender assignment – which must occur in all cases – follows early expert evaluation; evaluation and long-term management is to be performed at centers with an experienced multidisciplinary health-care team; open communication with patients and families is essential, and shared decision making is encouraged; and patient and family concerns and values should be taken into account when considering diagnosis, testing, and treatment – a cardinal principle of shared decision making.

With regard to the controversial topic of genital surgery, the Consensus Statement notes an important shift in emphasis from cosmetic appearance to functional outcome, including the goal of maintaining pleasurable sexual experience in the patient's adult life. Although the motivation to relieve parental distress through early surgery was acknowledged, conference participants agreed that systematic evidence for this belief – that early surgery for the infant actually relieves parental distress – is lacking.

Open communication with the parents, at the onset of ascertainment of a DSD, is emphasized because of the lasting influences of these early encounters with the health-care system. Helping the parents to differentiate the need to protect a child's privacy from the tendency to view DSD as shameful is stressed. Although the Consensus Statement is not prescriptive regarding the formula for sharing information about karyotype, gonadal status, and prospects for fertility with the affected

person, it recognizes that the process is ongoing and that planning for the education of the patient and family begins at the time of diagnosis.

The Consensus Statement addresses the structure of the health-care team responsible for delivering care noting that it will vary according to patient–family needs, DSD type, and local resources. A diverse professional composition of the team is intended to ensure comprehensive physical and psychosocial assessment and clinical care. A plan for clinical management of newly ascertained patients should be created by the team prior to conversations with the family regarding recommendations. An appointed liaison member of the team would provide comprehensive, synthesized, and consistent communication to the family as an essential element to patient- and family-centered care. Also recommended is ongoing consultation with the family's primary care physician, the medical home, and support system to ensure a successful transition from pediatric to adult care. Finally, the Consensus Statement acknowledges the important role that support groups play in facilitating positive quality of life outcomes for patients and their families; this is articulated in an appendix to the Consensus Statement (Support Groups) [1].

The Consensus Conference successfully articulated an achievable vision for patient- and family-centered care for persons and families affected by DSD. Current gaps in evidence of surgical, physical, and psychosocial outcomes, as well as the lack of consensus on desirable outcomes, point to the importance of the ethical principles outlined in the Consensus Statement. Next steps in improving health-care delivery for this population involve articulation and standardization of clinical practice, including the development of interdisciplinary teams incorporating patient- and family-centered care models into their daily practice. The decision processes for the management of DSD demand a team of specialists providing a variety of perspectives to appropriately educate and collaborate with the patient and family. Partnerships among interdisciplinary teams in multi-site clinical and translational research projects and long-term outcome studies can produce the information currently lacking for the integration of standardized, evidence-based protocols into clinical everyday practice.

From Consensus to Implementation

Accord Alliance (www.accordalliance.org) is a 501(c)(3) nonprofit organization that promotes comprehensive, integrated approaches to care that enhance the health and well-being of patients and families affected by DSD. The organization partners with patients and families, providers, researchers, and health-care administrators to facilitate communication and collaboration among all stakeholders interested in improving health-care delivery and medical and psychosocial outcomes in DSD.

A primary goal of Accord Alliance is to assist institutions in establishing successful interdisciplinary DSD teams provide a new standard of care and improve outcomes for patients and their families. This is accomplished by partnering with developing teams to articulate a mission and vision for DSD care tailored to the

organization in which the team is affiliated. Accord Alliance supports DSD teams in defining clinical objectives and identifying successes and opportunities for improvement. Moreover, Accord Alliance partners with teams to formulate strategic and programmatic goals, expand clinical services, and engage new patients through promotion.

In concert with the Consensus Statement's recommendations for future studies, Accord Alliance supports multi-center studies that can deliver larger numbers of cases for long-term outcome studies. In addition, this type of collaboration promotes the pooling of resources and sharing of information among teams and institutions, which helps to foster and spread best practices. Tools to support information sharing and shared decision making among health-care providers and patients and families can be mutually exchanged with the support of Accord Alliance, inevitably progressing research and clinical management of DSD.

A Case Study: Congenital Adrenal Hyperplasia (CAH) Demonstration Project

Accord Alliance, in collaboration with the Michigan Department of Community Health and the Department of Pediatrics & Communicable Diseases of the University of Michigan Medical School, has embarked on a clinical demonstration project to deliver comprehensive education to parents of children born with congenital adrenal hyperplasia (the most common cause of DSD in persons with a 46,XX karyotype) with the goal to reduce emotional stress and enable families to be informed participants in clinical care and decision making. For this 1-year project, Accord Alliance will (1) enhance existing educational content and make it web accessible to providers and families; (2) develop training workshops and supporting materials for health-care providers; and (3) conduct an evaluation of the feasibility, ease of use, and clinical value of the program and tools.

Resources for DSD Teams: Clinical Guidelines and Handbook for Parents

Accord Alliance maintains a web-based clearing house for educational tools and information about living with and caring for those with DSD which includes the *Clinical Guidelines for the Management of Disorders of Sex Development in Childhood* and *Handbook for Parents.* These handbooks were developed by the Consortium on the Management of DSD through a collaborative effort among three groups with a stake in improving care and outcomes for DSD: experienced clinical specialists, adults with DSD, and parents and family members of children with DSD. These handbooks are responsive to the charge for patient-centered care in the *Consensus Statement*. As a set, they provide a roadmap for providers, patients, and

families as they navigate the process of care for DSD together. When used effectively, they support an open flow of information and shared decision making with an eye toward improving care and outcomes.

The *Clinical Guidelines* is a resource for practitioners engaged in diagnosing, treating, educating, and supporting children born with DSD and their families. It can be used to inform clinical quality improvement discussions. Topics of interest include patient-centered care, reducing stigma related to medical care, treatment protocols, working with an interdisciplinary team, scripts for talking with parents and family members, and references for additional reading.

The *Handbook for Parents* covers questions common to parents as they learn about their child's DSD. It helps parents explore their concerns so they feel stronger, less confused, and less stressed. It provides information they can use to help their child adapt and thrive. Topics of interest include basic information about DSD; talking with health-care providers, family members, and teachers; how to find support for parents and children; and child development as it relates specifically to well-being for affected persons: physical, social and psychological.

In the absence of evidence-based practices in the area of DSD, principle-based tools such as the *Handbook for Parents* and *Clinical Guidelines* foster dialogue that may lead to more defined, evidence-based guidelines in the future. Currently, there is no confirmation that the use of these tools ensures good or desirable outcomes, just as there is a paucity of evidence regarding which DSD treatments result in which desirable outcomes. However, they not only exemplify tangible progress in the movement toward patient-centered care for DSD management but also fill a fundamental gap toward standardization of basic areas of psychosocial support for persons affected with DSD and their families as they begin to understand DSD and explore treatment options. As findings from long-term outcome studies accumulate, clinical guidelines expanding and elaborating on these tools can be developed and adopted more broadly.

Recommendations for Emerging Interdisciplinary DSD Teams

Collaboration among stakeholders helps frame discussion and information sharing and establishes an important foundation for consensus regarding approaches to ensure patient-centered care. The *Clinical Guidelines* and *Parents Handbook* offer balanced information based on a review of medical, sociological, and autobiographical literature on DSD. These tools suggest the following for caring for children with DSD and their families:

- Health-care providers should aim to create interdisciplinary teams that can meet the diverse needs of children with DSD and their families.
- Optimal care for DSD should include a behavioral health component and peer support.
- Providers should review and discuss with patients and families the various topics covered in the *Clinical Guidelines* and *Parents Handbook* so that families feel

comfortable with their understanding of DSD and their engagement in making decisions regarding DSD treatment options and their child's quality of life.
- Children with DSD should receive age-appropriate information about their condition, their bodies, and possible treatments so that they can participate, to every extent possible, in their own care.

Since no institution has yet to fully implement the new standard of DSD care, recent progress falls short of the objective of optimizing clinical care provided to people with DSD and their families. More progress needs to be made in long-term research efforts and collaboration across sites to provide ethical evidence-based care in the field of DSD. There is a strong need for an organization like Accord Alliance to assume the role of a convener of stakeholders across the health-care system and DSD communities in order to promote this new standard of care. Accord Alliance is filling this role by becoming the "go-to" organization for resources and information for health-care professionals and the community.

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Chapter 31
Assessing Health-Related Quality of Life in Disorders of Sex Development: Phase I – Item Generation

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Background

Disorders of sex development (DSD) are congenital conditions in which chromosomal, gonadal, or anatomic sex development is atypical [1]. Only anecdotal, retrospective reports exist regarding the experiences of children with DSD and their families, with no concurrently collected data on the specific stressors children and families face or on their processes of adaptation.

Objective

Develop validated parent proxy-report and parent self-report health-related quality of life (HRQoL) questionnaires that focus on issues specific to and shared by young patients (newborn to 6 years) with DSD and their families, which are not otherwise covered by generic HRQoL measures.

Design/Methods

Focus groups were conducted in-person or by conference call with key stakeholders. Participants included pediatric endocrinologists (two groups, $n = 10$); pediatric urologists (two groups, $n = 7$); mental health providers with DSD experience (one group, $n = 5$); parents of affected children, newborn to 6 years (three groups, $n = 11$); and patient advocates (two groups, $n = 4$). Because of the young age of the target group (newborn to 6 years with DSD), parents served as proxy informants for

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their child's experience. Focus groups were audio-recorded and transcribed for qualitative analysis. Transcriptions were coded by two independent raters into discrete segments reflecting areas of concern. Sampling of content was judged to be complete when transcripts from later focus groups failed to generate new topics of concern (i.e., reached 'saturation').

Results

Topics were categorized as either child or parent/family centered and grouped into domains (Tables 31.1 and 31.2). *Child-centered domains* included medications and procedures, physical functioning, gender concerns/body image, and emotional/social functioning. *Family/parent-centered domains* included diagnosis, medical management, anatomy and voiding, appearance, decision making, gender concerns, communication/information, role functioning/family activities, emotional functioning, social functioning, and future worries. Inter-coder agreement at the topic level ranged between $r = 0.63$ and 0.72 (mean $r = 0.66$).

Table 31.1 Selected child-centered domains with topic examples

	Frequency of topics endorsed by group				
Child centered	Endocrine	Urology	Mental health	Parents	Patient advocate
Medications and procedures					
Stress/embarrassment of genital exams	0	4	2	1	4
Repeated and/or painful medical procedures	1	0	3	3	2
Gender concerns/body image					
Child cannot identify with the same sex	1	1	0	2	1
Child displaying behavior of other gender	1	1	0	1	0
Emotional and social functioning					
Social difficulties	0	0	0	2	2
Child's condition limiting social activities	0	1	0	0	2

Stakeholder groups varied widely in their identification of important issues. The tables illustrate the variability in frequency with which respondents from different stakeholder groups identified these selected issues as important. Pediatric urologists expressed concerns regarding voiding and childcare most frequently. Patient advocates identified communication with healthcare providers as a central issue, and parents most frequently mentioned worries about the diagnosis, understanding the diagnosis, and remembering medications. None of the groups mentioned child emotional functioning/social difficulties among their top concerns.

Table 31.2 Selected parent/family-centered domains with topic examples

Parent/family centered	Frequency topic identified by group				
	Endocrine	Urology	Mental health	Parents	Patient advocate
Diagnosis					
Stress of uncertain diagnosis and treatment	1	1	4	5	3
Emotionally overwhelmed upon receiving diagnosis (stress, anxiety)	3	2	0	5	3
Medical management					
Making sure the child is getting the appropriate medications at the right time	0	6	0	4	2
Stress of remembering medications	0	0	2	7	3
Gender concerns					
Parental disagreement over gender assignment (with extended family, doctors)	2	0	4	0	0
Worry about gender identification (gender-appropriate toys/playmates)	0	5	0	2	3
Communication/information					
Managing communication with healthcare professionals	1	2	0	5	14
Stress of navigating conflicting medical information (doctors, Internet)	1	3	4	2	5
Role functioning and family activities					
Concerns about locating good child care	0	7	1	2	4
Concerned and sometimes upset about how the child voids/changing diapers	0	7	2	2	6

Conclusions

Qualitative analysis of focus groups with key DSD stakeholder groups reliably generated distinct child-centered and parent-centered issues. We identified substantial variability across stakeholder groups in their reporting of key issues and concerns for young children with DSD and their families. These differences should be considered when counseling families and planning treatment. The differing perspectives of healthcare provider groups reinforce the recommendation that patients with DSD be cared for in the context of well-integrated interdisciplinary teams.

Content from these focus groups has been used to generate items in the development of provisional HRQoL questionnaires for DSD. The utility of these questionnaires in comprehensively capturing situations and reactions relevant to young patients with DSD and their caregivers has recently been assessed through cognitive interviews in an independent parent sample. Cognitive interviewing is a procedure used to expose the thought processes respondents use when answering

questions [2]. Cognitive interviewing techniques are now widely used to gain information from respondents about how they understand and formulate their answers to questionnaires [2, 3].

Questionnaires were revised based upon parent/caregiver feedback and the final phase of questionnaire development is beginning. The project will conclude with establishing the psychometric properties of parent self-report and parent proxy-report questionnaires in a large national sample of parents of young children (newborn to 6 years) affected with a DSD. This project represents the first attempt to characterize subjective experiences of young patients and their families. Developing psychometrically sound quantitative measures to assess issues specific to DSD creates opportunities to systematically identify families' on-going needs for psychoeducational counseling and social and emotional support. The measures currently in development can also help gauge the influence of changes in the model of care proposed in the recent *Consensus Statement on Management of Intersex Disorders* [1] on the quality of life of patients and their caregivers.

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Chapter 32
Leydig Cell Hypoplasia due to Inactivating Luteinizing Hormone/Chorionic Gonadotropin Receptor Gene Mutation Presenting as a 46,XY DSD

Sunil K. Sinha, Amrit Bhangoo, Svetlana Ten, and Joerg Gromoll

Background

Leydig cell hypoplasia (LCH) due to inactivating mutations of luteinizing hormone receptor gene (LHCGR) is a relatively rare form of 46,XY disorder of sex development (DSD). LCH is inherited in an autosomal recessive manner, which leads to aberration of Leydig cell differentiation and ultimately subnormal androgen production both pre- and postnatally. In males, two distinct phenotypes have been described in the literature depending on the degree of remaining activity of LHCGR. Leydig cell hypoplasia type 1 (LCH type 1) represents more severe form with 46,XY DSD while Leydig cell hypoplasia type 2 (LCH type 2) is more diverse with micropenis to several degrees of undervirilization. We identified one kindred with 27-bp insertion in exon 1 causing 46,XY DSD.

Patients/Methods

The proband is a 16-month-old phenotypic female, who was referred to the pediatric endocrine clinic after gonads were identified in the inguinal region during presumed hernia repair. Parents are first cousins of Lebanese origin. On physical examination, she had no signs of external virilization. The LH was 0.8 mIU/ml and FSH was 2.74 mIU/ml, while her testosterone and DHT were 6 and 2 ng/dl, respectively. The karyotype from peripheral blood leukocytes revealed 46,XY. Pelvic MRI revealed bilateral gonads at inguinal region, with blind vaginal pouch but no mullerian derivatives. A younger 46,XY, phenotypic female sibling was evaluated at the second day of life, who was found to be unresponsive to HCG stimulation test, though ACTH stimulation test revealed appropriate adrenal function.

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Results

Molecular analysis by direct sequencing of LHCGR gene revealed a homozygous 27-bp in-frame insertion after nucleotide 54 at exon 1 in both siblings. Unaffected parents were heterozygous. Interestingly this is also found to be a common polymorphic site for 6-bp insertion (insLQ), especially in Caucasians [1]. These patients did not harbor this common 6-bp insertion polymorphism at this site, which is found in approximately 28% of the normal LH receptor alleles, especially in Caucasians. Previously, 33-bp insertion including polymorphic 6 bp at this site was reported in a compound heterozygous manner in two kindreds. This 27-bp insertion is located immediately upstream of the proposed signal peptide cleavage site and may therefore interfere with protein transport to the plasma membrane [2]. Previously in vitro expression of the insertion mutant showed complete absence of LH receptor function [3].

Conclusion

We report the first kindred with 27-bp in-frame insertion in exon 1. Two previously reported cases were in a compound heterozygous manner in conjunction with 6-bp polymorphic insertion [1, 3]. All reported five affected cases with 27/33-bp insertion regardless of homozygous or compound heterozygous state presented with LHC type 1 phenotype, whereas heterozygosity in parents did not affect phenotype or fertility either in male or female.

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Chapter 33
Inguinal Hernia in a Phenotypic Female Infant May Reveal a 46XY Sex Reversal, Supported by the Identification of a Novel SF1 Gene Mutation

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Background

Testicular development from bipotential gonad and its descent to the scrotal sac are thought to be dependent on multiple genetic, hormonal, and environmental factors. Presumed inguinal hernia in a phenotypic female is a common presenting sign of 46XY disorder of sexual differentiation (DSD) in infancy and childhood. Although androgen insensitivity syndrome (AIS) is the most frequently identified cause, evaluation of other rare genetic disruptions like SF1 should be considered based on clinical and biological phenotypes.

Design/Methods

The index case is a 4-year-old phenotypic female, presented for endocrine evaluation after gonads were identified during suspected inguinal hernia repair. On physical examination she was found to have apparently normal looking female genitalia without any evidence of clitoromegaly or other signs of external genital virilization. Hormonal evaluation at that time revealed FSH = 7.2 mIU/mL, LH = <0.3 mIU/mL, and testosterone = <10 ng/dL. Further hormonal analysis revealed AMH = 28 ng/mL, inhibin B = 41 ng/dL. Karyotype from peripheral blood revealed 46XY. Low basal testosterone level did not respond after stimulation with human chorionic gonadotropin (hCG) (5000 IU X3) but appropriate adrenal response was observed to ACTH stimulation test (from baseline cortisol 5.1–31 μg/dL).

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Results

Molecular analysis by direct sequencing of SF1 gene (*NR5A1*) revealed a novel heterozygous missense mutation (L361P). This gene abnormality was not identified in 100 controls, excluding a gene polymorphism. The replacement of leucine by proline likely leads to an abnormal SF1 function, as confirmed by the conserved leucin in position 361 among species. In vitro analysis of the mutated SF1 is in progress. We were unable to determine the mode of inheritance because samples were not available for evaluation from other family members due to social issue.

Conclusion

We identified a novel heterozygous mutation of SF1 gene causing 46XY DSD with preserved adrenal function. SF1 regulates the transcription of multiple genes involving adrenal, gonadal, and reproductive axes. Recently several studies with heterozygous loss-of-function mutations with similar phenotype have led to the speculation that the testis is more sensitive to partial loss of SF1 than adrenal gland in human.

Identification of a novel SF1 mutation provides further evidence that 46XY DSD with inguinal hernia with low testosterone response to HCG stimulation test warrants evaluation of rare genetic disruptions other than CIS, e.g., SF1, StAR protein mutation, and inactivation mutation of LH receptor with similar phenotypic presentation.

Chapter 34
University of Michigan Disorders of Sex Development (DSD) Research and Quality Improvement Symposium

David E. Sandberg, Anthony J. Asciutto, and Emily Haddad

Disorders of sex development (DSD) are "congenital conditions in which development of chromosomal, gonadal, or anatomic sex is atypical" [1]. For families, the birth of a child with a DSD, and the attendant uncertainty about the child's gender and future psychological and sexual development, is believed to be extraordinarily stressful. For health-care professionals, genital ambiguity and discordance among genotype, gonads, and anatomy can be the most challenging aspects of an already complex medical condition for which long-standing controversy over the most appropriate model of care exists [2].

Trends in DSD Clinical Management

Medical management of DSD ("intersexuality" formerly serving as the superordinate term) has evolved with enhanced understanding of the process of psychosexual differentiation. Until the mid-1950s, an individual's "true sex" was thought to be determined by the function of the gonads and that a person's gender identity and gendered behavior would naturally conform to their "true sex." The weakness of this approach is now readily apparent when, for example, considering the condition of complete androgen insensitivity syndrome (cAIS). Androgen insensitivity is characterized by an absent or defective androgen receptor gene. Despite the presence of XY sex chromosomes and normally functioning testes, affected persons appear as typical girls at birth resulting in a female sex announcement and gender of rearing. With one recently reported exception [3], women with cAIS characteristically develop and maintain a female gender identity throughout their lives [4].

Recognition that single biological markers of sex do not unequivocally predict gender identity, the "true sex" approach was replaced by the "optimal gender policy"

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which considers multiple aspects of outcome, most prominently potential for sexual function and appearance of the genitalia. This approach to the management of DSD was predicated on the assumptions that "gender identity" (i.e., identification of self as either girl/woman or boy/man) is not firmly established at birth and that a positive psychological adaptation requires that genital appearance match assigned sex, which often calls for early reconstructive genital surgery [2].

Beginning in the 1990s, the optimal gender policy began to draw organized criticism from several perspectives. The notion of "gender neutrality" at birth had been challenged on scientific grounds [5]. Additionally, affected adults expressed anger over their treatment, including the perceived lack of information or misinformation provided about their condition and feeling stigmatized and shamed by the secrecy surrounding their condition and its management [6]. Others also attributed poor sexual function to damaging genital surgery as well as insensitive genital examinations performed without informed consent [7–9]. Finally, social constructionists challenged the entire enterprise of medical management of DSD by arguing that such practices are rooted in history, language, politics, and culture and therefore are not universal scientific facts [10, 11]. According to this perspective, genital surgery performed in infancy can be considered less of a medical emergency than an elective adoption of a medical treatment in response to a cultural imperative to view the sexes as dichotomous.

Consensus Statement on the Management of DSD

Clinical care in DSD has been held back by a fragmented research agenda, leaving fundamental gaps in knowledge of the molecular basis of DSD and links between treatment options and desirable outcomes. Studies of small and incomplete patient samples, relying predominately on retrospective research designs, remain the standard and provide little insight for hypothesis-driven clinical research. The limited foundation of clinical evidence is likely to be associated with significant variation in diagnostic and treatment practices within and across medical, surgical, and behavioral health aspects of care for patients and their families.

In 2005, the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology convened a conference to review clinical management practices and data from longer term health and gender-related outcomes research and to identify key areas for future research. Invited conference participants included 48 clinicians and scientists specializing in this field and 2 participants with DSD serving as patient advocates. The Consensus Statement on Management of Intersex Disorders, published in 2006, identified features of optimal clinical care for DSD and the role of the "multidisciplinary team" in managing healthcare for the patient and family affected by DSD. This team would include not only various pediatric but also nursing, social work, and medical ethics subspecialists, if possible. Syndrome-specific guidance was provided regarding gender assignment based upon follow-up studies of gender identity stability. Because of the inherent risks to genital

sensitivity, surgery in infancy focusing on functional outcomes was emphasized over cosmetic appearance [1].

In addition to defining the composition of the ideal DSD health-care team, the Consensus Statement also recommended that one provider serve as liaison for communications between the team and the patient and family. Researchers and clinicians were also encouraged to examine a wider range of psychological outcomes (a shift away from the concentration on "gender" and "sexual orientation"), including consideration of "social and psychosexual adjustment, mental health, quality of life, and social participation" [1]. The Consensus Statement emphasized that cultural and social factors modulate outcomes in affected persons and, therefore, recommended that these influences be taken into account in clinical care and research design.

University of Michigan DSD Research and Quality Improvement Symposium

To keep pace with advances in basic and clinical DSD research, while accounting for the unique features of the US health-care system, there is an urgent need to establish a research infrastructure and collaborative network of researchers, health-care providers, and patient/family advocates in order to support basic, translational, and clinical research on DSD and other complementary fields of inquiry.

The DSD Research and Quality Improvement Symposium (Ann Arbor, MI, April 2009) was organized consistent with the goals of the 2005 International Consensus Conference on Intersex. Co-hosted by the Department of Pediatrics and Communicable Diseases of the University of Michigan and Accord Alliance (www.accordalliance.org), the symposium was structured to further articulate and put into action the roadmap for quality improvement in clinical care and research encouraged in the DSD Consensus Statement [1].

The aims of the symposium were to create a vision for research on caring for persons affected by DSD and guide recommendations for an interdisciplinary research and collaborative learning agenda. It was predicted that the active exchange of knowledge and ideas by participants of the symposium would help establish a broad network of collaborators capable of improving quality of care for DSD patients through research, enhanced clinical protocol design, and championing the practice of ethical and compassionate care.

Specific aims for the symposium were identified by the organizers and facilitators prior to sending invitations to over 100 potential attendees: develop consensus among stakeholders on focused areas for research and collaborative learning; develop recommendations to generate interest and support for initiatives to close the gap on the evidence and information available to providers and persons affected by DSD; and establish a network of researchers, providers, and patients and families with a stake in improving treatment and care for DSD.

Symposium Organization

The collaboration of subspecialists participating in the 2005 International Consensus Conference on Intersex Disorders was successful in advancing a framework for evidence-based practice in the management of DSD. However, the gap between consensus-based recommendations and the status quo for diagnosis and treatment in DSD is substantial. "Quality improvement collaboratives are an increasingly common strategy for implementing evidence-based practices in health care" [12]. Modeled after the Institute for Healthcare Improvement design, learning collaboratives provide a mechanism to systematically improve the quality of healthcare by data-driven, "structure–process" interactions involving affected persons and their families, clinicians, and various stakeholders in the conversation of quality improvement [13]. In utilizing the construct of *quality improvement learning collaboratives* for the DSD Research and Quality Improvement Symposium, the organizers designed an agenda around a collaborative, thematic structure to encourage broad participation of all attendees, especially those participants unfamiliar with a non-traditional conference design. The topics introduced were those for which clinical evidence remains provisional and controversy among stakeholders exists to varying degrees.

There is a risk that a traditional, hierarchal medical model discounts the importance of affected persons' and family perspectives in defining problems and designing remedies. Recent health-care management studies have shown that collaborative models of care result in higher levels of consumer satisfaction and effective team performance and care coordination, e.g., [14]. The aims and goals of the DSD Research and Quality Improvement Symposium were operationalized by first inviting a balanced representative community of stakeholders positioned to adopt and promote a collaborative learning network. Research indicates that heterogeneous groups can discuss complex topics and deliver important insights and recommendations when the quality of interpersonal interaction and teamwork is high [15]. The structure of this symposium, coupled with the diversity of its attendees, was indeed non-traditional in its approach to stakeholder engagement and facilitation.

Participants

Attendance at the 2005 International Consensus Conference on Intersex Disorders was relatively restricted in participant specialties and perspectives:

- *Providers:* pediatric endocrinology (23); pediatric urology (8); genetics (2); clinical psychology (6); psychiatry (2); pediatrician/adolescent medicine (1); oncology (1)
- *Researchers:* psychology (3) and neuroscience (2)
- Patient advocates (2)

In an effort to extend the previously established consensus to a broader list of stakeholders and to initiate first steps toward establishing a learning collaborative, attendance at the DSD Research and Quality Improvement Symposium included, in addition to DSD provider specialists and researchers, experts from other disciplines which can inform DSD care and research, a broader variety of affected persons and parents, representatives from DSD advocacy groups, a state health department, and the National Institutes of Health Office on Rare Diseases Research. Attendees represented the broadest range to date of stakeholders convening to discuss research and quality improvement in DSD:

- *Providers:* pediatric endocrinology (5); pediatric urology (7); genetics (3); adolescent gynecology (1); nursing (3); social work (2); clinical psychology (7); child and adolescent psychiatry (1); and child life (1)
- *Researchers:* genetics (2); pediatric urology (5); pediatric endocrinology (1); health economics (1); health-related quality of life (5)
- Affected persons (5)
- Parents of affected persons (4)
- DSD advocacy groups (5 organizations)
- Bioethics and legal experts (3)
- Medical writer (1)
- Michigan Department of Community Health (1)
- National Institutes of Health (1)

Symposium Facilitation Techniques

Two group facilitation techniques, Conversation Cafés and Open Space Technology, were used in combination to accommodate diverse participant backgrounds and perspectives as well as encourage active discussion, consensus building, and networking. Symposium participant name tags included first names only (rather than last names, titles, or degrees) so that conversations and workshop discussions would not be constrained by participants' assumptions of professional or familial roles or perceived level of expertise. Facilitators roamed among groups as participant observers to help discussions remain focused on the generation of research questions and the clinical management of DSD with an eye to optimal quality of life outcomes.

Conversation Cafés, which comprised the first half of the symposium agenda, are a semi-structured process for engaging people with diverse backgrounds and perspectives in meaningful discussion [16]. This technique was used to increase personal and group knowledge and awareness around important issues related to DSD. The symposium included a "warm-up" to the traditional Conversation Café structure in which content experts introduced topics that illustrated gaps in DSD research and clinical care. In accordance with the recommendations in the DSD Consensus Statement, these topics were targeted in order to spur research in areas in which salient gaps exist. The following topics were presented by content experts

and then explored further by all participants in Conversation Café sessions: psychological outcomes in DSD research, informed consent for research and clinical care, team-based healthcare, and shared decision making.

Following each content expert presentation, participants joined pre-assigned small groups (six to eight people) to explore and expand on each topic in brief (20 min) discussions. The small groups were arranged in advance by the organizers with the intent of bringing persons of diverse backgrounds together for effective and efficient collaborative learning. Discussions were then summarized and presented to all participants by a spokesperson nominated by the small group.

Organizers maintained open blocks of time in the agenda for additional unplanned discussions to emerge. The facilitators and some of the participants met after the first day to debrief and adapt the schedule according to the observed needs of the group. For example, during a scheduled break, participants were invited to bring a found object lacking monetary value that symbolized what they thought important in the discussions that had taken place during the first day. This activity was meant to promote openness and encourage participant dialogue; it also encouraged participants to think creatively about their experiences in the first day's discussions.

Following Conversation Cafés, a panel discussion was conducted involving representatives from various fields including social work, basic research, psychology, patient advocacy, and health-care team building. Panel representatives were asked to talk about their perspectives and roles in DSD quality improvement, research, or patient health and quality of life. This panel discussion, impromptu organized by symposium facilitators during the course of the first day, was introduced to illustrate the importance and differing perspective of each stakeholder group's contribution and interest in DSD. The panel discussion preceded Open Space Technology discussions with the intention of demonstrating the "process-oriented" and focused structure of Open Space Technology.

Open Space Technology is described as a dynamic, semi-structured process for meeting facilitation that functions "... best when the work to be done is complex, the people and ideas involved are diverse, and the passion for resolution and potential for conflict are high.... " It involves participants creating and managing their "own agenda of parallel working sessions around a central theme of strategic importance" [17]. Attendees were invited to nominate research, quality improvement, and outcomes-focused topics which were then rank ordered. Topics ranked "most important" by symposium attendees were explored in small participant-led break-out sessions in which the duration was left unspecified (sessions concluded when the group felt it had "exhausted" the topic). Multiple Open Space sessions were held simultaneously; attendees chose which sessions to attend and were encouraged to move between sessions based on personal interests. Participants were instructed to remain in a break-out group only as long as they felt they were involved in a dialogue that was accessible, fruitful, and engaging, in order to promote networking and collaborative learning. Scribes were present to record discussions and themes. Symposium facilitators again roamed among break-out groups to guide discussions toward recommendations for future research and inquiry.

Symposium Discussion Themes

The broad knowledge and experience base of participants created a dynamic and constructive dialogue during Open Space Technology discussion groups. Moreover, the sharing of anecdotal and empirical understandings dependent on the discipline and background of each participant greatly enhanced and furthered discussions. Open Space topics nominated and discussed by symposium participants included the following.

Research

Creating a research infrastructure. Utilize nascent interdisciplinary DSD teams at major US medical centers to participate in the formation of a multisite infrastructure to support data collection.

Long-term outcomes research. Patient-centered clinical research agendas linking psychosocial/sexual outcomes to diagnostic, medical management and physical health variables require the inclusion of psychometrically robust measures of psychological adaptation and health-related quality of life.

Research ethics. Institutional Review Boards and reviewers of research require grounding in DSD-specific medical procedures and experiences to conduct adequate assessments of potential risks and benefits.

Research that matters to patients and families. Clinical research should be a partnership with patients/families (participatory research model). Studies of genetic and hormonal predictors of psychosexual differentiation (i.e., gender identity, gender role, and sexual orientation) should be balanced by investigations of patient and family experiences related to diagnosis, medical management, and disclosure of medical information.

Complete androgen insensitivity syndrome. Studies are needed of the risks and benefits to affected girls of leaving the testes in place through spontaneous puberty. This work will benefit from retrospective examination of the pathology of testes removed from patients at various ages.

Clinical Practice

Patient- and family-centered care. Each family requires personalized care that accounts for their views and broader cultural practices. The behavioral health component of interdisciplinary teams must be sufficiently broad to integrate this aspect of care.

Transition from pediatric to adult care. The experiences of adults with DSD and recommendations of advocacy groups enhance our understanding of issues and

concerns faced by persons with DSD in their health-care management. These insights, in turn, can be used to develop plans for transition from pediatric to adult health-care systems.

Model of care and quality indicators. The DSD Consensus Statement specifies that optimal care for children with DSD requires an experienced multidisciplinary team. Through the systematic collection of process, procedural, and outcome data across multiple centers adhering to core clinical management principles, indicators (or predictors) of better health and quality of life outcomes will emerge. This approach has been effectively implemented for other rare conditions such as cystic fibrosis [18]and pediatric cancer [19].

Enhanced communication. Structured, face-to-face coordination of health-care delivery by members of the interdisciplinary DSD team will reduce the likelihood of miscommunications with the patient and family. Strategic education and communication with the family will be further promoted by designating a single member of the health-care team as liaison. The primary objective of this person is to ensure that the DSD team is aware of the patient's and parents' understanding and personal meaning of the condition and to ensure that all decisions made by the family are informed by balanced information.

Decision aids and informed consent. The uncertainties associated with quality of life outcomes in DSD have led to several stakeholders recommending a deliberative decision-making process that goes beyond what is typically encountered in standard clinical care [20–23]. Decision tools are recommended when there is high uncertainty of outcomes or multiple treatment options [24]. Algorithms (i.e., decision aids) are needed to guide the educational process for patients/families in DSD-related decision making. Such aids would enhance patient and parent (in the case of minors) understanding of the recommended medical or surgical intervention, their alternatives, and the chance of risks occurring. A major goal of such decision aids would be to promote a process that reduces the likelihood of decisional regret.

Shame and discomfort. Arguably, one of the greatest burdens associated with DSD is feelings of shame experienced by those affected and their families. Unfounded assumptions or personal biases regarding DSD and its management held by members of the health-care team may contribute to the development of or amplify such negative feelings in patients and families. Destigmatizing DSD can begin with the training of health-care providers and the general population.

Non-traditional DSD (Turner syndrome and Klinefelter syndrome). The new diagnostic nomenclature introduced in the consensus statement [1] is problematic for some patient groups because of the perceived stigma associated with the label "DSD" and the perception that this higher order classification does not add value to patient care. For example, in the case of Turner syndrome, practice guidelines (including an emphasis on interdisciplinary care) are widely accepted [25]. This break-out group recommended that the term DSD should be replaced by the more specific diagnosis whenever it is available.

Two conversations during the Open Space break-out sessions warrant special attention. Participants in the "Patient- and Family-Centered Care" group shared ideas and working strategies adopted at their own institutions to promote strong collaborations with families. Resources and tools provided to families that better coordinate the care of the child as well as document the processes of decision making with their health-care team were shared. Personal journals, summary reports, interactive web sites, and contact lists were a few of the practicable options offered to enhance patient- and family-centered care.

Also discussed was the value to health-care team function and family engagement in decision-making processes of designating one member of the team as lead contact for communications with the family. Participants in this break-out group perceived particular benefits of utilizing the behavioral health expert member of the team, knowledgeable in child development and family systems (e.g., psychologist, social worker), as the liaison between the interdisciplinary team and the family. This provider would simultaneously serve as a resource for the families and other members of the interdisciplinary team on topics such as the psychosocial aspects of atypical genital appearance or function and possible surgical interventions, hormone replacement therapy, and disclosure of medical information.

The Open Space break-out session devoted to "Long-term outcomes research" emphasized topics for study they believed were most relevant to health-care delivery and quality of life outcomes for persons with DSD. Although the value of basic research into the molecular basis of DSD and hormonal contributions to sex differences in behavior was acknowledged, participants in this group voiced a need to balance the research portfolio with studies of the health-care delivery process and its relationship to patient and family outcomes. The necessity of multisite collaborations following standardized clinical care protocols emerged from this discussion. Participants in this break-out group posited that high-quality psychosocial outcome data are dependent upon the trust experienced by the patient and family in the process of health-care delivery. The quality of the relationship between the health-care team and the patient/family will be an important determinant of the completeness of samples in follow-up studies.

Broad Consensus Items

Upon completion of the Open Space discussions, representatives from each group reported key content to all symposium participants. During this part of the agenda, referred to as "advancing the conversation," the entire group worked together to define the following list of broad consensus items:

- Parental understanding/adaptation to the child's diagnosis and prognosis is an important determinant of the child's health-related quality of life.
- Collaboration among affected adults, patient support groups, and interdisciplinary health-care teams will improve care and enhance outcomes.

- Outcome studies need to encompass psychological endpoints beyond psychosexual differentiation (i.e., gender identity, gender role, and sexual orientation).
- High-quality educational materials available for providers working with patients and families to promote better understanding are rare and difficult to access.
- No public curricula exist to correct misunderstandings and perceptions of stigma associated with DSD.
- Fragmentation of care, lack of coordination, and lack of support for parents reduce quality of care.
- Models of integrated interdisciplinary pediatric and adult healthcare exist that could inform development of DSD teams.
- Future DSD symposia should include participation of experts in health-care organization and funding because of existing barriers to reimbursement for integrated behavioral health services and coordination of care.

Next Steps

Successful collaborative learning discussions were established through this quality improvement-focused symposium agenda. This was enhanced by utilizing Conversation Cafés, Open Space Technology, and additional group facilitation techniques designed specifically for this diverse group of stakeholders. The decision to focus initial discussions through content expert-driven topic presentations accelerated the learning process for all attendees. The design of the symposium helped to emphasize that a structured, collaborative approach is the preferred manner in which advances in the standard of care for persons with DSD can be realized. Facilitation by trained consultants provided opportunities for persons affected with DSD, their families, providers, researchers, and other stakeholders to share knowledge and experiences resulting in consensus items that addressed areas in need of improvement. The Open Space discussion themes illustrate the abundance of domains in which the sharing of quality improvement strategies across institutions can advance not only clinical practice but also networking and collaboration toward evidence-based care.

Even before the symposium formally concluded, a group of attendees commenced grant preparation work to fund a multisite research and collaborative learning infrastructure necessary for hypothesis-driven research on mechanisms of sex development and evidence-based care for patients and families affected by DSD. The majority of the proposed sites for this network were represented at the symposium by health-care providers and researchers. Success in this collaborative venture promises to not only fill fundamental gaps in scientific discovery and health-care delivery for DSD but also serve as a model for accelerated clinical and translational research for other rare congenital conditions identified in the newborn period, notably the life-threatening and life-limiting conditions identified through newborn screening.

In conclusion, we have demonstrated that a conference designed in this fashion is an effective and efficient way in which research agendas and learning collaboratives

can be established. The important objectives of giving voice to the perspectives of diverse stakeholder groups while simultaneously engaging participants in important dialogue are reflected in the results of the symposium. The diversity in symposium participant backgrounds was essential to the generation of complex outcomes and consensus items and agreement for a multisite research collaborative.

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Chapter 35
The Challenge of Mistaken Sex Assignment in an 11-Year-Old with Virilizing CAH

Martha Taboada and Priscila Gagliardi

Background

Congenital adrenal hyperplasia is the most common form of 46,XX disorder of sexual development and can result in severe virilization of females. When identified in the newborn period these patients are assigned female gender, independent of the degree of virilization since with cortisol replacement, androgen levels decline and reproductive capacity is preserved. However, the identification of these disorders in older children presents a clinical and psychosocial management conundrum.

Objectives

To characterize the management challenge of unrecognized patients with DSD.

Case Presentation

- HPI: 11-0/12 previously healthy Haitian boy was brought for evaluation of ambiguous genitalia and early puberty. Upon adoption at the age of 7, the child was noted to have abundant pubic hair and was unable to urinate standing up. He had always been tall and was very athletic, the top player on his baseball team.
- PMH: Unknown birth history. No known history of protracted vomiting or shock.
- FH: No consanguinity. Father's height 187 cm (~75th percentile) and mother's height 165 cm (~50th percentile). MPH at the 75th percentile. Younger sibling, who also reportedly had abnormal genitalia, died in the newborn period in Haiti of unknown causes.

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- Social history: Born in Haiti, moved to the USA at 2 years. Informally adopted 3 years ago. Most of his care was received as uninsured at "walk-in" clinics for occasional exams.
- Physical exam:
  - BP 111/73, HR 79. Height 155 cm (95th percentile), weight 48.5 kg (91st percentile).
  - He was tall for age, had muscular body habitus and a deep voice.
  - The child had a moustache, some acne, hyperpigmentation on the palm creases, and abundant axillary hair.
  - Genitalia showed a 9-cm-large, phallic-like structure with chordee and third-degree scrotal hypospadias with partial fusion of the labioscrotal folds. There was one single opening with urethral meatus and no palpable gonads. The genitalia and pubic hair were Tanner 4.

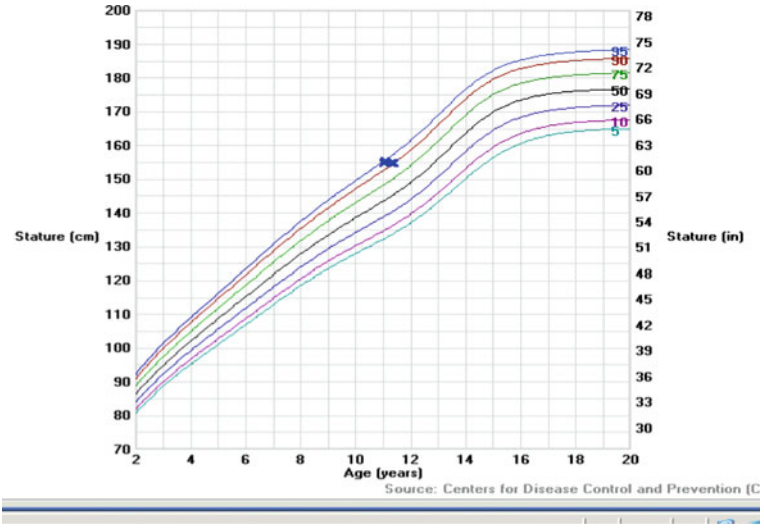

Fig. 35.1 Growth curve height

Fig. 35.2 External genitalia

Fig. 35.3 External genitalia

Pelvic US: revealed a prepubertal uterus and normal appearing ovaries with no cystic or solid adnexal mass. The adrenal region appeared normal

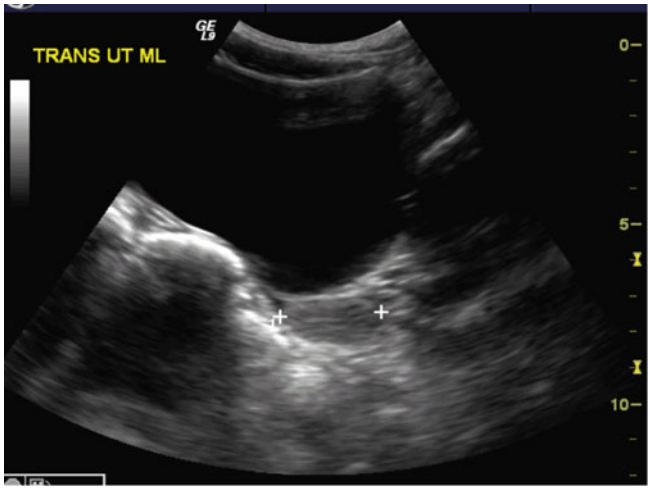

Fig. 35.4 Pelvic ultrasound. Bone age was 19 years

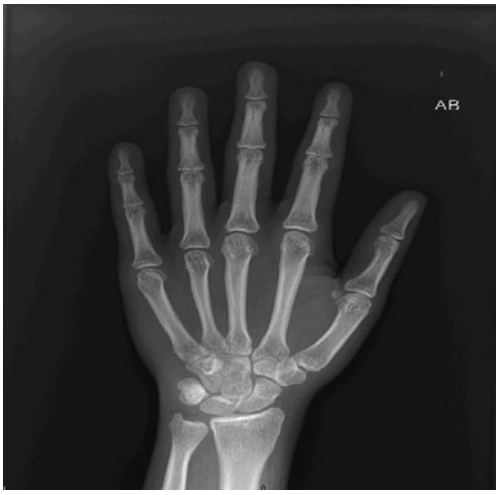

Fig. 35.5 Bone age. Laboratory analysis

Fig. 35.6 Laboratory results

Testosterone	280 ng/dl
LH	<0.2 mIU/L
FSH	<0.7 mIU/L
17 OH Progesterone	12,546 ng/dL (<200)
DHEAS	523 mcg/dL (<138)
Androstenedione	1465 ng/dL (<210)
AM Cortisol	1 mcg/dL (4–20)
Na	144 mmol/L (135–146)
K	4.5 mmol/L (3.8-5.1)
Karyotype	46, XX

Diagnosis

The patient is a genetic female with untreated congenital adrenal hyperplasia secondary to 21-hydroxylase deficiency of the simple virilizing form.

Clinical Course

Patient was started on glucocorticoid replacement and leuprolide acetate to prevent ovarian activation and feminization. Intense psychological therapy was implemented to assess gender identity and prepare the patient to participate in the decision making regarding sex assignment.

Follow-up levels were 17-OHP 629 ng/dL, androstenedione 46 ng/dL, and testosterone <10 ng/dL, after initiation of hydrocortisone treatment.

Discussion

Congenital adrenal hyperplasia (CAH) is a family of inherited disorders of adrenal steroidogenesis transmitted in an autosomal recessive fashion. The defects result in absence or decreased synthesis of cortisol from its cholesterol precursor, resulting in a reduced negative feedback with increased ACTH secretion and as a consequence overproduction of adrenal androgens. In a female fetus this leads to virilization of the external genitalia, which could range from mild to severe. Ninety percent of cases are due to 21-hydroxylase deficiency and three different variants have been recognized: classical salt wasting, simple virilizing, and non-classical or late-onset 21-hydroxylase deficiency. Our patient has the simple virilizing form.

Treatment of CAH of the simple virilizing type typically is straightforward with replacement of glucocorticoids and feminizing genitoplasty, preserving normal reproductive function in females. However, the fact that the diagnosis was not made

until our patient was 11 years old presented extraordinary challenges in management [1, 2]. Feminizing genitoplasty would preserve reproductive function and align phenotypic sex with genetic sex but override a well-established gender identity. On the other hand, virilizing genitoplasty would require hysterectomy, oophorectomy, and major genital reconstruction to make a functional phallic urethra and would remove all reproductive capacity [3]. The child has a very strong male identity and even shows sexual interest in girls [4, 5]. The patient has expressed concerns about his genitalia being different and is anxious to have surgery to allow normal male appearance and be able to urinate while standing. Unfortunately, all linear growth has been completed with an adult height of only 154.8 cm (60.9 in.). After extensive discussions with family, psychology, and our team the child is receiving treatment with glucocorticoid replacement and with monthly leuprolide acetate to suppress ovarian function until a decision regarding definitive sex assignment is made with more mature participation by the patient. Psychological evaluation and supportive care continues in preparation for disclosure of diagnosis once the child is old enough to participate in sex assignment decision.

Follow-up 11 months later shows a reasonably well-adjusted child with a continued strong male gender identity.

Conclusion

Late diagnosis of unrecognized 46,XX simple virilizing CAH raised as males presents a management challenge for pediatric endocrinologists. The ability to temporarily suppress gonadal axis affords everyone, especially the child, the opportunity to participate in life-changing decisions at a more mature time of development.

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Chapter 36
Report of Fertility in a Woman with a Predominantly 46,XY Karyotype in a Family with Multiple Disorders of Sexual Development

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Context

We report herein a remarkable family in which the mother of a woman with 46,XY complete gonadal dysgenesis was found to have a 46,XY karyotype in peripheral lymphocytes, mosaicism in cultured skin fibroblasts (80% 46,XY and 20% 45,X), and a predominantly 46,XY karyotype in the ovary (95% 46,XY and 5% 45,X).

Patients

A 46,XY mother who developed as a normal woman underwent spontaneous puberty, reached menarche, menstruated regularly, experienced two unassisted pregnancies, and gave birth to a 46,XY daughter with complete gonadal dysgenesis.

Results

Evaluation of the Y chromosome in the daughter and both parents revealed that the daughter inherited her Y chromosome from her father. Molecular analysis of the genes *SOX9*, *SF1*, *DMRT1*, *DMRT3*, *TSPYL*, *BPESC1*, *DHH*, *WNT4*, *SRY*, and *DAX1* revealed normal coding sequences in both the mother and daughter. An extensive family pedigree across four generations revealed multiple other family members with ambiguous genitalia and infertility in both phenotypic males and females and the mode of inheritance of the phenotype was strongly suggestive of X-linkage.

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Conclusions

The range of phenotypes observed in this unique family suggests that there may be transmission of a mutation in a novel sex-determining gene or in a gene that predisposes to chromosomal mosaicism.

Subject Index

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