



Disorders of
Sex Development in Indonesia:
Natural course and the implications
of a stepwise multidisciplinary approach

Achmad Zulfa Juniarto

Stellingen/Propositions

Disorders of Sex Development in Indonesia: Natural course and the implications of a stepwise multidisciplinary approach

1. In the Indonesian community the etiological spectrum of Disorders of Sex Development (DSD) is broad, 46,XYDSD being most common in contrast to observations in Western societies. (this thesis)
2. Determination of salivary androstenedione and 17-hydroxy progesterone levels is a useful and inexpensive alternative to measurements of those hormones in serum for monitoring of treatment of patients with congenital adrenal hyperplasia (this thesis)
3. The incidence of Germ Cell Cancer (GCC) is low in most Asian populations, while it is similarly high in Caucasian and Indonesian DSD patients indicating DSD to be a dominant risk factor for GCC (this thesis)
4. The full implementations of the current consensus statement on DSD (Hughes I et al 2006) in a resource – poor environment is very difficult and necessitates a tailored approach.(this thesis)
5. There is an urgent need for education of primary health care workers in Indonesia on recognition of DSD and urgent clinical assessment of DSD patients in order to prevent morbidity and mortality.(this thesis)
6. Social and community support and the existence of a support group are essential to help individuals with DSD to cope with social stigmatization and the prospect of poor quality of life.
7. The development of a robust and secure e-consultation service within the international community with the objective of providing expert opinion on a worldwide basis will broaden access to healthcare for all patients with rare diseases.
8. Cultural differences between the various tribes in Indonesia pose difficulties in handling and follow-up of patients.
9. Free availability of gluco- and mineralocorticoids is mandatory for a satisfactory treatment of Indonesian children suffering from adrenal insufficiency such as congenital adrenal hyperplasia.
10. The best of people are those that bring most benefit to the rest of mankind. (Al Hadits)
11. No one makes a lock without a key, that's why The one won't give you problems without solutions and diseases without cure.

Disorders of Sex Development in Indonesia: Natural course and the implications of a stepwise multidisciplinary approach

Stoornissen van seks ontwikkeling in Indonesië:
Natuurlijk beloop en de gevolgen van een stapsgewijze multidisciplinaire aanpak

Disorders of Sex Development in Indonesia: Natural course and the implications of a stepwise multidisciplinary approach

Stoornissen van seks ontwikkeling in Indonesië:
Natuurlijk beloop en de gevolgen van een stapsgewijze multidisciplinaire aanpak

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

15 January 2014 om 09.30 uur

Achmad Zulfa Juniarto
geboren te Semarang Indonesie



Promotiecommissie:

Promotores

Prof.dr. S.L.S. Drop
Prof.dr. F.H. de Jong
Prof.dr. S.M.H. Faradz

Overige leden:

Prof.dr. A.C.S. Hokken-Koelega
Prof.dr. S.W. J. Lamberts
Prof.dr.ir. A.P.N. Themmen

“The best of men are the most useful for others”
Hadith

For my family and teachers

Content

Chapter 1	9
General Introduction	
Chapter 2	15
Literature Review	
I. Physiology of Sex Development	
II. Disorder of Sex Development	
III. General Principles of DSD Management	
Chapter 3	57
Application of the new Classification on Patients with a Disorder of Sex Development (DSD) in Indonesia	
Chapter 4	79
Clinical evaluation of 286 patients with a Disorder of Sex Development (DSD) from Indonesiain relation to phenotype and genotype	
Chapter 5	105
Functional analysis of novel Androgen Receptor mutations in a unique cohort of Indonesian DSD patients	
Chapter 6	123
Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients	
Chapter 7	143
Correlation between androstenedione and 17-hydroxyprogesterone levels i saliva and plasma in treated patients with congenital adrenal hyperplasia.	
Chapter 8	155
Salivary 17-hydroxyprogesterone (17-OHP) and androstenedione in monitoring efficacy of treatment among Indonesian Congenital Adrenal Hyperplasia Patients	
Chapter 9	169
Virilization due to Androgen Hypersecretion in a Patient with an Ovarian Leydig Cell Tumor: Diagnostic and Psychosocial Implications	
Chapter 10	181
Gonadal malignancy in 13 consecutive collected patients with disorders of sex development (DSD) from Semarang (Indonesia)	
Chapter 11	197
General Discussion and Future Organization of Management on Disorder of Sex Development in Indonesia	
Chapter 12	209
Summary (in English and Bahasa Indonesia)	
Chapter 13	219
Acknowledgment	
Curriculum Vitae	222
List of Publications	223
PhD Portfolio	225
Abbreviations	226

Chapter 1

General Introduction

When a child is born, the question most often being asked is about its gender. Gender, whether male or female, affects almost every aspect of our lives. How profound the distinction between males and females is, and how these differences come about, are seemingly simple questions, however, are demanding a complex answers.

The process of development of the differences between males and females from an undifferentiated zygote is called sexual differentiation. As male and female individuals develop from zygotes into foetuses, infants, children, adolescents and eventually into adults, differentiation of sex and gender also develop at many levels: chromosomes, genes, gonads, hormones, anatomy, psychology, and social behaviours.

The purpose of sexual differentiation or the development of any male or female specific physical or behavioural characteristic is to equip the organism with the necessary anatomy and physiology to facilitate sexual reproduction.

Differences in sex may be absolute or may become only obvious by statistical analysis. Some aspects of development are typically sex-dichotomous characterizing one sex only. Examples of sex-dichotomous differences are the sex specific genital organs such as ovaries, uterus or phallic urethra. In contrast, sex-dimorphic differences may be mainly statistical differences, a matter of degree (e.g., size of phallus) with overlap between male and female populations. Nonetheless in the human, even sex-dichotomous differences are not an absolute matter. There are individuals who demonstrate exceptions, for example: males with a uterus, or phenotypic females with an XY karyotype, and those who exhibit biological and/ or behavioural characteristics of both sexes.

The very early phase of human differentiation seems to be quite similar in males and females. During the first weeks of life, the embryo has no anatomic or hormonal sex and can only be differentiated by karyotype. Specific genes initiate gonadal differentiation, gonads produce different hormones leading to anatomic differences as well as psychological and behavioural differences, some of which are innate while others are socially induced.

Disorders of Sex Development (DSD) in Indonesia

Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal or anatomical sex is atypical, and where previously referred to as intersex conditions (1). A new classification of DSD was proposed following a consensus meeting in Chicago in 2005. The primary root of this classification is based on the chromosomal constitution consisting of three main groups, namely; 46,XY DSD, 46,XX DSD, and sex chromosome DSD. Although this classification has its limitations and has been the subject of intensive discussions, it is considered less offensive than the use of different earlier terminologies and it prevents misunderstanding.

Babies born with DSD or children presenting with DSD at a later age are a challenge for both parents and physicians. Integrated handling, careful decision-making and counselling are indispensable for such patients.

In Indonesia, patients presenting with DSD most often do not receive a diagnostic evaluation and options for medical and surgical treatment are limited. A standardized management protocol for patients is not yet available. This makes the management of patients with DSD highly variable and dependent on the experience of the experts and on facilities available in a particular health centre.

The Sexual Adjustment Team (Tim Penyesuaian Kelamin) of the Faculty of Medicine, Diponegoro University-Kariadi Hospital in Semarang, Indonesia is one of six hospital based teams appointed by the Indonesian Government to handle patients with DSD. Since its founding in 1989, this team has been receiving patients with genital abnormalities from almost all over Indonesia.

The multidisciplinary team consist of specialists from various departments: paediatric endocrinology, urology, andrology, obstetric and gynaecology, genetics, psychology, psychiatry, social services, law and ethics. Significant progress has been made since the collaboration with the gender team of the Erasmus Medical Centre Rotterdam, The Netherlands starting in 2004. Out of the total patient number of 583 referred since 1989 not less than 347 patients were evaluated in the period 2004-2010. Due to inclusion criteria of the research, 286 of these patients were included in the present study.

Scope of the thesis

The final goal of management of DSD is to provide appropriate gender assignment and to optimize the quality of life of the patients. In the process of gender assignment of DSD patients many factors, both related to the patient him or herself and to the psycho-social environment of the patient should be taken into consideration.

For all of these reasons a stepwise diagnostic evaluation of patients with DSD referred to the Sexual Adjustment Team of the Faculty of Medicine, Diponegoro University-Kariadi Hospital in Semarang, Indonesia was initiated. This thesis reports not only on results of this evaluation but also on various aspects of management of DSD notably the management of congenital adrenal hyperplasia (CAH) and on gonadal tumor risk of patients with DSD. Furthermore, since in Indonesia, options for treatment of DSD are limited and often patients are referred at a later age, it was possible to study the natural course of DSD.

It is well recognised that DSD has a great impact on psychological and psychosexual development. Separately but in parallel with this thesis Mrs. Annastasia Ediati has made a study of these aspects focussing on the psychological and social aspects of DSD: gender development, psychopathology, stigmatization, quality of life and cognitive functioning. From this study conclusions are drawn on the psychological consequences in patients with DSD which should be taken into consideration for further intervention.

Aims of the study

The aims of the various studies reported in this thesis can be summarized as follows:

A. Stepwise diagnostic evaluation of a cohort of patients with DSD

1. Diagnostic spectrum

- What is the diagnostic spectrum of DSD in Indonesia based on clinical and hormonal data?
- What is the spectrum of gene mutations within each of the 3 diagnostic DSD categories?

- Are there any novel mutations in the well-known loci or other loci in suspected genes?

2. Value of clinical and laboratory parameters

- Which clinical and laboratory parameters are most contributory in the diagnostic process?

B. Congenital Adrenal Hyperplasia, diagnosis and management

- What is the clinical spectrum of CAH in Indonesia?
- What is the spectrum of gene mutations among CAH patients in Indonesia?
- How should CAH patients be treated comprehensively?

C. Gonadal tumor risk

- Does Germ Cell Cancer (GCC) occur in the Indonesian DSD population and if so what types of gonadal cancer can be found?
- What is the gonadal cancer risk per diagnostic category?
- Is it safe for certain diagnostic groups to leave the gonads in place?

D. Management of DSD

- What is the outcome of the implementation of comprehensive management of DSD in Indonesia?
- What are essential requirements for the management of DSD?

Reference

1. Hughes IA, Houk C, Ahmed SF, Lee PA. Consensus statement on management of intersex disorders. *J Pediatr Urol.* 2006 Jun;2(3):148-62.

CHAPTER 2

Disorders of Sex Development

A Literature Review

I. PHYSIOLOGY OF SEX DEVELOPMENT

As one of the most important physiological processes, reproduction leads to the production of new organisms and the perpetuation of the species. Effective reproduction requires a normal development of the sex organs which is strongly influenced by genetic composition and gonadal development.

A. CHROMOSOMAL SEX

Genetic sex, indicated by karyotype, is the chromosomal basis of sex determination and defines the sex-determination system. In man, the normal number of chromosomes is 46, 44 of which are autosomes while 2 distinct chromosomes are acknowledged as the sex chromosomes which define the sex of an organism and various sex-related characteristics. An individual normally has a single pair of sex chromosomes in each cell where males have one Y chromosome and one X chromosome while females have two X chromosomes, making females the homogametic sex. However, other karyotypes do exist.

During meiosis, or the process of gamete formation, the autosomal chromosomes are arranged in arrays and exchange of genetic material between the members of each pair is possible by recombination. In the sex chromosomes, this process is restricted to small regions on the distal ends of the X and the Y, which are called the 'pseudoautosomal' regions.

Nowadays, it is generally accepted that the X and Y chromosomes have evolved from an ancestral pair of homologous autosomes. The Y chromosome is considered to have evolved sometime between 170 and 310 million years ago when a sex-determining locus arose on one of the proto-sex chromosomes which led to the accumulation of other sex-specific alleles nearby. This resulted in deletion of large parts of the Y chromosome, with the shrinking of this chromosome (1). One might think that the Y chromosome would sooner or later lose all homology with the X. However, mice, and humans appear to possess a vital requirement for the preservation of X and Y pairing for the meiotic process and XY chromosomal segregation during spermatogenesis (2).

A.1. X chromosome

The sex chromosomes form one of the 23 pairs of chromosomes in each human cell. Named for its distinctive characteristics by early researchers, the X chromosome was the first of the two sex chromosomes to be described. The other sex chromosome, which was discovered later, was named Y chromosome, representing the next letter in the alphabet. The X chromosome spans about 165 million DNA building blocks (base pairs), and represents approximately 5 percent of the total DNA. It is likely to contain between 900 and 1.500 genes which have a variety of roles in the body. Female individuals have two X chromosomes (one from each parent) while males inherit a single maternal X chromosome. Gene expression on one of the X chromosomes in female somatic cells is deactivated early in development by the process of X-inactivation and this chromosome remains inactive thereafter (3). This X-inactivation warrants that females have one functional copy of the X chromosome in each body cell similar to males. In normal females, due to the random nature of X –inactivation, the X chromosome inherited from the mother may be active in some cells and that inherited from the father in other cells.

A.2. Y chromosome

As one of the two sex chromosomes in the human, the Y chromosome spans about 60 million base pairs and represents almost 2 percent of the total DNA in cells. It contains about 50 or 52 genes (1, 4). The genes on this chromosome are involved in male sex determination and development, which is stimulated by the expression of the *SRY* gene, a testis-determining factor. As one of the important genes related to male fertility, the *SRY* gene is placed in the non-recombining portion of the Y chromosome (NRY), a specific region that encompasses 95% of the human Y chromosome (5). Other genes on the Y chromosome are also important for male fertility.

It is expected that at least 27 distinct proteins or protein families are encoded by the NRY region. Most of these contribute to sex determination and specific male functions e.g. spermatogenesis (6).

Defects or deletions of the *SRY* gene are likely to be the cause of the clinical picture of XY females in 15% of the cases. In contrast, males with an XX karyotype usually present with a tiny piece of the Y chromosome, containing the *SRY* gene, added to the X chromosome. This sex reversal is often associated with other developmental malformations (7).

Abnormalities in the sex chromosomes will lead to disorders of sex development. The most widely known sex chromosomal abnormalities are Turner syndrome (45,X) and Klinefelter Syndrome (47,XXY). Although both abnormalities represent variations in sexual differentiation, they are often not categorized as intersex conditions, since there is usually no discordance between elements of sexual differentiation. Variant karyotypes of these syndromes are also possible, in which different cells from the same individual have different karyotypes (mosaicism) such as 45,X/46,XY; 45,X/47,XYY.

B. PHENOTYPIC SEXUAL DIFFERENTIATION

Determined by endocrine influences, phenotypic sex includes gonadal sex, defined by the presence of ovarian or testicular gonads and morphological sex, determined by the morphology of the external and internal genitalia.

Hormones secreted by the gonads determine the secondary sex characteristics, which will affect the physical phenotype outside the gonads. A female body possesses a vagina, cervix, uterus, oviducts, and mammary glands, and a male body features a penis, seminal vesicles, and a prostate gland (8).

B. 1. Sexual differentiation of the internal genitalia

The first phase of gonadal development is marked by the appearance of the indifferent, bipotential gonad, or genital ridge which is identical in males and females and remains so until the 7th week of human gestation, when the migration of the primordial germ cells begins.

The next stage is the development of the indifferent gonad into a testis or an ovary (9). The bipotential gonad consists of four presumptive cell lineages: the germ cells (which have an extra-embryonic origin) and three types of somatic cells.

These somatic elements include the supporting cell precursors, which will create Sertoli cells in the male or granulosa cells in the female; secondly the steroidogenic precursors, which differentiate into Leydig cells (male) and theca cells (female), and finally the connective tissue cells which accommodate other important structures (10).

This process of gonadal sex determination marks the first step in sexual development and includes a unique decision to make a testis or an ovary out of a bipotential primordial gonad. The differentiation process of the gonad is the primary step on which all other aspects of sexual differentiation depend. This has been known as the 'central dogma' of the sexual differentiation process (11).

In male fetuses, at the 7th week of gestation the testicular development begins. A vital event in this process is marked by *SRY* gene expression in cells destined to differentiate into Sertoli cells (12-14), which in turn coordinate the differentiation of all other cell types in the testis. These cells also affect spermatogenesis, which is followed by the differentiation of seminiferous tubules and the formation of Leydig cells (9, 15). Later in life, Sertoli cells will nurture the development of germ cells into sperm. Concurrent with the differentiation of Sertoli cells, the size of gonad increases significantly because of increased proliferation and migration of cells from the adjacent mesonephros. This progression occurs only in males, after the inception of *SRY* expression (16).

The development of the primordial germ cells in the testis stops at the stage of pro-spermatogonia as a result of paracrine action of mediators secreted by the Sertoli cells (17). At the same time, anti-Müllerian Hormone (AMH) is also secreted by the Sertoli cells. AMH is a member of the transforming growth factor β (TGF β) family and is also referred to as Müllerian inhibiting substance (18). It elicits male sexual differentiation by signaling via two cell-surface receptors: AMH receptors I and II (18).

Formed by invagination of a tube from the surface epithelium of the mesonephros, the Müllerian (or paramesonephric) ducts are the precursor of the female ductal system whereas the Wolffian (or mesonephric) ducts are the progenitors of the male duct system. The Müllerian duct runs parallel to the Wolffian duct in both male and female embryos (Figure 1). The

differentiation of an indifferent gonad into a testis or ovary will determine which one of the two duct systems will develop. In the human embryo, this process starts at the 7th week of gestation.

In the absence of AMH, the Müllerian ducts will develop and differentiate into oviducts, uterus, and upper third of the vagina; but on the development of male sex, AMH causes these ducts to regress instead of developing (18). At the 8th week of gestation, the masculinization process of the male fetus is initiated when Leydig cells appear in the differentiating testis and start secreting androgens. Under the influence of testosterone, the Wolffian ducts develop into epididymis, vasa deferentia and seminal vesicles(19).

There are two stages of testicular descent during sexual development. The first is caused by development of the gubernacula rather than actual movement and the testes reach the internal inguinal ring by week 24. Insulin-like Factor 3 (INSL3), produced by Leydig cells, regulates this trans-abdominal phase of descent by stimulating the development of the gubernaculum (20). In the last two months of fetal life, the second stage occurs where the testis passes through the inguinal canal to reach the scrotum. Androgens are a primary requirement for this migration and in 97% of normal infants, the testes are in scrotal position at the time of birth (19).

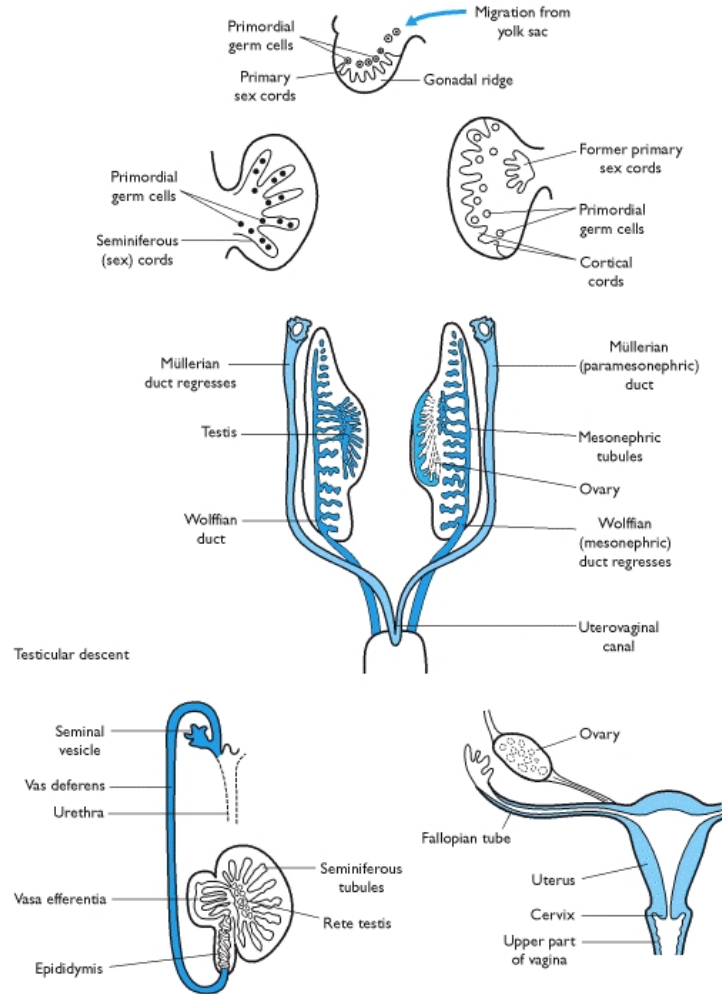


Figure 1. Schematic overview of the differentiation of the internal male (bottom left) and female (bottom right) reproductive tracts from the Wolffian and Müllerian ducts (19).

The two main roles of the ovary are firstly to produce hormones and secondly to generate mature oocytes that are capable of being fertilized and of developing into an embryo.

Surrounded by granulosa and theca cells, the ovarian follicle is the functional component of the ovary, where the oocyte matures (9). Unlike the seminiferous tubule, the functional part of the testis, the ovarian follicles commence differentiation only after birth.

The development of the ovary seems to be based upon a number of genes and transcription factors: *DAX1* and *WNT4* are known as genes that are likely to suppress testicular development (21, 22).

B. 2. Sexual differentiation of the external genitalia

Similar to the development of the gonads, but unlike that of the internal reproductive tracts, the external genitalia of both female and male are formed from the same initially identical structures: The genital or labioscrotal swelling, the genital or urethral folds, the genital tubercle and the urogenital sinus.

Testosterone is essential in the development of the male external phenotype. However, to allow normal differentiation, it must be converted into its active metabolite, 5α -dihydrotestosterone (DHT) within the cells of the anlagen by the enzyme steroid 5α -reductase type 2 (SRD5A2) (19).

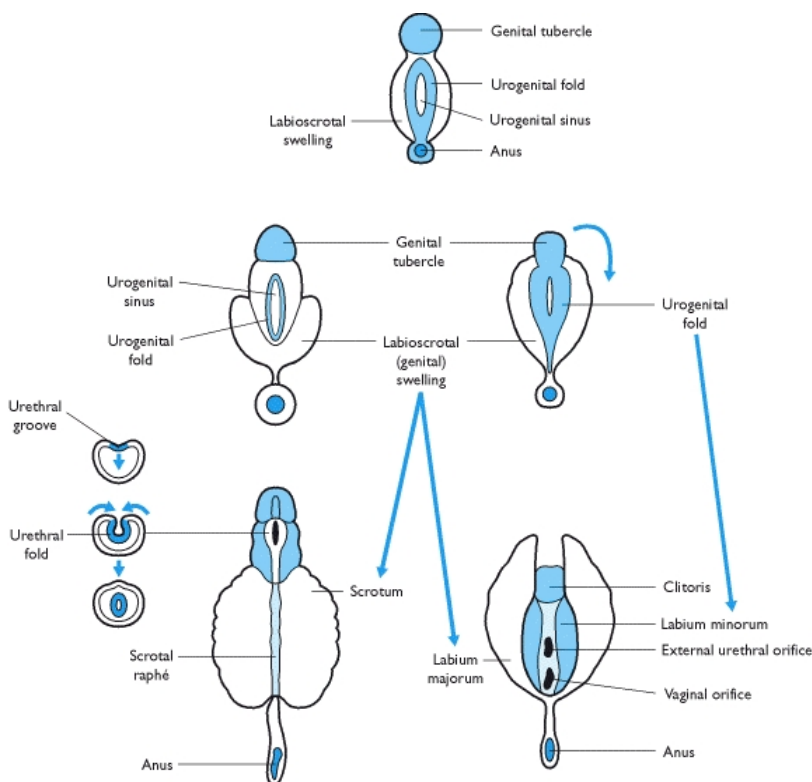


Figure 2. Diagram of the development of the male (bottom left) and female (bottom right) external genitalia.

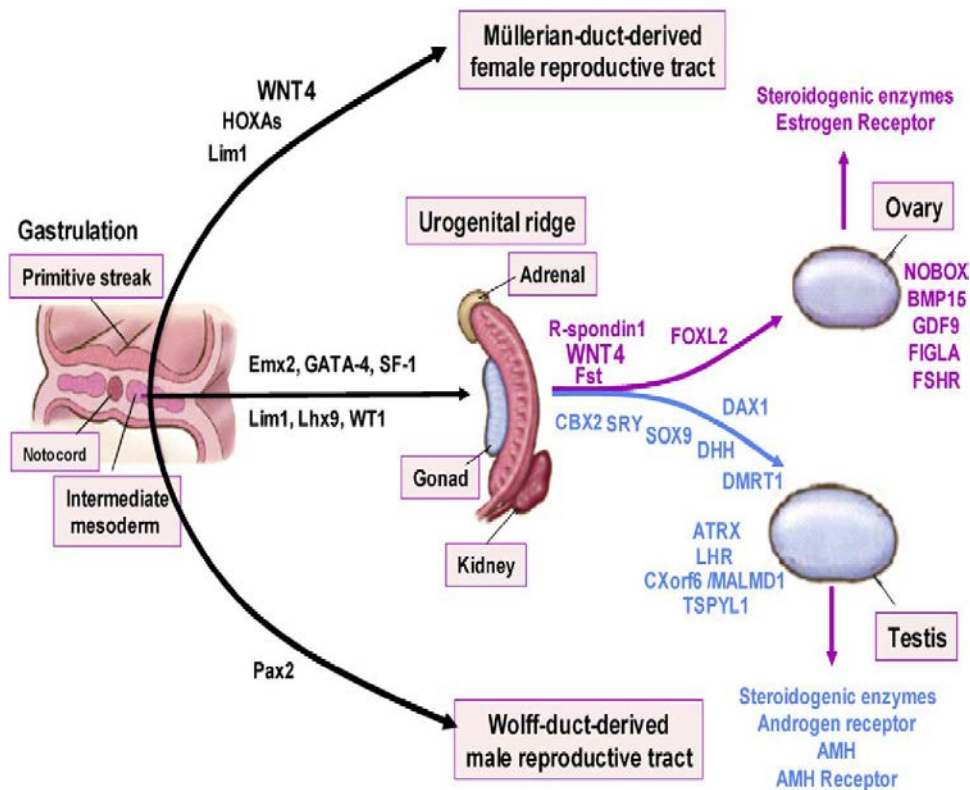
In males, the genital tubercle grows and forms the shaft and glans of the penis. The urogenital sinus becomes continuous with a groove that develops on the caudal face of the genital tubercle and this groove closes to become the penile part of the urethra while the fused urogenital folds enclosing the sinus become the prostate part of the urethra. In the most distal part of the penis, invagination of a cord of epithelial cells covering the glans meets the penile urethra and when this cord canalizes the formation of the urethra is complete. The line of fusion along the urethra and scrotum is called the raphé. The labioscrotal folds form the scrotum (19).

In the female fetus, the genital tubercle folding inferiorly will form the clitoris, whereas the urogenital folds become the labia minora and the genital swellings develop into the labia majora. The vagina and the urethra open into the vestibule of the urogenital sinus. Until 15 weeks of gestation the size of external genitalia of the male and female is the same. In the male the growth of the external genitalia is not to begin until the last two thirds of pregnancy which will be followed by the descent of the testes into the scrotal sac (19).

C. GENES INVOLVED IN SEX DETERMINATION AND DIFFERENTIATION

The process of an undifferentiated gonad passing through a complicated series of steps to develop into a testis or an ovary is called the differentiation of the gonad and depends on the sex-determining pathway succeeding in determining the sex (23).

Numerous investigators have tried to discover genes that are vital in sex determination and differentiation. Their work led to the conclusion that a number of genes are essential for normal sex differentiation. Later studies revealed that mutations of some of these genes perturb the normal differentiation process and can be causative for various forms of Disorders of Sex Development (DSD).



Modified from McLaughlin, NEJM 2004, 350: 367

Figure 5. Summary of genes that code for transcription factors that induce differentiation of a bipotential gonad into either a testis or an ovary and subsequent differentiation of the internal reproductive tracts (24)

C.1. Undifferentiated state (bipotential)

Pax2 : *Pax2* is a transcriptional regulator of the paired-box family and is extensively expressed in the development of ductal and mesenchymal components of the urogenital system (24). Expression of *Pax2* within the mesonephros is limited to the Wolffian ducts and mesonephric tubules. *Pax2* homozygous mutant newborn mice are found to be lacking kidneys, ureters and genital tracts. These defects might be associated with dysgenesis of both ductal and mesenchymal parts of the developing urogenital system (25). In either sex in *Pax2*^{-/-} mice, no defects in gonadal development have been found, indicating that the formation of the mesonephric tubules is not a prerequisite for gonadal development. Genetic defects in *PAX2* in humans are known the in renal-coloboma syndrome, without genitourinary abnormalities (9, 24).

Emx2 : *Emx2* (Empty spiracles homolog 2) encodes a homeobox-containing transcription factor that is a homolog of the *Drosophila* head gap “empty spiracles” gene and is expressed in the developing dorsal telencephalon and in the epithelial parts of the urogenital system (26). In *Emx2*^{-/-} mice, the migration of the primordial germ cells (PGCs) occurs as normal. However, the first stage of the gonadal development which is marked by the thickening of the coelomic epithelium, is poor: the Wolffian ducts degenerate and the Müllerian ducts never form. Thus the mutants have neither gonads nor genital tract (27). Mutations in *EMX2* are a rare cause of schizencephaly. Somatic mutations in *EMX2* have been found in endometrial carcinomas (28). However, there is no report on defects of human sex development due to *EMX2* mutations until now.

Lhx9 : At embryonic day 9.5, the expression of the gene *Lhx9*, a member of the LIM homeobox gene family, commences in the urogenital ridges of male mice. It is also localized in the interstitial region during morphological differentiation. In mice lacking functional *Lhx9*, somatic cells of the genital ridge are somehow unable to proliferate and a discrete gonad fails to form despite normal migration of germ cells. The steroidogenic factor 1 (*Sf1*) expression which is essential for gonadogenesis, is reduced to minimal levels in the *Lhx9*-deficient genital ridge. Biochemical analysis shows that *Lhx9* can attach directly to the *Sf1* promoter and has an additive effect to its *Wt1*-induced activation in vitro (29). These results indicate that *Lhx9* may be an initial point for the coordination of a network of gene regulation during the undifferentiated stage of gonad development which may lie upstream of *Sf1* in a developmental cascade. The absence of testosterone and AMH will make a genetically male mouse to have the phenotype of a female. *Lhx9* mutants do not show additional major developmental defects. Despite the fact that *LHX9* mutations may underlie certain forms of isolated gonadal agenesis in humans, no such mutation has been found to date (30).

WT1 : The *WT1* gene encodes a zinc finger DNA-binding protein that is expressed widely throughout the urogenital ridge, in the mesonephros, the kidney, and the gonad (9).

Both mutation analysis in human patients and genetic experiments in mice indicate that *WT1* plays an important role in development as well as in tumor suppression. In the development of the gonad, *Wt1* is expressed in both the coelomic epithelial cell layers and the developing Sertoli cells in males and in granulosa cells in females. Although the gonadal primordium is visible in 11 dpc *Wt1*^{-/-} mutant mouse embryos, it degenerates afterwards due to increased apoptosis (9). Because the gonads and adrenal cortex share a common primordium, the adrenal glands are also affected in *Wt1*^{-/-} mice. Expression of *Sf1* and expression of *Wt1* in the gonad begins concurrently and might be interdependent (31). In vitro, a number of genes including *Sf1*, are activated by *Wt1* and in research with transgenic mice, it was found that *Sf1* is likely to be a genuine *Wt1* target (29). *Wt1* might also stimulate an *Sf1*-independent pathway necessary for adrenogenital survival, including the anti-apoptotic factor *Bcl2* (24). In gonad formation, *WNT-4*, *DAX 1* and AMH also appear to be activated by WT1. It appears that *WT1*'s function as a transcriptional activator is vital for the survival and differentiation process of gonadal cells (9). As an influencing gene in male sexual development in mouse and man, *Dax1* was proposed to be a repressor of the *AMH* promoter that could remove *Wt1* from its complex with *Sf1* (24, 32). Mutations in the *WT1* gene have been discovered in patients with a variety of disorders such as Wilms' tumor and isolated diffuse mesangial sclerosis (IDMS), as well as in complex diseases such as aniridia and genito-urinary anomalies. In addition, *WT1* mutations have been described in mental retardation syndrome (WAGR syndrome), Frasier syndrome, Denys–Drash syndrome (DDS) and Meacham syndrome (24).

GATA-4 : *GATA-4*, a member of the GATA family of transcription factors, is found in the gonad and possibly plays a role as a regulator of gonadal gene expression. It is vital for the establishment of Sertoli cell localization as well as for proper testis cord formation. *GATA-4* is found in the developing somatic cell lineages specifically (Sertoli cells in testis and granulosa cells in ovary) but not in primordial germ cells (23, 24).

C.1. a. Testes

SRY : *SRY* belongs to a large family of nuclear proteins characterized by a DNA-binding domain known as high mobility group (HMG) box, which functions as a transcription factor (33). To activate testis determination and consequent development of male sexual characteristics, *SRY* alone is necessary and sufficient. Whenever *SRY* is impaired or non-existent, ultimately an ovary will be formed. In the mouse embryo, a restricted and limited spatiotemporal profile of expression of *Sry* has been demonstrated in the precursors of Sertoli cells of the XY gonad (34). Previous literature shows that *Sry* is expressed initially around 10.5 days post coitum (dpc) in the mouse, soon after the genital ridges appear, and reaching maximal levels of expression at 11.5 dpc shortly after 12.5 dpc expression begins to regress. *SRY* obviously instigates a molecular switch to perform a male-specific cascade of molecular events. However, for these events to surface, continued expression of *SRY* is not required (24). *SRY* appears to affect the expression of one single gene, *SOX9*, which is then rapidly enhanced by positive regulatory loops. Subsequently, *SOX9* induces Sertoli cell formation which leads to testis differentiation. Whenever *SRY* fails to act timely, or when it is totally absent, *SOX9* expression is shut down and follicular cells start to develop resulting in ovarian differentiation. The condition of 46,XY DSD caused by an *SRY* gene mutation leads to pure gonadal dysgenesis, originally referred to as 46,XY, female with gonadal dysgenesis (35).

SOX9 : *SOX9* is a member of the *SOX* family of transcription factors and is proposed to be the best candidate for a direct *SRY* target gene for the following reasons (36). First, *SOX9* is initially expressed at low levels in the indifferent gonad, and gradually expression is increasing until it becomes up-regulated in the Sertoli cells immediately after the start of *SRY* expression, whereas it is down-regulated in the ovary. *SOX9* expression is preserved in the Sertoli cells until birth.

Second, *SRY*-positive cells exclusively become *SOX9*-positive Sertoli cells. Third, in most cases of campomelic dysplasia XY patients have male to female sex reversal. It is well known that the sole factor that accounts for this human skeletal malformation syndrome is a heterozygous

mutation of *SOX9*. In the male sexual development process, *SOX9* is not only necessary but also sufficient (9). *SOX9* expression in the genital ridge is not entirely reliant on *SRY*, the normal gonadal *SOX9* transcriptional regulation consists of an *SRY*-independent initiation; an *SRY*-dependent up-regulation and finally an *SRY*-independent maintenance in adult life (24). *SFI* is a suitable candidate to instigate or sensitize *SOX9* expression further. It initiates a low level of expression in the genital ridge in both sexes at 10.5 dpc in the mouse. *SFI* also activates *SRY* expression, presumably jointly with other factors such as *WT1* (24). By joint action of *SRY* and *SFI*, *SOX9* expression is up-regulated. In contrast, it is down-regulated in the female. This down-regulation tends to be an active process, caused by the presence of one or more currently unknown repressors. When the transient expression of *SRY* is finished, high levels of *SOX9* are controlled by its direct auto-regulation and via *FGF9* signaling (24). *DAX1* and *FGF9* are also affecting the up-regulation of *SOX9* expression in pathways parallel to *SRY*. Up until now, there is only limited information on the genes which are stimulated by *SOX9* and are important in testis determination. One study revealed that joint effects of *Sox9* and *Sfi* are responsible for the regulation of anti-Müllerian hormone (*Amh*) gene expression. However, where *Sox9* is vital in the initiation of *Amh* transcription, *Sfi* appears to act as a quantitative regulator of the level of *Amh* transcription (37).

SFI/NR5A1 : The expression of *SFI* in the developing urogenital ridge, hypothalamus, and the anterior pituitary gland, emphasizes its importance for the development of the hypothalamic-pituitary-gonadal axis. After the completion of sexual differentiation, *SFI* can be detected in steroidogenic (Leydig) and non-steroidogenic (Sertoli) cells in the testis (9). The pattern of *SFI* expression, starting in the human adrenal gland and bipotential gonad at 32-33 dpc, is consistent with its pivotal task, which is to regulate adrenal development, and gonadal determination and differentiation. In the hypothalamic-pituitary system it controls reproduction and metabolism (24). From approximately day 42 onwards, *SFI* expression in the human is consistently preserved in the somatic cells of the early testis. Jointly with *SRY*, *SFI* plays a critical role in supporting *SOX9* expression (24, 36). In Sertoli cells of the developing male fetus, *SFI* is involved in regression of Müllerian structures due to activation of *AMH* expression from around

7 weeks of gestation. In Leydig cells, *SFI* activates the expression of steroidogenic enzyme systems from 8 weeks of gestation, which leads to androgenisation of the external genitalia (24). *SFI* can also be detected in somatic cells (granulosa and theca cells) of the female adult ovary (38). The phenotypic spectrum caused by mutation in the human *SFI/NR5A1* gene ranges from complete testicular dysgenesis with Müllerian structures, through individuals with mild clitoromegaly or genital ambiguity to severe penoscrotal hypospadias or anorchia (39, 40). A recent study found that heterozygote mutations of the *SFI* gene are associated with ovarian insufficiency although it is not essential for ovarian determination (41). Like *SFI/NR5A1*, *NR5A2/LRHI* also plays an important role in induction of steroidogenic enzymes and factors. This liver receptor homolog is strongly expressed in the Leydig cells of the testis but only weakly in Sertoli cells, where *SFI* predominates (42, 43).

DAX1/NR0B1 : As an orphan member of the nuclear hormone receptor family of transcriptional regulators, *DAX1* is encoded by an X-chromosomal gene. The key model of the molecular mode of action of *DAX1* is presumably related with that of other nuclear receptors such as *SFI*, recruiting co-repressors or co-activators to the appropriate transcriptional complex, thus affecting nuclear receptor-mediated transcriptional activation (44, 45). Interaction between *DAX1* and *SFI* during gonadogenesis occurs in a similar manner as seen during steroidogenesis. A common function for *DAX1* as a repressor of *SFI* 'trans'-activation has been implied in both adrenal and gonadal tissues. Expression of *DAX1* is found in the developing adrenal gland, the gonads, the hypothalamus, and the pituitary (46). In testicular development in mice, *Dax1* is expressed in somatic cells prior to *Sry* expression, with a strong up-regulation in Sertoli cells by 12.5 dpc, which declines subsequently. Inactivating mutations in *DAX1* lead to adrenal insufficiency with glucocorticoid and mineralocorticoid deficiency. These mutations account for most patients with adrenal hypoplasia congenita (AHC) (47). Duplication of the region in the X chromosome containing *DAX1* leads to dosage-sensitive sex reversal (24, 48). Therefore, it is unexpected that loss of function in female mice had no reproductive consequences, while the testicular development in XY animals was impaired (49). The molecular mechanism underlying this testis-

promoting versus anti-testis function of *DAX1* is not clear. In gonadal differentiation of man and mice, dosage sensitivity seems to be crucial, in which a proper balance of *DAX1* levels at the right time could tip the balance in one of the two ways (50).

DMRT1: One of the characteristics found in patients with XY gonadal dysgenesis, Swyer Syndrome, is the absence of *DMRT1*, which maps to a region of chromosome 9p (51). This is supported by evidence that *DMRT1* expression in humans is detected only in the developing gonads, and at higher levels in testes compared with ovaries, usually in the late sex-determining or early testis-differentiation period (52). However, null mutant mice show only a moderately mild phenotype in postnatal testis differentiation which signifies that either *Dmrt1* is not crucial for primary sex determination or that other factors are able to compensate for its loss (53). However, results of a more recent study indicate that *DMRT1* is essential to maintain testis determination in mammals (54). Differentiation of Sertoli cells into granulosa cells can be reprogrammed by deletion of *DMRT1* in adults through activation of *FOXL2* (13).

SOX8: *SOX8* is also a member of the family of SOX proteins. The temporal and spatial expression patterns in the developing gonad are similar to those of *Sox9* and the expression is up-regulated in Sertoli cells 12 h after *Sox9*, but prior to *Amh* expression. Similar to *Sox9*, *Sox8* can attach to the *Amh* promoter and jointly with *Sfl* it activates *Amh* expression in vitro (55).

FGF9: *FGF9* is a signaling molecule secreted by Sertoli cells, an essential factor in influencing Sertoli cell differentiation. Broadly expressed in the mouse embryo with a sex-specific pattern in the developing gonad, *Fgf9* can be found from 11.5 dpc in male and female mouse fetuses (56). Later, from 12.5 dpc onwards, it is expressed only in the testis cords of the XY gonad, but not in the XX gonad or the mesonephros of both sexes. XY sex reversal is mostly believed to be due to a reduced proliferation rate, presumably combined with impaired differentiation of pre-Sertoli cells, where the threshold number required to direct the differentiation of other cells in the gonad is not sufficient in completing the testis differentiation process. *Fgf9* receptor type 2 (*Fgfr2*) is considered a candidate testis-determining gene. This gene encodes a potential receptor for *Fgf9*

during male sex determination and knockout mice of the other FGF receptors such as *Fgfr1*, *Fgfr3* and *Fgfr4* show no obvious gonadal phenotype whereas FGFR2 protein shows male-specific localization in the nuclei of Sertoli cell precursors during the period between 11.0-12.5 dpc (56).

DHH: Desert hedgehog (*DHH*) appears to have a critical role in regulating Leydig and peritubular myoid cell function. Emerging at 11.5 dpc in somatic cells of the testes in XY mice, its expression continues afterwards in Sertoli cells. However, in the developing ovary, no expression is detected at any stage. *Dhh* is attached to its receptor Patched 1 (PTCH1), the expression of which comes shortly later being under the positive control of *Dhh* in Leydig and peritubular myoid cells (57). In mice, null mutation of *Dhh* causes disrupted formation of testis cords due to abnormal peritubular tissue. Furthermore, *Dhh* appears to have an important role in Leydig cell differentiation by upregulating *Sfl* (58). Mutations of the *DHH* gene are associated with the presence of 46,XY complete pure gonadal dysgenesis in men (59).

ATRX: Diseases associated with deletions or mutations of *ATRX* are found in alpha thalassemia and mental retardation, X-linked (**ATRX**) syndromes. The involvement of *ATRX* in the development of the human testis was hypothesized because of *ATRX* gene mutations displaying varying degrees of gonadal and urogenital abnormalities (60), ranging from small testes to ambiguous external genitalia in 46,XY individuals. The biochemical role of *ATRX* still remain unclear.

CBX2/M33: Known as the murine counterpart of the *Polycomb* gene in *Drosophila*, *M33* maintains a repressed state of homeotic and other developmentally regulated genes. This is done by compressing the chromatin, preventing the binding of transcription activators. Homozygous *M33*^{-/-} mice display a male to female sex reversal in most XY animals with perfectly normal female gonads and genitalia but with invariable sterility (24, 61). It is assumed that *M33* plays a significant role in early gonadal development, i.e. before the time of sex determination. Recent research showed that *M33* is involved in the regulation of *Sfl* expression in spleen and adrenal

glands (62). It is assumed to have a similar function in gonadal development, however, its cellular roles or molecular functions are yet to be verified (9).

TSPYLI: Testis-specific protein Y-like-1 (*TSPYLI*) is a member of the TSPY-SET-NAP1L1 family of chromatin modifiers which is involved in nucleosome assembly. *TSPYLI* on chromosome 6p22 lacks introns, and it is assumed to be originating from retroposition of a *TSPY* transcript (63). Mutations in *TSPY* were found to disturb beta globin regulation and to be related to the syndrome of Sudden Infant Death with Dysgenesis of the Testes (SIDDT). SIDDT can occur in genotypic males with fetal testicular dysgenesis and ambiguous genitalia, intra-abdominal dysplastic testes, and deficient fetal testosterone production, but fusion and rugation of the gonadal sac and partial development of the penile shaft can also be found (24, 64, 65).

The sexual development in an 46,XX infant with a *TSPYLI* mutation was reported to be normal at birth. However the child developed signs of viscer-autonomic dysfunction early in life, followed by death prior to the age of 12 months due to abrupt cardio-respiratory distress. One should notice that no mutation in *TSPYLI* has been detected in cases of sudden infant death without testicular dysgenesis, indicating the testis-specific role of this protein (65, 66).

MAMLDI: Mastermind-Like Domain-Containing Protein 1 (*MAMLDI*), also called Chromosome X Open Reading Frame 6: *CXORF6*, localized on chromosome Xq28, is expressed in several tissues including fetal Sertoli and Leydig cells around the critical period for sex development (67). Most patients with a deletion of this region of chromosome X also had ambiguous genitalia. *MAMLDI* (*CXORF6*) seems to have an important role in the testosterone production around the critical period for sex development. The protein localizes to the nuclear bodies and has a transactivation function for *Hes3*, increasing testosterone production, assumed to be due to regulation of *Sfi*(68). Recent findings show that a gain of function mutation in *MAMDL1* was present in a 46,XX DSD woman with virilization (clitoromegaly, streak gonads and small uterus), indicating this protein's involvement in ovarian development (24, 69).

PGD2S: Lipocalin-type prostaglandin D2 synthase (L-PGDS) and haematopoietic prostaglandin D2 synthase (H-PGDS) are expressed in the testes and are involved in the nuclear translocation

and expression of SOX9. Knockout of *l-* and *h-Pgds* in male mice results in delayed testicular organisation and a uni- or bilateral testis migration defect. Shortly after *SRY* and SOX9 expression emerge, *Pgd2s* is expressed in mouse embryonic Sertoli cells. *Pgds* up-regulation is mediated by *SOX9*, but not by *SRY* (70). A large part of the mechanism causing testicular descent is still not understood but several factors, such as Hoxa-10, epidermal growth factor (EGF) and calcitonin gene-related peptide (CGRP), together with androgens and insulin-like factor 3 (Insl3), have been proposed to have a regulatory role (71, 72).

C.1. b. Ovaries

RSPO1: In mice, the ovarian phenotype of XX, R-spondin1 (*Rspo1*) *-/-* mutants is identical to that of *Wnt4**-/-* female mice, marked by the similar formation of coelomic vessels and the presence of functional steroidogenic cells. This implies that the ovarian phenotype in *Rspo1* *-/-* mice is at least in part because of the failing expression of *Wnt4* in XX gonads. *Wnt4**-/-* mice show adrenal or uterine abnormalities, in contrast to *Rspo1* *-/-* female mice, because of the fact that *Rspo1* is not expressed in adrenals or mesonephros. This indicates that activation of *Wnt4* in these organs is independent of *Rspo1* (73). The primary function of R-spondin1 and *WNT4* may be to synergize in XX gonadal (ovarian) development to stabilize beta-catenin (74).

WNT4: Recent findings in a mouse model contradict the theory of a passive, default pathway of ovarian development. *Wnt4* *-/-*XX mice display a partial sex reversal, indicating that *Wnt4* acts to positively regulate ovary differentiation. *Wnt4* may also stimulate the proliferation of female germ cells, as shown by the lower number of oocytes in the ovaries of these knock-out mice. Produced in ovarian somatic cells (pre-granulosa cells), *WNT4* is a member of the *WNT* family of secreted molecules acting in a paracrine manner. *WNT4* has a significant role in affecting a number of developmental changes. *Wnt4* up-regulates *Dax1*, a gene known to antagonize *Sfl*, and thereby inhibits steroidogenic enzymes (75). Initially detected in mice in the mesonephric mesenchyme and coelomic epithelium from 9.5 dpc onwards, *Wnt4* is also expressed in the mesenchyme of the indifferent gonad and the mesonephros of both sexes at 11 dpc. However, it

is downregulated in the male gonad 12 hours later. The expression is present in both the mesonephroi and in the female gonads as well as the mesenchyme surrounding the Müllerian ducts (76). It appears that *Wnt4* has several functions in sexual differentiation. It is needed in the bipotential stage for initial Müllerian duct morphogenesis in both sexes (76). *Wnt4* also is an inhibiting factor in the development of the male specific vascularization in the ovary and is vital in separation of adrenal steroidogenic cells from the male and female gonadal primordium (77).

FOXL2: *FOXL2* is a single-exon gene encoding a nuclear forkhead/winged helix (fkh) transcription factor. In murine gonads, its expression starts from 12.5 dpc in a female specific manner and its pattern is conserved between different phyla. It is first expressed in mesenchymal pregranulosa cells and subsequently in granulosa cells (78). *FOXL2* is suspected to contribute to granulosa cell differentiation and ovary maintenance because it is still strongly expressed in postnatal and adult follicular cells. However, there are only a few transcriptional targets described until now despite the significance of *FOXL2* in ovarian development and maintenance (79). In the context of the pituitary, the expression of the gonadotropin-releasing hormone (GnRH) receptor appears to be stimulated by *FOXL2* (79). Human blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) is caused by a mutation in the *FOXL2* gene. It has been proposed that in males, *SRY* (or *SOX9* itself) would repress *Z*, a repressor of the male pathway. Without *Z*, *SOX9* will be expressed, testis will develop and XX sex reversal will begin. (10, 80, 81)

C.1. c. Müllerian ducts

AMH: This hormone signals via two transmembrane receptors, type II (which is specific) and type I, (which is shared with the bone morphogenetic proteins). Produced by fetal Sertoli cells, AMH is responsible for regression of the Müllerian ducts , Mutations in *AMH* and *AMH* receptor type II (*AMHR-II*) genes will cause the uterus and Fallopian tubes to persist in males and are therefore involved in the Persistent Müllerian Duct Syndrome (82).

HOXA10: As a member of a family of conserved regulatory molecules, *HOXA10* controls aspects of morphogenesis and cell differentiation in the development of the embryo. It is vital for the development of the Müllerian tract in the embryonic period, but is also expressed in the adult uterus. *Hox* gene expression is regulated by sex steroids during embryonic life and during endometrial development in the menstrual cycle. *Emx2* and β_3 -integrin acting downstream of *Hoxa10* are likely involved in both of these developmental processes (83).

Lim1/LHX1: *Lim1/LHX1* encodes a LIM-class homeodomain transcription factor that is necessary for head and kidney development. In the developing urogenital system, *Lim1* expression is detected in the Wolffian (mesonephric) duct, the mesonephros, metanephros and fetal gonads. *Lim1* expression in the Müllerian duct is related to its formation and differentiation in females and its regression in males. Female *Lim1*-null neonates mice have ovaries; however, they lack a uterus and oviducts, indicating that *Lim1* is required for Müllerian duct epithelium formation (84). The effect of *Lim1* mutations in human have not yet been described. (85)

II. DISORDERS OF SEX DEVELOPMENT

A. Nomenclature and definition

The term Disorder of Sex Development (DSD) is used for any congenital condition associated with atypical chromosomal, gonadal or anatomical sex. In recent publications proposed subcategories are 46,XY DSD, 46,XX DSD and sex chromosome DSD. More specific terms are preferred over general terms but this depends on the availability of a further diagnostic classification, for instance based on a primary genetic defect (86).

As shown in figure 7, the primary root is based on the karyotype, the shaded row explains the disorders as the secondary root, and actual diagnoses are listed under each secondary root. The actual diagnosis is often based on the results of molecular, biochemical, or histological examination (87). Research in past decades has contributed a lot in explaining the physiology of sexual development and differentiation. This has made a significant input in understanding the mechanisms involved in gonadal maldevelopment as several genes have been discovered to be

vital in the development of male and female gonads. Mutation analysis of genes associated with these factors in patients with genital disorders has been shown to play an important role.

Figure 7. DSD classification

Sex chromosome DSD	46,XY DSD	46,XX DSD
A: 45,X (Turner Syndrome and variants)	A: Disorders of gonadal (testicular) development 1. Complete gonadal dysgenesis (Swyer syndrome)	A: Disorders of gonadal (ovarian) development 1. Ovotesticular DSD
B: 47,XXY (Klinefelter Syndrome and variants)	2. Partial gonadal dysgenesis 3. Gonadal regression 4. Ovotesticular DSD	2. Testicular DSD (e.g. <i>SRY</i> β , dup <i>SOX9</i>) 3. Gonadal dysgenesis
C: 45,X/46,XY (mixed gonadal dysgenesis, ovotesticular DSD)	B: Disorders in androgen synthesis or action 1. Androgen biosynthesis defect (e.g. 17-hydroxysteroid dehydrogenase deficiency, 5 α reductase deficiency, StAR mutations)	B: Androgen excess 1. Fetal (e.g. 21 hydroxylase deficiency, 11 β hydroxylase deficiency) 2. Fetoplacental (aromatase deficiency, POR)
D: 46,XX/46,XY (chimeric, ovotesticular DSD)	2. Defect in androgen action (e.g. CAIS, PAIS) 3. LH receptor defects (e.g. Leydig cell hypoplasia, aplasia) 4. Disorders of AMH and AMH receptor (Persistent Mullerian Duct Syndrome)	3. Maternal (luteoma, exogenous, etc.)
	C: Other (e.g. severe hypospadias, cloacal extrophy)	C: Other (e.g. cloacalextrrophy, vaginal atresia, MURCS, other syndromes)

A.1. 46,XY DSD

46,XY DSD patients are distinguished by ambiguous or undervirilized external genitalia due to incomplete intrauterine masculinization and the presence or absence of Mullerian structures. Patients with complete absence of virilization have normal female external genitalia, but due to the absence of breast development and/or to primary amenorrhea, these patients are often discovered only at a pubertal age (88).

46,XY DSD due to gonadal dysgenesis

As a genetically heterogeneous disorder with autosomal, X and Y-linked forms, gonadal dysgenesis occurs as the result of an impairment during formation of the primordia of the gonads related to the two major elements of the fetal testes, the Sertoli cells that secrete AMH and the Leydig cells that secrete testosterone. Gonadal dysgenesis covers clinical conditions with abnormal fetal gonad development which include both complete and partial forms. The characteristics of complete gonadal dysgenesis are displayed as female external and internal genitalia, lack of secondary sexual characteristics, normal or even tall stature with no Turner syndrome stigmata and bilateral dysgenetic (streak) gonads. The partial form includes conditions where external genitalia may be ambiguous with impaired testicular development also resulting in presence or absence of Müllerian structures. A similar phenotype can also result from a mosaic 45,X/46,XY karyotype (88). 46,XY gonadal dysgenesis is a heterogeneous disorder, which can be caused by *SRY* deletions or point mutations, dosage sensitive sex (DSS) locus duplication on the X chromosome or mutations in autosomal genes (88). Very often no gene mutation is found but candidate genes observed on the basis of knowledge of the phenotype of mouse knockout models include *DAX1*, *SF1*, *WNT4*, *SOX3*, *LHX9* and *FOG2* (89).

46,XY DSD due to Androgen synthesis or action defects

Aforementioned as disorders of androgen production, these conditions are caused by inability to produce testosterone due to defects in any of the enzymes required to synthesize testosterone from cholesterol. It could occur in all stages of testosterone biosynthesis and secretion carried out by the fetal Leydig cells, as well as in the conversion of testosterone into

5 α -dihydrotestosterone (DHT) (90). Patients with 46,XY DSD secondary to defects in androgen production reveal a variable phenotype, which depends strongly on the specific mutated gene. Generally, these conditions are detected at birth because of the ambiguity of external genitalia. However, the diagnosis for patients with extremely undervirilised genitalia is often not made until late childhood or even adulthood (90).

The typical phenotype of 46,XY DSD due to impaired androgen synthesis varies from female external genitalia to male external genitalia in which specific characteristics are cause-dependent.

Table 2. Classification of 46,XY DSD caused by impaired androgen production (90)

-
- 46,XY DSD due to Impaired Leydig cell differentiation (LHCGR defects)
 - 46,XY DSD due to Testosterone synthesis defects
 - 46,XY DSD due to Cholesterol synthesis defects
 - Smith-Lemli-Opitz syndrome
 - 46,XY DSD due to Enzymatic defects in adrenal and testicular steroidogenesis
 - StAR deficiency
 - P450scc deficiency
 - 3 β -hydroxysteroid dehydrogenase type II deficiency
 - 17-hydroxylase and 17,20 lyase deficiency
 - 46,XY DSD due to enzymatic defects in testicular steroidogenesis
 - Isolated 17,20-lyase deficiency
 - 17 β -hydroxysteroid dehydrogenase type III deficiency
 - 46,XY DSD due to altered steroidogenesis due to disrupted electron transfer
 - P450 oxidoreductase defect
 - cytochrome b5 defect
 - 46,XY DSD due to testosterone metabolism defects
 - 5 α -reductase type 2 deficiency
-

The complete form of 46,XY due to impaired or inactivated Leydig cell differentiation features female external genitalia leading to female sex assignment, without any development of sexual characteristics at puberty, undescended testes, which are slightly smaller than normal with rather conserved seminiferous tubules and the absence of mature Leydig cells. Epididymis and vas deferens are present but uterus and fallopian tubes are absent (90).

Partial inactivation of the hCG/LH receptor could cause a broad range of phenotypes. The majority of cases with this condition have predominantly male external genitalia with micropenis and/or hypospadias. Testes are undescended or in the scrotum (91). In contrast, activating mutations of this receptor will lead to familial male limited gonadotropin-independent precocious puberty (FMPP) or testotoxicosis (92).

The major androgens acting in the androgenic cascade are testosterone and DHT. The 5 α -reductase type II (SRD5A2) deficiency is a defect in the conversion of T to DHT in (mostly) extratesticular target tissues, which is known as a part of the ‘androgen insensitivity syndromes’ (AISs). However, at present, 5 α -reductase deficiency is classified as a special form of androgen-biosynthesis defect.

Predominantly female phenotype at birth followed by virilization at puberty is a characteristic of mutation of *SRD5A2* gene (93). Ideally the diagnosis 5 α -reductase type 2 deficiency should be made as early as possible in infancy using biochemical techniques and confirmed by molecular genetic analysis. Ratio of plasma testosterone: dihydrotestosterone (T:DHT) >8.5 has been indicated for identifying 5 α -reductase type 2 deficiency, especially during the neonatal period (93, 94). The phenotypes of newborn 46,XY DSD with partial androgen insensitivity syndrome or 17-hydroxysteroid dehydrogenase deficiency may be indistinguishable from the phenotype of 5 α -reductase type 2 deficiency (95).

As the main player in the translation of androgen action, the androgen receptor (AR) is a nuclear transcription factor that binds various androgenic steroids as ligands and acts through differential DNA targeting and genetic control afterward. The complete form of androgen

insensitivity syndrome (CAIS) is a condition of absolute absence of androgen action, whereas the partial form (PAIS) occurs as a result of variable degrees of androgen action impairment (88).

Since the description of the genetic structure of the AR, it is commonly acknowledged that most 46,XY patients with a presumed defect of androgen action carry mutations in the coding regions of the AR gene. This has been confirmed in the majority of patients with CAIS, but patients who were clinically assigned as PAIS or minimal AIS (MAIS) to a certain extent do not carry relevant mutations in the AR (96).

Patients with AIS will have a clinical phenotype that is consistent with the extent of remaining activity of the AR. However, because testicular function appears to be normal in AIS, the size and histology of the testes may be normal in childhood. Due to normal AMH synthesis and action, Müllerian structures are absent. Thus during clinical investigation, in childhood AIS cannot be differentiated from disorders of testicular androgen biosynthesis.

In CAIS, the external genitalia appear to be female. The gonads may be seen in the labia majora or in the inguinal region, but they may also be impalpable during clinical investigation as they may be located intra-abdominally. In infancy, a diagnosis is made on the fact that Müllerian structures (i.e., oviducts, uterus and the upper part of the vagina) are absent, in addition to phallic hypospadias or absence of androgen-dependent Wolffian structures, mainly the lack of epididymis as well as the prostate during either ultrasound investigation or surgical exploration.

The phenotype of PAIS is highly variable, ranging from minor signs of virilization, shown by an elongated ano-vaginal distance, through explicit signs of androgen action with rugation of labio-scrotal folds, urogenital sinus, phallic enlargement to a feature of predominantly male phenotype with appearance of normal male phallic size, despite moderate to severe hypospadias. However, in most of PAIS cases, reduction of phallic size is observed in conjunction with severe, mostly penoscrotal to perineal hypospadias. The specific clinical appearance of AIS becomes more visible during puberty when serum levels of testosterone, LH and estradiol are elevated. Due to dysfunction of androgen action, the negative feedback regulation of LH is impaired, resulting in elevated LH levels despite increased testosterone levels. Estradiol levels increase due to aromatisation of testosterone. This is the key feature of

pubertal development is the feminization that occurs in most of the patient with CAIS and PAIS with gonads in situ (96, 97).

Non-classified forms

Hypospadias *e causa ignota* is a rather common phenomenon, where 40% of the cases are related to other defects of the urogenital system. Generally, it is a sporadic phenomenon, but familial cases do exist. When hypospadias is present, it indicates that an alteration in testosterone secretion or action may have occurred during intra-uterine development. Fukami et al. explained this phenotype on basis of mutations in an X-linked gene, *CXorf6*, in three boys with penoscrotal hypospadias and micropenis (87). The presence of penoscrotal hypospadias along with decreased penis size requires assessment of testicular function in order to rule out causes such as defects in testosterone synthesis and action, which require not just surgical treatment, but also hormonal treatment and genetic counseling (88).

The time trends of prevalence of hypospadias in countries worldwide are not consistent. Some countries like Australia (98), Denmark (99), and the United Kingdom (100) have reported increasing trends of prevalence, whereas Japan (101), Scotland (102), and the U.S.A. states of New York (103), Washington (104), and California (105) have reported no significant increases.

Regional differences in hypospadias prevalence have also been reported. The prevalences of overall and isolated hypospadias in China were 9.03 and 7.64 per 10.000 male births, respectively, and lower than reported for other countries or regions (106). For example, the total prevalence of hypospadias was 34.9 per 10.000 live births in New York in 1992 to 2005 (Fisch et al., 2009), 29 per 10.000 male births in Bulgaria (Kumanov et al., 2007), 31 per 10.000 male births in northern England in 1993 to 2000 (Abdullah et al., 2007)

A.2. 46,XX DSD

46,XX DSD consists of disorders of ovarian development, disorders of androgen synthesis and other conditions affecting sex development (107). In rare cases, such as ovotesticular DSD, the developing ovary may contain some testicular tissue. The developing ovary may also lead to a functioning testis that secretes ample amounts of testosterone for

adequate virilization and AMH for regression of the Müllerian ducts, which is called testicular DSD (87).

46,XX ovotesticular or Testicular DSD

The developing ovary may contain testis tissue (ovotesticular) or may function as a testis that secretes amounts of testosterone and AMH which could make adequate virilization and müllerian regression possible (testicular DSD). Ambiguous genitalia are an initial manifestation in almost all cases of ovotesticular DSD with gradation of internal genitalia between male and female.

46,XX testicular DSD commonly results in a normal male phenotype or a relatively mild abnormality of the male genitalia, such as distal or mid-shaft hypospadias. In adulthood, testosterone synthesis is not affected even though spermatogenesis is usually severely affected (87).

Ovarian dysgenesis.

Ovarian dysgenesis is commonly associated with sex chromosome aneuploidy such as Turner syndrome and related variants. However, the condition does not cause physical abnormalities of sex development in infancy (87).

46,XX DSD due to disorders of androgen synthesis

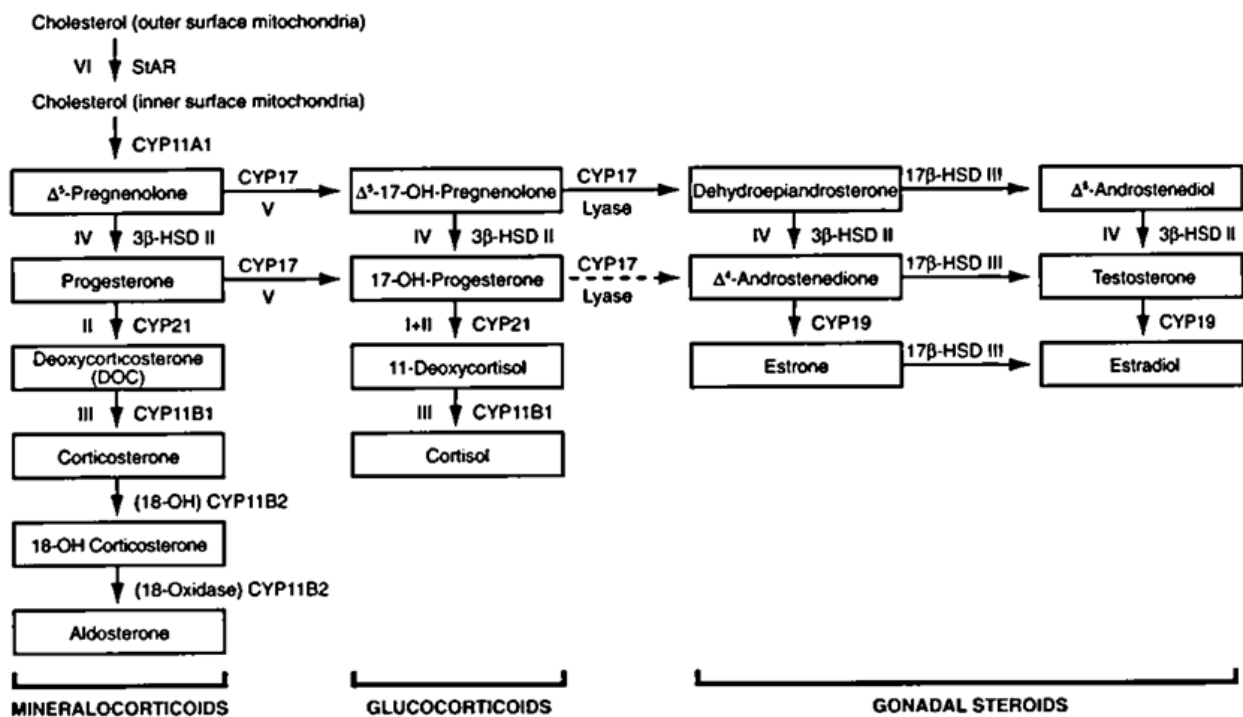
A fetus with a 46,XX chromosomal complement and normal ovarian organogenesis could be exposed to excessive amounts of androgens originating either from the fetus itself or from the mother. The congenital adrenal hyperplasias (CAHs) are disorders of impaired cortisol biosynthesis and the most frequent cause of ambiguous genitalia of the newborn with 46,XX karyotype (89). Low levels of cortisol are causing reduction of feedback inhibition and therefore increased ACTH secretion, which results in hyperplasia of the adrenals and in disorder of steroidogenesis.

Clinical signs of these diseases are based on three conditions: the transformation of cortisol precursors to steroids normally produced in much lower amounts; the biological

activities of these cortisol precursors, which accumulate prior to the block and the deficiency of cortisol and other steroids normally produced by the adrenal and sometimes also the gonads (108). Most of the cases of CAH are caused by the 21-hydroxylase deficiency. A newborn girl with this disease may show virilization to a certain extent. High serum concentrations of 17-hydroxyprogesterone (>300 nmol/l) are present after the first 48 h of life and high androstenedione and testosterone in the early neonatal period are the biochemical characteristic of this condition. Due to a deficiency of mineralocorticoid synthesis, more than 75% of these infants will be salt-losers and the condition of salt-losing crisis will present in the second or third week of life (87).

The manifestation in each CAH patient varies depending on the severity of the enzyme deficiency, the other steroid pathways that are disrupted, the ethnic background, and additional genetic and environmental factors, which are correlated to the level of severity of the disease, many of which are unidentified (108).

Figure 8. Steroidogenesis scheme



Virilization in a female fetus can also be caused by a maternal source of elevated androgens. Ovarian tumours such as luteomas of pregnancy, arrhenoblastomas, hilar-cell tumours, masculinising ovarian stromal cell tumours and Krukenberg tumours are all androgen producing tumours. Even if the mother is overproducing androgens, the female offspring is exposed to relatively minimal androgenic effects. The less active virilization is due to placental aromatase activity, converting androgens into estrogens. Another factor inducing fetal virilization can be caused by the maternal ingestion of androgens, progestagens or other drugs.

Exogenous steroids consumed during pregnancy may result in posterior fusion of the labia, clitoral enlargement and even increased degrees of androgenization (87). Glucocorticoid resistance, although it is a rare case, leads to increased ACTH production, which in turn causes an increase in androgen production (109).

46,XX DSD due to other conditions affecting sex development

Complex urogenital abnormalities such as cloacal anomalies may occur in both sexes and require major reconstructive surgery. Disorders of müllerian development can be related to renal, cardiac or spinal abnormalities as part of the Mayer–Rokitansky–Kuster–Hauser (MRKH) syndrome or müllerian, renal, cervical spine syndrome (MURCS) (87, 110). In special cases, the absence of müllerian structures, and the presence of co-existing hyper- androgenaemia, has been associated with a mutation in the *WNT4* gene (111).

III. GENERAL PRINCIPLES OF DSD MANAGEMENT

Either in the newborn baby or in the newly presenting adolescent, a comprehensive management is required once a patient with a disorder of sex development (DSD) is detected. The complexity of the treatment poses a difficult challenge for health-care professionals (86, 110, 112).

Based on consensus, the primary recommendations to manage the patient with DSD can be summarized as follows:

1. Sex assignment of all newborn infants with DSD should be based on consultation and diagnosis of a multidisciplinary team that includes an endocrinologist, urologist / surgeon, psychologist / psychiatrist, geneticist, neonatologist and gynecologist and, if possible, social workers, nursing and ethics specialists.
2. If the diagnosis is in doubt, a hasty decision about sex assignment must be avoided and expert evaluation should be sought.
3. When an infant with DSD is born, all specialists of the neonatal units are expected to manage the critically unwell newborn, while an experienced multidisciplinary team conducts a holistic evaluation and develops a long-term management strategy at a specialized center.
4. The specialized center is expected to complete first-line investigations sufficient for sex assignment and exclusion of immediate medical concerns; afterwards, the center should develop a plan for second-line investigations which will result in a long-term management plan for the child.
5. Implementing a holistic patient centered approach and individualized management, the team should make evidence based decisions as far as possible. Patient's family should be informed of decisions that are not evidence based.
6. Patient and family concerns should be respected and addressed in strict confidence.
7. The relationship between team and family should include open communication, active involvement in decision making, attention to concerns and respect for privacy.
8. The multidisciplinary specialist team should be able to arrange or even provide a long-term care from infancy to adulthood for the patients.

A. Clinical evaluation of the infant with a suspected DSD

It is crucial that all professionals involved with the management of newborn babies (during labor and in the postnatal ward) are well-informed about the procedure of treating an infant with indeterminate sex. Health care professionals should regularly inform the family about the situation, taking into account the level of understanding as well as their religious and cultural beliefs (112).

An extensive investigation must be conducted, when the appearance of the external genitalia is sufficiently ambiguous to make sex assignment impossible; or when the phenotype is not consistent with prenatal genetic tests (107).

Gathering related information about the family is vital, such as consanguinity, stillbirths, multiple miscarriages, fertility problems, genital abnormalities, hernias, delayed puberty, genital surgery, unexplained deaths and the need for steroid replacement. In addition, maternal health and drug exposure during pregnancy and the pregnancy history should be collected, for it may hold important information. The patient is expected to have a general physical examination that focuses on any related dysmorphic features, and an assessment of the genital anatomy.

Information on the following points should be included at referral for further investigation by a specialist: overt genital ambiguity (cloacal exstrophy); female genitalia with an enlarged clitoris, posterior labial fusion, or an inguinal/labial mass; male genitalia with bilateral undescended testes, isolated micropenis, isolated perineal hypospadias, or mild hypospadias with undescended testes; any form of familial hypospadias and those who have a combination of genital anomalies with an External Masculinization Score (EMS) of <11 which is based upon the site of urethral opening, the severity of the micropenis, the degree of scrotal fusion, and the position of the gonads (figure 9), family history of DSD such as CAIS; a discordance between genital appearance and a prenatal karyotype (107).

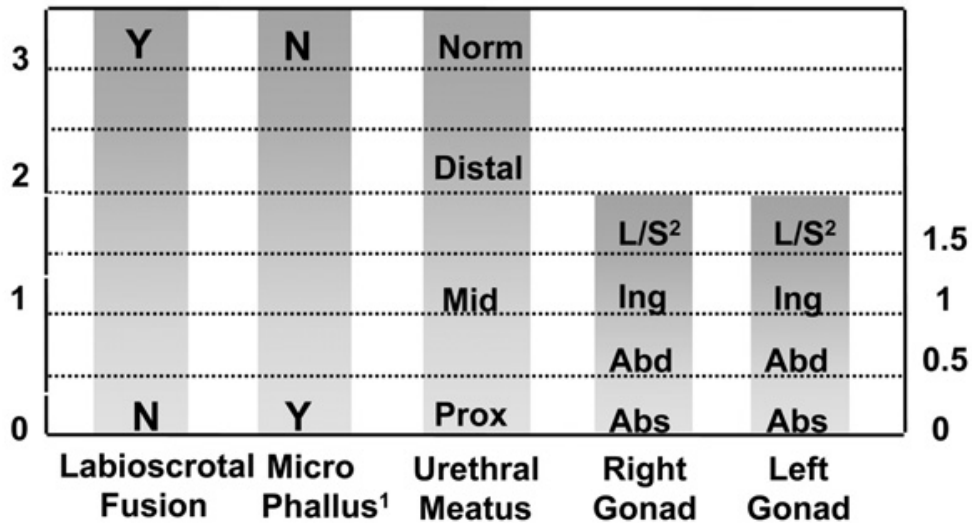


Figure 9. External masculinization score (EMS) for assessing the degree of under androgenization in an individual with 46,XY DSD (87)

B. Surgical management

In this procedure, the surgeon (pediatric surgeon or pediatric urologist) will sketch the surgical sequence and its consequences from infancy to adulthood. Information obtained at early evaluation will accommodate the surgeon in envisaging an image of the genitalia at presentation, with regard to all the changes that may occur in the early months of life. Subsequently, the surgeon will decide on what investigations are the most appropriate for the diagnosis, as well as to guide decisions about the sex of rearing (cystoscopy, vaginoscopy, laparoscopy and so on).

Finally, the surgeon will consider about the type and the quantity of surgical procedures needed as well as the involvement of other surgical specialists (e.g., gynecologist) in order to create the desired outcome. The fundamental aim of delivering functional outcome should be taken into account, rather than a strictly cosmetic appearance. Additionally, it is recommended that the surgeons performing these procedures are only those who have an expertise in the care of children and specific training in the surgery of DSD (110, 112).

C. Psychosocial management

It is known that physical and psychological health for DSD patients are closely interrelated. Psychosocial care should not be separated from the management of the DSD patient, in order to promote positive adaptation and to allow parents to express and resolve their concern. Skill in psychosocial care is beneficial in facilitating team decisions about gender assignment/reassignment, timing of surgery, and sex hormone replacement (110, 112). Due to the degree of complexity and sensitivity of the disease, an experience of working with individuals and families with DSD is advantageous in dealing with the issues that commonly arise. Psychological input should be treated as a process instead of an event; therefore, the contact with a psychology team is implemented. Specific tasks may be required at different stages of development; therefore the family should be involved in planning from an early stage. Pacing and timing of disseminating the information and making decisions is specific to each individual circumstance. Studies in other chronic medical disorders and of adoptees reveal that disclosure should be correlated with or is dependent on patient's improving psychosocial adaptation (112, 113).

References

1. Marshall Graves JA. The rise and fall of SRY. *Trends Genet.* 2002 May;18(5):259-64.
2. Kauppi L, Barchi M, Baudat F, Romanienko PJ, Keeney S, Jasin M. Distinct properties of the XY pseudoautosomal region crucial for male meiosis. *Science.* 2011 Feb 18;331(6019):916-20.
3. Reik W, Ferguson-Smith AC. Developmental biology: the X-inactivation yo-yo. *Nature.* 2005 Nov 17;438(7066):297-8.
4. Navarro-Costa P. Sex, rebellion and decadence: The scandalous evolutionary history of the human Y chromosome. *Biochim Biophys Acta.* 2012 Apr 20.
5. de Carvalho CM, Santos FR. Human Y-chromosome variation and male dysfunction. *J Mol Genet Med.* 2005;1(2):63-75.
6. Skaletsky H, Kuroda-Kawaguchi T, Minx PJ, Cordum HS, Hillier L, Brown LG, et al. The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature.* 2003 Jun 19;423(6942):825-37.
7. Knowler KC, Kelly S, Harley VR. Turning on the male--SRY, SOX9 and sex determination in mammals. *Cytogenet Genome Res.* 2003;101(3-4):185-98.
8. Svechnikov K, Landreh L, Weisser J, Izzo G, Colon E, Svechnikova I, et al. Origin, development and regulation of human Leydig cells. *Horm Res Paediatr.* 2010;73(2):93-101.
9. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev.* 2007 Jan;87(1):1-28.
10. Veitia RA. FOXL2 versus SOX9: a lifelong "battle of the sexes". *Bioessays.* 2010 May;32(5):375-80.
11. Vilain E. Anomalies of human sexual development: clinical aspects and genetic analysis. *Novartis Found Symp.* 2002;244:43-53; discussion -6, 79-85, 253-7.
12. Krone N, Hanley NA, Arlt W. Age-specific changes in sex steroid biosynthesis and sex development. *Best Pract Res Clin Endocrinol Metab.* 2007 Sep;21(3):393-401.
13. Matson CK, Murphy MW, Sarver AL, Griswold MD, Bardwell VJ, Zarkower D. DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature.* 2011 Aug 4;476(7358):101-4.
14. Uhlenhaut NH, Jakob S, Anlag K, Eisenberger T, Sekido R, Kress J, et al. Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell.* 2009 Dec 11;139(6):1130-42.
15. Barsoum IB, Yao HH. Fetal Leydig cells: progenitor cell maintenance and differentiation. *J Androl.* 2010 Jan-Feb;31(1):11-5.
16. Schmahl J, Eicher EM, Washburn LL, Capel B. Sry induces cell proliferation in the mouse gonad. *Development.* 2000 Jan;127(1):65-73.
17. Turnpenny L, Spalluto CM, Perrett RM, O'Shea M, Hanley KP, Cameron IT, et al. Evaluating human embryonic germ cells: concord and conflict as pluripotent stem cells. *Stem Cells.* 2006 Feb;24(2):212-20.
18. Orvis GD, Jamin SP, Kwan KM, Mishina Y, Kaartinen VM, Huang S, et al. Functional redundancy of TGF-beta family type I receptors and receptor-Smads in mediating anti-Mullerian hormone-induced Mullerian duct regression in the mouse. *Biol Reprod.* 2008 Jun;78(6):994-1001.
19. Nussey S, Whitehead S, editors. *Endocrinology: An Integrated Approach* 2001: BIOS Scientific Publishers.
20. Tremblay JJ. What signals testis descent? *Biol Reprod.* 2010 Nov;83(5):687-9.

21. Pleskacova J, Snajderova M, Lelbl J. [Disorders of sex differentiation: genes responsible for development of genital system and final phenotype]. *Ceska Gynekol*. 2009 Jun;74(3):192-7.
22. Baillet A, Mandon-Pepin B. Mammalian ovary differentiation - a focus on female meiosis. *Mol Cell Endocrinol*. 2012 Jun 5;356(1-2):13-23.
23. Piprek RP. Molecular and cellular machinery of gonadal differentiation in mammals. *Int J Dev Biol*. 2010;54(5):779-86.
24. Biason-Lauber A. Control of sex development. *Best Pract Res Clin Endocrinol Metab*. 2010 Apr;24(2):163-86.
25. Torres M, Gomez-Pardo E, Dressler GR, Gruss P. Pax-2 controls multiple steps of urogenital development. *Development*. 1995 Dec;121(12):4057-65.
26. Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, et al. Emx1 and Emx2 functions in development of dorsal telencephalon. *Development*. 1997 Jan;124(1):101-11.
27. Miyamoto N, Yoshida M, Kuratani S, Matsuo I, Aizawa S. Defects of urogenital development in mice lacking Emx2. *Development*. 1997 May;124(9):1653-64.
28. Noonan FC, Mutch DG, Ann Mallon M, Goodfellow PJ. Characterization of the homeodomain gene EMX2: sequence conservation, expression analysis, and a search for mutations in endometrial cancers. *Genomics*. 2001 Aug;76(1-3):37-44.
29. Wilhelm D, Englert C. The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. *Genes Dev*. 2002 Jul 15;16(14):1839-51.
30. Birk OS, Casiano DE, Wassif CA, Cogliati T, Zhao L, Zhao Y, et al. The LIM homeobox gene Lhx9 is essential for mouse gonad formation. *Nature*. 2000 Feb 24;403(6772):909-13.
31. Hatano O, Takakusu A, Nomura M, Morohashi K. Identical origin of adrenal cortex and gonad revealed by expression profiles of Ad4BP/SF-1. *Genes Cells*. 1996 Jul;1(7):663-71.
32. Wagner KD, Wagner N, Schedl A. The complex life of WT1. *J Cell Sci*. 2003 May 1;116(Pt 9):1653-8.
33. Cunha JL, Soardi FC, Bernardi RD, Oliveira LE, Benedetti CE, Guerra-Junior G, et al. The novel p.E89K mutation in the SRY gene inhibits DNA binding and causes the 46,XY disorder of sex development. *Braz J Med Biol Res*. 2011 Apr;44(4):361-5.
34. Wilhelm D, Martinson F, Bradford S, Wilson MJ, Combes AN, Beverdam A, et al. Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Dev Biol*. 2005 Nov 1;287(1):111-24.
35. Das DK, Sanghavi D, Gawde H, Idicula-Thomas S, Vasudevan L. Novel homozygous mutations in Desert Hedgehog gene in patients with 46,XY complete gonadal dysgenesis and prediction of its structural and functional implications by computational methods. *Eur J Med Genet*. 2011 Jul 23.
36. Sekido R, Lovell-Badge R. Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature*. 2008 Jun 12;453(7197):930-4.
37. Arango NA, Lovell-Badge R, Behringer RR. Targeted mutagenesis of the endogenous mouse *Mis* gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. *Cell*. 1999 Nov 12;99(4):409-19.
38. Murayama C, Miyazaki H, Miyamoto A, Shimizu T. Involvement of Ad4BP/SF-1, DAX-1, and COUP-TFII transcription factor on steroid production and luteinization in ovarian theca cells. *Mol Cell Biochem*. 2008 Jul;314(1-2):51-8.
39. Lin L, Achermann JC. Steroidogenic factor-1 (SF-1, Ad4BP, NR5A1) and disorders of testis development. *Sex Dev*. 2008;2(4-5):200-9.

40. Barbaro M, Cools M, Looijenga LH, Drop SL, Wedell A. Partial deletion of the NR5A1 (SF1) gene detected by synthetic probe MLPA in a patient with XY gonadal disorder of sex development. *Sex Dev.* 2011;5(4):181-7.
41. Lourenco D, Brauner R, Lin L, De Perdigo A, Weryha G, Muresan M, et al. Mutations in NR5A1 associated with ovarian insufficiency. *N Engl J Med.* 2009 Mar 19;360(12):1200-10.
42. Bertolin K. Orphan nuclear receptor regulation of reproduction. *Anim, Reprod.* 2010 Jul./Sep.;7(n.3):146-53.
43. Pezzi V, Sirianni R, Chimento A, Maggiolini M, Bourguiba S, Delalande C, et al. Differential expression of steroidogenic factor-1/adrenal 4 binding protein and liver receptor homolog-1 (LRH-1)/fetoprotein transcription factor in the rat testis: LRH-1 as a potential regulator of testicular aromatase expression. *Endocrinology.* 2004 May;145(5):2186-96.
44. Suzuki T, Kasahara M, Yoshioka H, Morohashi K, Umesono K. LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1. *Mol Cell Biol.* 2003 Jan;23(1):238-49.
45. Zhang H, Thomsen JS, Johansson L, Gustafsson JA, Treuter E. DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. *J Biol Chem.* 2000 Dec 22;275(51):39855-9.
46. Hanley NA, Hagan DM, Clement-Jones M, Ball SG, Strachan T, Salas-Cortes L, et al. SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. *Mech Dev.* 2000 Mar 1;91(1-2):403-7.
47. El-Khairi R, Martinez-Aguayo A, Ferraz-de-Souza B, Lin L, Achermann JC. Role of DAX-1 (NR0B1) and steroidogenic factor-1 (NR5A1) in human adrenal function. *Endocr Dev.* 2011;20:38-46.
48. McCabe E. *Adrenal hypoplasias and aplasias.* 8th ed. New York: McGraw-Hill; 2001.
49. Bouma GJ, Albrecht KH, Washburn LL, Recknagel AK, Churchill GA, Eicher EM. Gonadal sex reversal in mutant Dax1 XY mice: a failure to upregulate Sox9 in pre-Sertoli cells. *Development.* 2005 Jul;132(13):3045-54.
50. Ludbrook LM, Harley VR. Sex determination: a 'window' of DAX1 activity. *Trends Endocrinol Metab.* 2004 Apr;15(3):116-21.
51. Veitia RA, Nunes M, Quintana-Murci L, Rappaport R, Thibaud E, Jaubert F, et al. Swyer syndrome and 46,XY partial gonadal dysgenesis associated with 9p deletions in the absence of monosomy-9p syndrome. *Am J Hum Genet.* 1998 Sep;63(3):901-5.
52. DeFalco TJ, Verney G, Jenkins AB, McCaffery JM, Russell S, Van Doren M. Sex-specific apoptosis regulates sexual dimorphism in the Drosophila embryonic gonad. *Dev Cell.* 2003 Aug;5(2):205-16.
53. Raymond CS, Murphy MW, O'Sullivan MG, Bardwell VJ, Zarkower D. Dmrt1, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. *Genes Dev.* 2000 Oct 15;14(20):2587-95.
54. Barrionuevo FJ, Burgos M, Scherer G, Jimenez R. Genes promoting and disturbing testis development. *Histol Histopathol.* 2012 Nov;27(11):1361-83.
55. Schepers G, Wilson M, Wilhelm D, Koopman P. SOX8 is expressed during testis differentiation in mice and synergizes with SF1 to activate the Amh promoter in vitro. *J Biol Chem.* 2003 Jul 25;278(30):28101-8.
56. Schmahl J, Kim Y, Colvin JS, Ornitz DM, Capel B. Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. *Development.* 2004 Aug;131(15):3627-36.

57. Yao HH, Capel B. Disruption of testis cords by cyclopamine or forskolin reveals independent cellular pathways in testis organogenesis. *Dev Biol.* 2002 Jun 15;246(2):356-65.
58. Yao HH, Whoriskey W, Capel B. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev.* 2002 Jun 1;16(11):1433-40.
59. Canto P, Soderlund D, Reyes E, Mendez JP. Mutations in the desert hedgehog (DHH) gene in patients with 46,XY complete pure gonadal dysgenesis. *J Clin Endocrinol Metab.* 2004 Sep;89(9):4480-3.
60. Tang P, Park DJ, Marshall Graves JA, Harley VR. ATRX and sex differentiation. *Trends Endocrinol Metab.* 2004 Sep;15(7):339-44.
61. Katoh-Fukui Y, Tsuchiya R, Shiroishi T, Nakahara Y, Hashimoto N, Noguchi K, et al. Male-to-female sex reversal in M33 mutant mice. *Nature.* 1998 Jun 18;393(6686):688-92.
62. Katoh-Fukui Y, Owaki A, Toyama Y, Kusaka M, Shinohara Y, Maekawa M, et al. Mouse Polycomb M33 is required for splenic vascular and adrenal gland formation through regulating Ad4BP/SF1 expression. *Blood.* 2005 Sep 1;106(5):1612-20.
63. Vinci G, Brauner R, Tar A, Rouba H, Sheth J, Sheth F, et al. Mutations in the TSPYL1 gene associated with 46,XY disorder of sex development and male infertility. *Fertil Steril.* 2009 Oct;92(4):1347-50.
64. de Andrade TG, Peterson KR, Cunha AF, Moreira LS, Fattori A, Saad ST, et al. Identification of novel candidate genes for globin regulation in erythroid cells containing large deletions of the human beta-globin gene cluster. *Blood Cells Mol Dis.* 2006 Sep-Oct;37(2):82-90.
65. Puffenberger EG, Hu-Lince D, Parod JM, Craig DW, Dobrin SE, Conway AR, et al. Mapping of sudden infant death with dysgenesis of the testes syndrome (SIDDT) by a SNP genome scan and identification of TSPYL loss of function. *Proc Natl Acad Sci U S A.* 2004 Aug 10;101(32):11689-94.
66. Hering R, Frade-Martinez R, Bajanowski T, Poets CF, Tschentscher F, Riess O. Genetic investigation of the TSPYL1 gene in sudden infant death syndrome. *Genet Med.* 2006 Jan;8(1):55-8.
67. Laporte J, Guiraud-Chaumeil C, Vincent MC, Mandel JL, Tanner SM, Liechti-Gallati S, et al. Mutations in the MTM1 gene implicated in X-linked myotubular myopathy. ENMC International Consortium on Myotubular Myopathy. European Neuro-Muscular Center. *Hum Mol Genet.* 1997 Sep;6(9):1505-11.
68. Fukami M, Wada Y, Okada M, Kato F, Katsumata N, Baba T, et al. Mastermind-like domain-containing 1 (MAMLD1 or CXorf6) transactivates the Hes3 promoter, augments testosterone production, and contains the SF1 target sequence. *J Biol Chem.* 2008 Feb 29;283(9):5525-32.
69. Brandao MP, Costa EM, Fukami M, Gerdulo M, Pereira NP, Domenice S, et al. MAMLD1 (mastermind-like domain containing 1) homozygous gain-of-function missense mutation causing 46,XX disorder of sex development in a virilized female. *Adv Exp Med Biol.* 2011;707:129-31.
70. Wilhelm D, Hiramatsu R, Mizusaki H, Widjaja L, Combes AN, Kanai Y, et al. SOX9 regulates prostaglandin D synthase gene transcription in vivo to ensure testis development. *J Biol Chem.* 2007 Apr 6;282(14):10553-60.
71. Foresta C, Zuccarello D, Garolla A, Ferlin A. Role of hormones, genes, and environment in human cryptorchidism. *Endocr Rev.* 2008 Aug;29(5):560-80.
72. Shono T. [Molecular and anatomical studies of testicular descent]. *Hinyokika Kyo.* 2007 Jul;53(7):505-8.
73. Chassot AA, Gregoire EP, Magliano M, Lavery R, Chaboissier MC. Genetics of ovarian differentiation: Rspo1, a major player. *Sex Dev.* 2008;2(4-5):219-27.
74. Parma P, Radi O, Vidal V, Chaboissier MC, Dellambra E, Valentini S, et al. R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nat Genet.* 2006 Nov;38(11):1304-9.

75. Jordan BK, Mohammed M, Ching ST, Delot E, Chen XN, Dewing P, et al. Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans. *Am J Hum Genet.* 2001 May;68(5):1102-9.
76. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. *Nature.* 1999 Feb 4;397(6718):405-9.
77. Heikkila M, Prunskaitė R, Naillat F, Itaranta P, Vuoristo J, Leppaluoto J, et al. The partial female to male sex reversal in Wnt-4-deficient females involves induced expression of testosterone biosynthetic genes and testosterone production, and depends on androgen action. *Endocrinology.* 2005 Sep;146(9):4016-23.
78. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, et al. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development.* 2004 Feb;131(4):933-42.
79. Pannetier M, Fabre S, Batista F, Kocer A, Renault L, Jolivet G, et al. FOXL2 activates P450 aromatase gene transcription: towards a better characterization of the early steps of mammalian ovarian development. *J Mol Endocrinol.* 2006 Jun;36(3):399-413.
80. Crisponi L, Deiana M, Loi A, Chiappe F, Uda M, Amati P, et al. The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nat Genet.* 2001 Feb;27(2):159-66.
81. Pannetier M, Elzaïat M, Thepot D, Pailhoux E. Telling the story of XX sex reversal in the goat: highlighting the sex-crossroad in domestic mammals. *Sex Dev.* 2012;6(1-3):33-45.
82. Lang-Muritano M, Biason-Lauber A, Gitzelmann C, Belville C, Picard Y, Schoenle EJ. A novel mutation in the anti-mullerian hormone gene as cause of persistent mullerian duct syndrome. *Eur J Pediatr.* 2001 Nov;160(11):652-4.
83. Benson GV, Lim H, Paria BC, Satokata I, Dey SK, Maas RL. Mechanisms of reduced fertility in Hoxa-10 mutant mice: uterine homeosis and loss of maternal Hoxa-10 expression. *Development.* 1996 Sep;122(9):2687-96.
84. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. *Development.* 2004 Feb;131(3):539-49.
85. Guertl B, Senanayake U, Nussold E, Leuschner I, Mannweiler S, Ebner B, et al. Lim1, an embryonal transcription factor, is absent in multicystic renal dysplasia, but reactivated in nephroblastomas. *Pathobiology.* 2011;78(4):210-9.
86. Barthold JS. Disorders of sex differentiation: a pediatric urologist's perspective of new terminology and recommendations. *J Urol.* 2011 Feb;185(2):393-400.
87. Ahmed SF, Rodie M. Investigation and initial management of ambiguous genitalia. *Best Pract Res Clin Endocrinol Metab.* 2010 Apr;24(2):197-218.
88. Mendonca BB, Domenice S, Arnhold IJ, Costa EM. 46,XY disorders of sex development (DSD). *Clin Endocrinol (Oxf).* 2009 Feb;70(2):173-87.
89. Hughes IA. Disorders of sex development: a new definition and classification. *Best Pract Res Clin Endocrinol Metab.* 2008 Feb;22(1):119-34.
90. Mendonca BB, Costa EM, Belgorosky A, Rivarola MA, Domenice S. 46,XY DSD due to impaired androgen production. *Best Pract Res Clin Endocrinol Metab.* 2010 Apr;24(2):243-62.
91. Richter-Unruh A, Verhoef-Post M, Malak S, Homoki J, Hauffa BP, Themmen AP. Leydig cell hypoplasia: absent luteinizing hormone receptor cell surface expression caused by a novel homozygous mutation in the extracellular domain. *J Clin Endocrinol Metab.* 2004 Oct;89(10):5161-7.

92. Boot AM, Lumbroso S, Verhoef-Post M, Richter-Unruh A, Looijenga LH, Funaro A, et al. Mutation analysis of the LH receptor gene in Leydig cell adenoma and hyperplasia and functional and biochemical studies of activating mutations of the LH receptor gene. *J Clin Endocrinol Metab.* 2011 Jul;96(7):E1197-205.
93. Walter KN, Kienzle FB, Frankenschmidt A, Hiort O, Wudy SA, van der Werf-Grohmann N, et al. Difficulties in diagnosis and treatment of 5alpha-reductase type 2 deficiency in a newborn with 46,XY DSD. *Horm Res Paediatr.* 2010;74(1):67-71.
94. Perry RJ, Novikova E, Wallace AM, Donaldson MD. Pitfalls in the diagnosis of 5alpha-reductase type 2 deficiency during early infancy. *Horm Res Paediatr.* 2011;75(5):380-2.
95. Ko JM, Cheon CK, Kim GH, Kim SH, Kim KS, Yoo HW. Clinical characterization and analysis of the SRD5A2 gene in six Korean patients with 5alpha-reductase type 2 deficiency. *Horm Res Paediatr.* 2010;73(1):41-8.
96. Werner R, Grotsch H, Hiort O. 46,XY disorders of sex development--the undermasculinised male with disorders of androgen action. *Best Pract Res Clin Endocrinol Metab.* 2010 Apr;24(2):263-77.
97. Hellmann P, Christiansen P, Johannsen TH, Main KM, Duno M, Juul A. Male patients with partial androgen insensitivity syndrome: a longitudinal follow-up of growth, reproductive hormones and the development of gynaecomastia. *Arch Dis Child.* 2012 May;97(5):403-9.
98. Nassar N, Bower C, Barker A. Increasing prevalence of hypospadias in Western Australia, 1980-2000. *Arch Dis Child.* 2007 Jul;92(7):580-4.
99. Lund L, Engebjerg MC, Pedersen L, Ehrenstein V, Norgaard M, Sorensen HT. Prevalence of hypospadias in Danish boys: a longitudinal study, 1977-2005. *Eur Urol.* 2009 May;55(5):1022-6.
100. Abdullah NA, Pearce MS, Parker L, Wilkinson JR, Jaffray B, McNally RJ. Birth prevalence of cryptorchidism and hypospadias in northern England, 1993-2000. *Arch Dis Child.* 2007 Jul;92(7):576-9.
101. Kurahashi N, Murakumo M, Kakizaki H, Nonomura K, Koyanagi T, Kasai S, et al. The estimated prevalence of hypospadias in Hokkaido, Japan. *J Epidemiol.* 2004 May;14(3):73-7.
102. Ahmed SF, Dobbie R, Finlayson AR, Gilbert J, Youngson G, Chalmers J, et al. Prevalence of hypospadias and other genital anomalies among singleton births, 1988-1997, in Scotland. *Arch Dis Child Fetal Neonatal Ed.* 2004 Mar;89(2):F149-51.
103. Fisch H, Lambert SM, Hensle TW, Hyun G. Hypospadias rates in new york state are not increasing. *J Urol.* 2009 May;181(5):2291-4.
104. Porter MP, Faizan MK, Grady RW, Mueller BA. Hypospadias in Washington State: maternal risk factors and prevalence trends. *Pediatrics.* 2005 Apr;115(4):e495-9.
105. Carmichael SL, Shaw GM, Nelson V, Selvin S, Torfs CP, Curry CJ. Hypospadias in California: trends and descriptive epidemiology. *Epidemiology.* 2003 Nov;14(6):701-6.
106. Li Y, Mao M, Dai L, Li K, Li X, Zhou G, et al. Time trends and geographic variations in the prevalence of hypospadias in China. *Birth Defects Res A Clin Mol Teratol.* 2011 Sep 30.
107. Faisal Ahmed S, Achermann JC, Arlt W, Balen A, Conway G, Edwards Z, et al. UK Guidance On The Initial Evaluation Of An Infant Or An Adolescent With A Suspected Disorder Of Sex Development. *Clin Endocrinol (Oxf).* 2011 Apr 16.
108. Auchus RJ, Chang AY. 46,XX DSD: the masculinised female. *Best Pract Res Clin Endocrinol Metab.* 2010 Apr;24(2):219-42.

109. Mendonca BB, Leite MV, de Castro M, Kino T, Elias LL, Bachega TA, et al. Female pseudohermaphroditism caused by a novel homozygous missense mutation of the GR gene. *J Clin Endocrinol Metab.* 2002 Apr;87(4):1805-9.
110. Lee PA, Houk CP, Ahmed SF, Hughes IA. Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics.* 2006 Aug;118(2):e488-500.
111. Sultan C, Biason-Lauber A, Philibert P. Mayer-Rokitansky-Kuster-Hauser syndrome: recent clinical and genetic findings. *Gynecol Endocrinol.* 2009 Jan;25(1):8-11.
112. Brain CE, Creighton SM, Mushtaq I, Carmichael PA, Barnicoat A, Honour JW, et al. Holistic management of DSD. *Best Pract Res Clin Endocrinol Metab.* 2010 Apr;24(2):335-54.
113. Committee on Paediatric AIDS AAO. Disclosure of illness status to children and adolescents with HIV infection. American Academy of Pediatrics Committee on Pediatrics AIDS. *Pediatrics.* 1999 Jan;103(1):164-6.

Chapter 3

Application of the New Classification on Patients with a Disorder of Sex Development (DSD) in Indonesia

Achmad Zulfa Juniarto, Yvonne G. van der Zwan, Ardy Santosa, Remko Hersmus, Frank H. de Jong, Renske Olmer, Hennie T. Bruggenwirth, Axel P.N. Themmen, Katja P. Wolffenbuttel, Leendert H.J. Looijenga, Sultana MH Faradz, Stenvert L.S. Drop

Int J Endocrinol. 2012;2012:237084. doi: 10.1155/2012/237084. Epub 2011 Dec 29.

PMID: 22253624

Abstract

Disorder of Sex Development (DSD) patients in Indonesia most often does not receive a proper diagnostic evaluation and treatment. This study intended to categorize 88 Indonesian patients in accordance with the new consensus DSD algorithm. Diagnostic evaluation including clinical, hormonal, genetic, imaging, surgical and histological parameters was performed. Fifty-three patients were raised as male and 34 as female. Of 22 patients with 46,XX DSD, 15 had congenital adrenal hyperplasia while in one patient an ovarian Leydig cell tumor was found. In all 58 46,XY DSD patients, 29 were suspected of a disorder of androgen action (12 with an androgen receptor mutation), in 9 gonadal dysgenesis was found and in 20 severe hypospadias e.c.i. Implementation of the current consensus statement in a resource-poor environment is very difficult. The aim of the diagnostic work up in developing countries should be to end up with an evidence based diagnosis. This is essential to improve treatment and thereby to improve the patients' quality of life.

INTRODUCTION

The sequential expression of many genes is essential for gonadal development in the male as well as in the female (1, 2). In addition, timely secretion and action of hormones such as androgens and Anti-Müllerian Hormone (AMH) are crucial for normal male development (3). Mutation analysis of genes related to these factors in patients with genital disorders has substantiated their essential role (4-7). Therefore, in a number of cases a specific diagnosis can be made by mutation analysis. Disorders of sex development (DSD) are defined as congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (8). In patients categorized under the term 46,XY or 46,XX DSD with anomalies of gonadal development, often no specific etiology can be established (9). Yet, the establishment of a specific diagnosis is relevant with regard to proper gender assignment as well as regarding hormonal and surgical treatment. Moreover, patients with various forms of 46,XY DSD and Chromosomal DSD are at a substantially increased risk of developing gonadal germ cell tumors (10).

In Indonesia, patients presenting with DSD most often do not receive a diagnostic evaluation and there are limited options for medical and surgical treatment. In this study we performed a diagnostic evaluation in 88 patients with DSD referred to a major Centre in Semarang, Indonesia. The aim of this study was to categorize the patients in accordance with the nomenclature proposed in the new consensus statement (8). Therefore we performed a diagnostic evaluation including clinical and hormonal parameters. Furthermore results from imaging and surgery as well as genetic and histological parameters were evaluated.

SUBJECT AND METHODS

Subjects

Eighty-eight patients (from 84 index patients) with various forms and degrees of DSD were evaluated consecutively. They were referred for chromosomal analysis by clinicians of the departments of Urology, Pediatrics, Internal Medicine and Obstetrics of the Dr Kariadi Hospital, Semarang, Indonesia. Referral took place between 2004 and 2006 to the department of Human

Genetics Center for Biomedical Research, Faculty of Medicine Diponegoro University (FMDU), Semarang. Reason of referral was the presence of ambiguous genitalia or any anatomical abnormality of external or internal genitalia, including penoscrotal hypospadias, with or without descended testes. Patients with sex chromosome aberrations were included except patients with classical Klinefelter (47, XXY) and Turner syndromes (45, XO). In addition four patients with cloacal malformation were excluded.

All patients were recalled to the hospital for a physical examination, pedigree construction and collection of blood for hormonal and gene mutation analyses. The age at initial presentation was also the age of investigation and the start of follow up in 87 patients, one patient with presumptive CAH already received suppletion therapy for 9 months. The local medical ethics committee approved this study and informed consent was obtained from all participants, their parents or guardians.

Methods

A stepwise diagnostic approach (figure 1) was used in order to determine the diagnosis in each patient. First of all the patients were clinically evaluated, a detailed description of the external genitalia was obtained and the genitalia were staged according to Quigley (11). The assigned gender was also recorded. Subsequently chromosomal patterns were determined and based on the results the patients were categorized according to the primary root of the recent classification (8): 46,XY DSD, 46,XX DSD or chromosomal DSD. In all patients a blood sample was obtained for hormonal and gene analysis; in patients with 46,XY DSD or Y containing chromosomal DSD an additional blood sample was obtained 72 hr after the intramuscular injection of 1500 IU hCG (Pregnyl ®Organon,Oss, The Netherlands). The hCG test was not performed in 11 patients for the following reasons: the gonads had been removed (n=3), logistic reasons (n=7) and in one patient hCG therapy had been started by the referring doctor in order to enlarge the penis. Subsequently imaging was offered to all the patients as well as surgery in the form of a laparoscopy or cystoscopy whenever needed for diagnostic options or for gonadectomy in case of a high tumor risk. Based on the results, a differential diagnosis was made followed by

gene mutation analysis. Finally gonadal samples were analyzed when they were available in order to complete the classification.

Chromosome analysis

Karyotype was established using a G-banding technique in the Molecular and Cytogenetics Laboratory of the Center for Biomedical Research of FMDU (Semarang). G - banding was also performed for confirmation of the presence of the Y chromosome.

Serum hormones

Serum determinations of Inhibin B, AMH, LH and FSH in the basal serum samples were performed in the endocrine laboratory of Erasmus MC (Rotterdam) as described previously (12). Testosterone was determined in serum collected before and after injection of hCG using the Coat-a-Count radioimmunoassay purchased from Siemens (Los Angeles, CA). (12). Androstenedione, dehydroepiandrosterone sulphate and progesterone were also measured in these samples using the Immulite 2000 (Siemens). Finally, 17-hydroxyprogesterone levels were estimated using an in house method (13). Reference values were used as described earlier (12, 14).

Gene analysis

DNA was extracted from leucocytes of EDTA blood using the salting out method as described earlier (15). Based on the clinical and hormonal information, specific genes were analysed such as *CYP21A2*(16), and *LHR* (6). *AR*, *SRY* and *WNT4* were analysed by direct sequence analysis of the coding exons and exon-flanking intronic regions (reference sequence *AR* (17, 18): nm_000044 (numbering according to Gottlieb et al (18), *SRY*: X53772.1 and *WNT4*: nm 030761. Primer sequences and locations are available upon request.

Pathology:

Histopathological assessments were performed by means of hematoxylin and eosin stainings and immunohistochemistry for various markers of germ cells, e.g. OCT3/4, TSPY,

VASA, SCF (including double staining for OCT3/4-TSPY or VASA); as well as SOX 9 and FOXL2 for supportive cells (19).

RESULTS

For a stepwise diagnostic approach the algorithm shown in figure 1 was followed.

1. Clinical evaluation and chromosome determination

All 88 patients were categorized according to their karyotype; there were 22 patients with 46,XX DSD, 58 patients with 46,XY DSD and eight patients with chromosomal DSD.

Data on age, sex of rearing and Quigley stage (11) of the 88 patients are provided in table 1. The majority of patients were older than two years at the time of referral; only six patients were referred below the age of one year (figure 2). The group of 88 patients contains four families with one or more patients with DSD (a total of nine). There was no known consanguinity of the parents. Fifty-three patients were raised as male and 34 as female while one patient's gender was undetermined due to early age; 16 patients (19%) were raised discordant with their genotype (two males with karyotype 46,XX and 14 females with karyotype 46,XY). Two 46,XY patients changed gender from female into male during the study and one patient with a 46,XX karyotype decided to live as a male. One patient with presumed androgen excess died one week after inclusion without gender assignment.

Table 1: Phenotype (Quigley stage) in correlation with the DSD classification, and sex of rearing. NA: not assigned, patient died after inclusion but before gender assignment

Classification	Quigley stage						sex of rearing		
	1	2	3	4	5	6	male	female	NA
46, XX DSD		3	5	6	4	4	2	19	1
46 XY DSD	4	22	22	6	1	3	45	13	
Chrom. DSD	1	3	0	2	0	2	5	3	

											88 DSD patients							
1											Clinical Evaluation							
			46 XX DSD N = 22 (F 19, M 2, NA 1)									46 XY DSD N = 58 (F 13, M 45)						chromosomal DSD N = 8 (F 3, M 5)
2	Hormonal Assesment 17 O H Prog Androstenedione		Imaging Ultrasound MRI	Hormonal Assesment AMH/INHB HCG test	Imaging Ultrasound MRI	Surgery laparoscopy gonadal biopsy	Hormonal Assesment AMH/INHB HCG test	Imaging Ultrasound MRI	Surgery laparoscopy gonad biopsy									
3	CAH N = 15	androgen Excess N = 2	MRKH N = 4	XX-M N = 1	Androg Act N = 29	Hypospadias N = 20	Gon Dysgen N = 9		Gon Dysgen N = 8									
4	CYP 21 100%	androgen Excess 50%	WNT4 0%	SRY 0%	AR 41,4%	AR 0%	Gene mut 0%		SRY 12,5%									
5	Gonadal History																	
6	Final Classification																	

Figure 1: Stepwise diagnostic approach. An algorithm used with the aim to classify the patients following the new classification system (8). Row number 1 represents the clinical evaluation of the patients and classification following the primary root; number 2 the hormonal analysis and imaging followed by the secondary root classification. Row number 4 shows the percentage mutations that were found and 5 the gonadal histology leading to the tertiary root and final classification of the patients. Explanation of abbreviations: GR: glucocorticoid receptor; MRS: Mayer – Rokitansky Syndrome; XX-M; XX male; OV-TU; ovotesticular DSD.

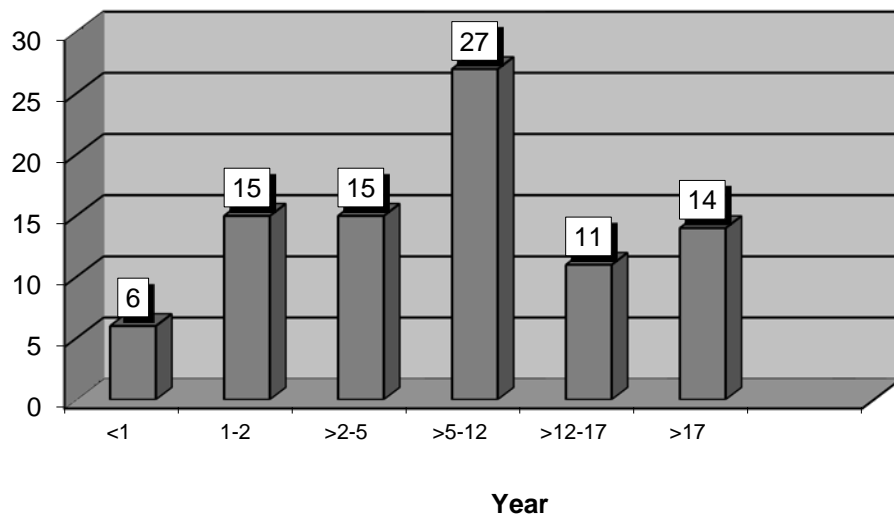


Figure 2: Age distribution of the patients at presentation

2. Hormonal Analysis, imaging and surgery

46,XX DSD

Hormonal analysis showed that out of the 22 46,XX patients, 15 patients (68%) had serum values of adrenal steroids suggesting CAH. Fourteen of these 15 patients showed marked elevation of levels of 17-hydroxyprogesterone, androstenedione and testosterone. In the remaining patient these values were in the normal range as she already received corticosteroid supplementation therapy before referral. No patients with the salt wasting form of CAH were found. As expected, AMH and inhibin B levels were all in normal range for females (data not shown). In one patient with no history of steroid medication, adrenal steroid levels including cortisol were suggestive of cortisol resistance. This could not be confirmed by sequencing the glucocorticoid receptor gene, however.

Extremely high testosterone levels (basal level of 59.9 nmol/l) and slightly increased values of gonadotrophins (LH: 12.3 IU/L, FSH: 8.1 IU/L) were identified in one 33 years-old patient with Quigley stage 4, phallus length 2.5 cm, and a low voice. Four patients had normal

ovarian and adrenal hormone levels and in view of the clinical presentation were suspected of having the Mayer – Rokitansky Syndrome (20).

One 11 years-old patient with the 46,XX male syndrome with palpable gonads in the scrotum had normal FSH and testosterone levels but low LH, AMH and Inhibin B levels for boys of this age. Testosterone rose from 0.3 to 3.4 nmol/l in response to hCG, suggesting that there were functional Leydig cells.

Only in 12 of the 22 patients that were eligible, a diagnostic ultrasound was performed. In seven patients surgery was advised, again only in two (28 %) patients diagnostic surgery in the form of a laparoscopy or cystoscopy was done. The remaining patients refused because of economic reasons.

46,XY DSD

In all 58 patients basal hormonal measurements were obtained. LH levels were elevated in nine patients, decreased in 35 patients and normal for age in 14. FSH levels were elevated in 18 patients and in the normal range in the remaining 40. Finally testosterone levels were elevated in 15 patients, decreased in 36 patients and normal in seven patients.

Twelve patients showed elevated levels of AMH, 27 patients had levels in the normal range and 17 patients had decreased levels of AMH. In one patient, after gonadectomy, AMH was not determined.

Inhibin B levels were elevated in 17 patients, normal in 27 patients and decreased in 14 patients. An hCG test was performed in 47/58 patients (see methods), 45 patients showed a sufficient response of testosterone and its precursors. In two patients with a decreased response Leydig cell hypoplasia was suspected. Due to lack of material we could not test for DHT.

Diagnostic ultrasound was performed in 11 out of 58 (19%) patients and only in five (9 %) patients diagnostic procedures in the form of a cystoscopy were performed. In two (9 %) of the 21 eligible patients a gonadectomy was performed. The remaining patients refused because of economic and cultural reasons.

Chromosomal DSD

The karyotype of the eight patients is provided in table 2. The basal level of LH was increased for age in one patient. The level of FSH was elevated in two patients (age 2 and 33 years) with low levels of Inhibin B in comparison with male values, while AMH and Inhibin B were increased in comparison to normal female levels.

Ultrasound was performed in five out of eight (62.5 %) patients. In one (12.5%) patient a diagnostic procedure in the form of a cystoscopy was done. In only one (12.5%) of the eight eligible patients a gonadectomy was performed. The remaining patients refused because of economic and cultural reasons. Efforts to follow up on these patients have been performed.

Table 2: Karyotype of patients with Chromosomal DSD

Karyotype	Number
XXY/XY	2
XY/X	2
XX/XXq-	2
XY/XX	1
XX/XXY	1
Total	8

3. Secondary root classification

Based on the hormonal evaluation, imaging and diagnostic surgery, the following secondary root categorization was made.

46,XX DSD

Fifteen patients were suspected of congenital adrenal hyperplasia, one patient of a glucocorticoid receptor defect, four patients with Mayer – Rokitansky Syndrome, one patient with XX male syndrome and one patient with Ovotesticular DSD.

46,XY DSD

Twenty nine patients were suspected of a disorder of androgen action, nine with a disorder of gonadal development and 20 with severe hypospadias. We did not establish the

diagnosis testosterone synthesis disorders in any of the patients, but we cannot rule out the presence of 5-alpha-reductase deficiency in our patients without a definitive diagnosis and a normal response of 5 α -dihydrotestosterone (DHT) levels to hCG.

Chromosomal DSD

All patients were categorized under the second root diagnosis disorders of gonadal development (Gonadal dysgenesis).

4. Mutation analysis

Based on these results mutation analysis was performed.

46,XX DSD

Based on their phenotype, hormonal and chromosomal analysis, *CYP21A2* analysis was performed in 15 patients and indeed *CYP 21* gene mutations were found in all of them (16). In four patients, clinically suspected of having Mayer – Rokitansky syndrome *WNT4* gene analysis was negative. One patient was suspected of a glucocorticoid receptor defect, however, a mutation in the sequence of this receptor was not found.

46,XY DSD

Mutation analysis of the androgen receptor (AR) gene was performed in 29 patients who were suspected of having a disorder of androgen action and in 20 patients with severe hypospadias with a normal response to hCG. In two index patients (four patients) pathogenic AR mutations were found, R840H and 902insA. In an additional two patients the sequence variant V730M was found, of which it is unlikely that it is causing the phenotype; functional studies showed that this variant is an activating mutation. It has been described as a somatic variant in patients with prostate cancer (21, 22). In five index patients (six patients) unclassified variants were found, I603N, 2170T>A; P671S, 2373C>T; C175G, 885T>G and Q738R, 2575A>G; only one of these four variants (C175G) has been described at the nucleotide level . The three novel

sequence variants (I603N, P671S and Q738R) were functionally investigated (23). Further mutation analysis for the SRD5A2 should be performed for patients without AR gene mutation.

In the 20 patients with severe hypospadias no AR mutations were found. Two patients suspected of having Leydig cell hypoplasia were analyzed for a mutation in the LH receptor gene, but no mutation was found

Chromosomal DSD

An SRY deletion was found in one patient with mosaic Klinefelter XX/XXY. In the remaining seven patients no SRY deletions were detected.

5. Gonadal Histology

Histology of the gonads was available in four patients as shown in table 3: one with 46,XX DSD (biopsy as mentioned earlier), two with 46,XY and one with chromosomal DSD (46,XY/46,XX) .

One patient with 46,XX DSD had an ovarian Leydig cell tumor. In this patient, ultrasonography did not reveal abnormalities. However, a diagnostic laparoscopy showed normal adrenal glands and large ovaries. During laparoscopy a biopsy was obtained. In a 23-year-old 46,XY patient, the testis showed Leydig cell hyperplasia and atrophy of most seminiferous tubules but no evidence of CIS. A thirteen-year-old 46,XY boy was found to have Carcinoma *in situ* (CIS), the precursor lesion for malignant germ cell tumors, as reported recently (24). Ovarian tissue with multiple cysts including primordial follicles and granulosa cells was found without evidence of malignancy in one patient with mosaic XX/XY.

6. Final Classification

Based on the above mentioned steps in the diagnostic workup in patients with DSD syndromes a final classification was made following the current consensus statement (8). Data are shown in table 4. In sixteen 46,XX DSD patients (72%) a tertiary root classification was made, in patients with 46,XY DSD this was the case in 12 patients (21%) and in the group of patients with chromosomal DSD a tertiary root classification was made in one patient (12.5%).

Of course the last category is a special one because the chromosomal abnormalities itself are an explanation for the etiology. In the remaining patients without an identified genetic or pathologic cause of DSD, the tertiary root category had to be 'other'.

The 6 patients aged less than one year were diagnosed as follows: androgen action disorder (2), excess androgen (2), and unknown male undermasculinization (2).

The two 46,XY patients who changed gender from female to male had the final diagnosis of androgen action disorder, while one 46,XX CYP21A2-deficient patient decided to live as a male.

Table 3: Additional clinical data in patients with known histology results.

Number	Age	Gender	Karyotype	Quigley Stage	Hormonal Analysis	Imaging	Surgery	Mutation	Histology	Diagnosis
1	33	Female	46 XY	4	very high testosterone slightly increased values of gonadotrophines	no abnormalities	laparoscopy	no	Ovarian Leydig cell tumor lacking histological signs of malignancy	Disorder of Androgene excess
2	23	Female	46 XY	6	high basal testosterone level	no abnormalities	gonadectomy	AR neg	Leydic cell hyperplasia and atrophy of most seminiferous tubules but no evidence of CIS	Disorder of Androgene action
3	13	Male	46 XY	3	high basal testosterone level	gonads not in situ*	laparoscopy	AR neg	CIS	PAIS
				no uterus and adnexa		gonadectomy				
				1/3 vagina present						
4	3	Male	46 XX/XY	2	hCG test: good response testosterone	no abnormalities	gonadectomy	SRY neg	Ovarian tissue with multiple cysts including primordial follicles and granulosa cells	Gonadal Dysgenesis
*Gonads already removed before ultrasound										

Table 4: Final classification

DSD classification	Number of	Total
46,XX DSD		
Disorder of androgen excess, other	2	22
Disorder of androgen excess, 21 hydroxylase deficiency	15	
Defect of mullerian development, other	4	
Disorder of gonadal development, ovotesticular DSD	1	
46,XY DSD		
Disorder of gonadal development, other	9	58
Disorder of androgen action, PAIS	12	
Disorder of androgen action, other	17	
Male undermasculinisation of unknown aetiology	20	
Chromosomal DSD		
46 XX/46 XY DSD, Disorder of gonadal development, ovotesticular DSD	1	8
47 XXY DSD, disorder of gonadal development, other	3	
45X/46XY DSD, disorder of gonadal development, other	2	
Other, disorder of gonadal development, other	2	

DISCUSSION

Reports on presentation and age distribution of DSD patients in Asian countries are scarce and are mostly limited to CAH patients (25-27). The age of presentation of the DSD patients in our study differs greatly from the age of presentation in the western world. More than 75% of the patients were over two years old. In India 58% of the patients are referred within the first year of life (26). Reasons for this late clinical referral are a lack of awareness among primary care providers, limited diagnostic and therapeutic facilities as well as socio-economic problems. Moreover, parents are reluctant to discuss sexual issues, even with medical professionals (28). Thailand has started the multidisciplinary management of ambiguous genitalia in 1979 (27) while in Semarang, Indonesia a start was only made in 1999.

Out of 88 patients nine (10.2%) were related, spread over four families. There was no known consanguinity of the parents in any of the cases. Thus in only nine patients a familiar background suggestive of an inherited disease could be established. One family with 2 affected

children and their cousin from mother's side had the same *AR* gene mutations (R840H). Identical mutations on *CYP21A2* were found in 2 siblings in one family (IVS2-12A>G).

Based on physical examination, chromosomal analysis, and hormonal data and in a limited set of patients imaging and laparoscopy, the patients were categorized in accordance with the current consensus statement (8). A secondary root diagnosis was made in all patients, however, it should be noted that the secondary root includes male-under masculinization of unknown etiology, which was assigned in 20 patients (34%). However, we cannot rule out 5 α -reductase deficiency in these patients.

In 15 out of 22 patients with 46,XX DSD the diagnosis CAH based on *CYP21A2* mutations was made, including two familial cases. As a result of the diagnostic procedure 12 patients are presently under steroid treatment. Three patients remained untreated (parents' request): two of them are sibs, and one of them showed a rather severe form of virilization. This patient was raised as a male and when the diagnosis of CAH was made at the age of 17 years he chose to continue to live as a male after full explanation was given. The parents decided to leave the 46,XX CAH sib (age 3 years) also untreated and are raising this child as a male. Parents of the third patient did not choose for hormonal treatment for economic reasons. One patient with presumed CAH died one week after inclusion. Because of lack of diagnostic and treatment options, it is suspected that patients with 46,XX DSD may have died from a crisis before coming to medical attention due to salt losing CAH (29, 30).

Interestingly, one patient at first thought to have CAH turned out to have an androgen producing ovarian tumor with the histology of Leydig cell tumor. This demonstrates the value of histological examination of abdominal lesions in these patients.

One patient was categorised as 46,XX, gonadal dysgenesis. Gene mutation analysis was done in a patient suspected of a glucocorticoid receptor defect, but no mutant sequence was found (31), and in none of the four patients with the clinical diagnosis of Mayer –Rokitansky Syndrome a *WNT4* mutation was detected (20).

Normal development and function of Sertoli cells and Leydig cells are essential for hormone – mediated sex differentiation of male internal and external genitalia.

In order to diagnose 46,XY DSD, determination of LH, FSH, gonadal steroids, AMH and Inhibin B levels are essential. Leydig cell activity is examined by hCG stimulation. In our patients measurement of testosterone precursors such as androstenedione, 17-OH progesterone, progesterone and DHEA did not give evidence of a testosterone synthesis disorder such as 17 β -HSD or 17-20 Lyase deficiency (5, 7). Only in two patients no rise of testosterone and its precursors was observed after hCG stimulation, suggesting an LH receptor defect. However, no mutation of the LH receptor gene was found (32). In most of the patients with the clinical phenotype of Leydig cell hypoplasia no causative mutations are found (6).

In pre-pubertal patients, low AMH levels indicate malfunctioning Sertoli cells in the testis. The best marker to evaluate the presence of functional testis after puberty is inhibin B (33). Circulating concentrations of AMH remain high until puberty when they fall in response to the effect of testosterone. For this reason, we decided to categorize normal AMH values based on testosterone levels (34). We confirmed that increased AMH levels after puberty (testosterone level >6 nmol/l) are suggestive for a disorder of androgen action or synthesis. The combination of high LH and testosterone levels in undermasculinized patients also supports a defect in androgen action. However in only 12 (25 %) of the in total 48 patients with 46,XY DSD with clinical and hormonal features compatible with altered androgen sensitivity an AR mutation was found. Two of the mutations were pathogenic and four mutations were unclassified variants which in later investigation were found to be pathogenic (23). All of these patients showed the typical features of partial androgen insensitivity. It is noteworthy that 11 patients are being raised as males and only one as female. In none of the patients classified as severe (penoscrotal) hypospadias an AR mutation was found.

All patients with Chromosomal DSD had a chromosomal mozaicism. Three patients had a Klinefelter variant; all of them were raised as males.

Although imaging procedures are highly informative for the establishment of a second root diagnosis, sonography was only performed in 28/88 patients (31.8%) and a diagnostic laparoscopy or cystoscopy was done in only nine of the patients (10.2%). Limited facilities and economic problems are the main reason for these relatively low numbers. In 30/58 patients with

46,XY DSD (51.7%), genital surgery was performed such as hypospadias correction, gonadectomy and mastectomy. The rest of the patients (n=28) remained untreated mostly for economic reasons. Some patients or parents refused the advice of the gender team and just dropped out (n=9). One assumption is that the cultural reasons are of influence. Decision-making is not just based on what is recommended by the doctor, but is influenced by the family.

The risk of developing a malignant germ cell tumor is increased in patients with DSD containing Y chromosomal material, known as the Gonadoblastoma locus on the Y chromosome (GBY). This phenomenon is probably related to the expression of the *TSPY* gene on the Y chromosome (19). It is important to mention that a non-scrotal position of the gonad increases this risk. Pro-active clinical interference, like orchidopexy, biopsy or even gonadectomy, is recommended in patients with 46,XY DSD with maldevelopment of the testes (with or without known gene mutation such as *WT1*) and in addition, in patients with PAIS, especially when the gonads are in a non-palpable position (10). It is noteworthy that in our small sample of four gonadectomized patients already one patient with 46,XY DSD had developed carcinoma *in situ* (CIS). This is the known precursor of malignant germ cell tumors, which will progress to invasiveness in about 70% within seven years. A biopsy was not performed in all patients at risk because of the limited resources. This raises the question whether a prophylactic gonadectomy in all patients at risk for a malignancy should be performed. At the moment research is conducted focussing on the identification of factors to estimate the actual cancer risk in the individual patient to prevent unnecessary prophylactic gonadectomy (10).

A point of debate is the inclusion of the histology and the genetic analysis in a diagnostic workup. Of course mutation analysis provides confirmation, whereas the histology is also an important prognostic parameter as a base for further treatment. We used the tertiary root only if a mutation was found or if gonadal histology was known. This was the case in 29 out of 88 patients (32%).

In conclusion, in daily practice the implementation of the current consensus statement in a resource – poor environment is very difficult. Especially the tertiary root classification that is based on molecular genetic or histopathology diagnostics is in many cases not feasible.

Therefore we recommend the following stepwise approach: As a first step a careful clinical evaluation, karyotyping of peripheral blood and sonographic imaging of the internal genitalia should be performed in all patients.

Subsequently, in the 46 XX patients rapid determination of 17-hydroxyprogesterone in serum or saliva (35) are needed in the first week of life in order to recognize a salt-losing CAH and prevent a life-threatening crisis. An increased level is highly suggestive of the diagnosis CAH and needs to be followed by immediate initiation of life-saving treatment. In patients with 46, XY or Y chromosome containing DSD, determination of gonadotropins, testosterone, DHT, inhibin B and AMH is to be performed. Dependent on age and stage of puberty, a second root working diagnosis can be made allowing gender assignment and planning for further diagnostic procedures and management in collaboration with global DSD centers of excellence.

This implies the need for education of primary health care workers on how to recognize DSD as a clinical feature that requires urgent assessment to prevent morbidity and mortality in some cases. Protocols on referral pathways should be implemented.

Unfortunately, in Indonesia several factors such as patient's and general society's opinion on DSD problems, economic background of DSD patients and lack of access to health insurance can affect the complex management of DSD in a negative way.

References

1. MacLaughlin DT, Donahoe PK. Sex determination and differentiation. *N Engl J Med*. 2004 Jan 22;350(4):367-78.
2. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev*. 2007 Jan;87(1):1-28.
3. Rey R. How to evaluate gonadal function in the cryptorchid boy. Lessons from new testicular markers. *J Pediatr Endocrinol Metab*. 2003 Mar;16(3):357-64.
4. Boehmer AL, Brinkmann AO, Sandkuijl LA, Halley DJ, Niermeijer MF, Andersson S, et al. 17Beta-hydroxysteroid dehydrogenase-3 deficiency: diagnosis, phenotypic variability, population genetics, and worldwide distribution of ancient and de novo mutations. *J Clin Endocrinol Metab*. 1999 Dec;84(12):4713-21.
5. Boehmer AL, Brinkmann O, Bruggenwirth H, van Assendelft C, Otten BJ, Verleun-Mooijman MC, et al. Genotype versus phenotype in families with androgen insensitivity syndrome. *J Clin Endocrinol Metab*. 2001 Sep;86(9):4151-60.
6. Richter-Unruh A, Martens JW, Verhoef-Post M, Wessels HT, Kors WA, Sinnecker GH, et al. Leydig cell hypoplasia: cases with new mutations, new polymorphisms and cases without mutations in the luteinizing hormone receptor gene. *Clin Endocrinol (Oxf)*. 2002 Jan;56(1):103-12.
7. Van Den Akker EL, Koper JW, Boehmer AL, Themmen AP, Verhoef-Post M, Timmerman MA, et al. Differential inhibition of 17alpha-hydroxylase and 17,20-lyase activities by three novel missense CYP17 mutations identified in patients with P450c17 deficiency. *J Clin Endocrinol Metab*. 2002 Dec;87(12):5714-21.
8. Hughes IA, Houk C, Ahmed SF, Lee PA, Lawson Wilkins Pediatric Endocrine Society /European Society for Paediatric Endocrinology Consensus G. Consensus statement on management of intersex disorders. *J Pediatr Urol*. 2006 Jun;2(3):148-62.
9. Hughes IA. Disorders of sex development: a new definition and classification. *Best Pract Res Clin Endocrinol Metab*. 2008 Feb;22(1):119-34.
10. Cools M, Drop SL, Wolffenbuttel KP, Oosterhuis JW, Looijenga LH. Germ cell tumors in the intersex gonad: old paths, new directions, moving frontiers. *Endocr Rev*. 2006 Aug;27(5):468-84.
11. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev*. 1995 Jun;16(3):271-321.
12. Pierik FH, Deddens JA, Burdorf A, de Muinck Keizer-Schrama SM, Jong FH, Weber RF. The hypothalamus-pituitary-testis axis in boys during the first six months of life: a comparison of cryptorchidism and hypospadias cases with controls. *Int J Androl*. 2009 Oct;32(5):453-61.
13. Lamberts SW, Bons EG, Bruining HA, de Jong FH. Differential effects of the imidazole derivatives etomidate, ketoconazole and miconazole and of metyrapone on the secretion of cortisol and its precursors by human adrenocortical cells. *J Pharmacol Exp Ther*. 1987 Jan;240(1):259-64.

14. Andersson AM, Juul A, Petersen JH, Muller J, Groome NP, Skakkebaek NE. Serum inhibin B in healthy pubertal and adolescent boys: relation to age, stage of puberty, and follicle-stimulating hormone, luteinizing hormone, testosterone, and estradiol levels. *J Clin Endocrinol Metab.* 1997 Dec;82(12):3976-81.
15. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988 Feb 11;16(3):1215.
16. Goossens K, Juniarto AZ, Timmerman MA, Faradz SM, Wolffenbuttel KP, Drop SL, et al. Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients. *Clin Endocrinol (Oxf).* 2009 Nov;71(5):628-35.
17. Androgen Receptor Gene Mutations Database [database on the Internet]. Available from: <http://androgendb.mcgill.ca/AR23C.pdf>.
18. Gottlieb B, Beitel LK, Trifiro MA. Variable expressivity and mutation databases: The androgen receptor gene mutations database. *Hum Mutat.* 2001 May;17(5):382-8.
19. Hersmus R, de Leeuw BH, Wolffenbuttel KP, Drop SL, Oosterhuis JW, Cools M, et al. New insights into type II germ cell tumor pathogenesis based on studies of patients with various forms of disorders of sex development (DSD). *Mol Cell Endocrinol.* 2008 Sep 10;291(1-2):1-10.
20. Philibert P, Biason-Lauber A, Rouzier R, Pienkowski C, Paris F, Konrad D, et al. Identification and functional analysis of a new WNT4 gene mutation among 28 adolescent girls with primary amenorrhea and mullerian duct abnormalities: a French collaborative study. *J Clin Endocrinol Metab.* 2008 Mar;93(3):895-900.
21. Newmark JR, Hardy DO, Tonb DC, Carter BS, Epstein JI, Isaacs WB, et al. Androgen receptor gene mutations in human prostate cancer. *Proc Natl Acad Sci U S A.* 1992 Jul 15;89(14):6319-23.
22. Peterziel H, Culig Z, Stober J, Hobisch A, Radmayr C, Bartsch G, et al. Mutant androgen receptors in prostatic tumors distinguish between amino-acid-sequence requirements for transactivation and ligand binding. *Int J Cancer.* 1995 Nov 15;63(4):544-50.
23. Elfferich P, Juniarto AZ, Dubbink HJ, van Royen ME, Molier M, Hoogerbrugge J, et al. Functional analysis of novel androgen receptor mutations in a unique cohort of Indonesian patients with a disorder of sex development. *Sex Dev.* 2009;3(5):237-44.
24. Cools M, van Aerde K, Kersemaekers AM, Boter M, Drop SL, Wolffenbuttel KP, et al. Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilization syndromes. *J Clin Endocrinol Metab.* 2005 Sep;90(9):5295-303.
25. Armstrong KL, Henderson C, Hoan NT, Warne GL. Living with congenital adrenal hyperplasia in Vietnam: a survey of parents. *J Pediatr Endocrinol Metab.* 2006 Oct;19(10):1207-23.
26. Bajpai A, Kabra M, Menon PS. 21-Hydroxylase deficiency: clinical features, laboratory profile and pointers to diagnosis in Indian children. *Indian Pediatr.* 2004 Dec;41(12):1226-32.

27. Nimkarn S, Likitmaskul S, Sangacharoenkit P, Pathomvanich A, Sawathiparnich P, Wacharasindhu S, et al. Ambiguous genitalia: an overview of 22 years experience and the diagnostic approach in the Pediatric Department, Siriraj Hospital. *J Med Assoc Thai.* 2002 Aug;85 Suppl 2:S496-505.
28. Warne GL, Raza J. Disorders of sex development (DSDs), their presentation and management in different cultures. *Rev Endocr Metab Disord.* 2008 Sep;9(3):227-36.
29. Speiser PW, Azziz R, Baskin LS, Ghizzoni L, Hensle TW, Merke DP, et al. Congenital adrenal hyperplasia due to steroid 21-hydroxylase deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab.* 2010 Sep;95(9):4133-60.
30. Strnadova KA, Votava F, Lebl J, Muhl A, Item C, Bodamer OA, et al. Prevalence of congenital adrenal hyperplasia among sudden infant death in the Czech Republic and Austria. *Eur J Pediatr.* 2007 Jan;166(1):1-4.
31. van Rossum EF, Lamberts SW. Glucocorticoid resistance syndrome: A diagnostic and therapeutic approach. *Best Pract Res Clin Endocrinol Metab.* 2006 Dec;20(4):611-26.
32. Piersma D, Verhoef-Post M, Berns EM, Themmen AP. LH receptor gene mutations and polymorphisms: an overview. *Mol Cell Endocrinol.* 2007 Jan 2;260-262:282-6.
33. Tuttelmann F, Dykstra N, Themmen AP, Visser JA, Nieschlag E, Simoni M. Anti-Mullerian hormone in men with normal and reduced sperm concentration and men with maldescended testes. *Fertil Steril.* 2009 May;91(5):1812-9.
34. Rey R. Anti-Mullerian hormone in disorders of sex determination and differentiation. *Arq Bras Endocrinol Metabol.* 2005 Feb;49(1):26-36.
35. Juniarto AZ, Goossens K, Setyawati BA, Drop SL, de Jong FH, Faradz SM. Correlation between androstenedione and 17-hydroxyprogesterone levels in the saliva and plasma of patients with congenital adrenal hyperplasia. *Singapore Med J.* 2011 Nov;52(11):810-3.

Chapter 4
**Clinical evaluation of 286 patients with a Disorder of Sex
Development (DSD) from Indonesia in relation to phenotype and
genotype**

A. Zulfa Juniarto, Yvonne G. van der Zwan, Ardy Santosa, Annastasia Ediati, Stefanie Eggers, Remko Hersmus, Axel P.N. Themmen, Hennie T. Bruggenwirth, Katja P. Wolffenbuttel, Jacky Hewitt, Erin Turbitt, Andrew Sinclair, Stefan J White, Leendert H.J. Looijenga, Sultana MH Faradz, Frank H. de Jong, Stenvert L.S. Drop

Submitted

Abstract

Objective: This study determined the etiological spectrum of DSD in a large cohort of patients and investigated which clinical, biochemical and genetic parameters are most useful in the diagnosis and management of patients with DSD in an underprivileged population.

Methods: Two hundred and eighty six patients with atypical external and/or internal genitalia from Semarang, Indonesia, previously undiagnosed and referred between 2004 and 2010 were included. The diagnostic evaluation included clinical, imaging, hormonal, molecular genetics, and histological parameters. Patients were categorized according to the recent consensus statement on DSD.

Results: The age at presentation was 0-0.5 years in 41 cases (14.3%), >0.5-12 years in 181 cases (63.3%), and >12 years in 64 cases (22.4%). 46,XY DSD was most common (68.2%, n=195), while 46,XX DSD was found in 23.4% of the patients (n=67) and sex chromosome DSD in 8.4 % (n=24) of cases. In 61.2 % of the 46,XX DSD patients, 17.9% of the 46,XY DSD patients and all sex chromosome DSD patients (27% in total) a final diagnosis was reached based on genetic (karyotype and/or gene mutation analysis) or histological evaluation. Familial DSD was reported in eleven cases from five families with 46,XX DSD, and in 11 cases from five families with 46,XY DSD.

No consanguinity of parents of any of the patients was reported. Serum determination of 17-hydroxyprogesterone and androstenedione were the most distinctive parameter in 46,XX DSD patients in whom congenital adrenal hyperplasia was the most common diagnosis (59.7%). Mutations in *CYP21A2* or *CYP11B1* were found in all of these. In the remaining 46,XX DSD patients determination of LH, FSH, testosterone and AMH were the subsequent parameters to establish gonadal function. In 46,XY DSD the following diagnostic groups were identified based on external masculinization score (EMS): androgen action disorder (AAD), unknown male undermasculinization (UMU) and gonadal dysgenesis (GD). Serum levels of LH, FSH and basal testosterone differed between AAD and UMU versus GD, particularly in post pubertal patients. hCG tests were of no additional value as no patients with androgen synthesis disorders were found. *AR* mutations were found in only 24.5% and 1.8 % of patients with AAD and UMU,

respectively. In 16% of 46,XY GD patients causative copy number variants of several genes were found. The karyogram of 18 out of the 24 patients with sex chromosome DSD contained a Y-chromosome. Hormonal profiles of the patients with a Y-chromosome containing karyotype showed high levels of LH and FSH, and low levels of AMH, inhibin B, and testosterone.

Conclusion: A stepwise practical diagnostic approach in a large cohort of DSD patients in Indonesia led to a genetically or histologically proven final diagnosis in 27 % of the patients. The most helpful parameters were serum levels of 17-hydroxyprogesterone and androstenedione in 46,XX DSD patients and LH, FSH and basal testosterone levels in 46,XY DSD patients. New Massively Parallel Sequencing techniques, which provide a higher resolution, are expected to improve the percentage of patients who can be given a molecular diagnosis

INTRODUCTION

Sex determination and differentiation are regulated by a complex developmental network. Chromosomal sex determines gonadal sex, which in turn determines phenotypic sex (1). This has been referred to as the "central dogma" of sex differentiation (2). Sex determination results from the expression of genes that cause the bi-potential gonads to develop into either testes or ovaries.

Testicular differentiation occurs after the onset of the expression of sex-determining region Y (*SRY*) in a subset of somatic cells. This leads to their differentiation into Sertoli cells, which in turn produce anti-Müllerian hormone (AMH). These cells are the supportive cell lineage surrounding the developing germ cells. AMH inhibits the differentiation of the Müllerian ducts into a uterus and other Müllerian structures. Furthermore, the developing Leydig cells start secreting testosterone, which causes the stabilization of the Wolffian ducts. In females, there is no obvious single dominant gene that determines the sex of somatic and germ cells in the ovary (3-5).

Disorders of sex development (DSD) cover many different phenotypes of atypical sex anatomy, which result from faults in the underlying network that regulates gonadal development and differentiation. Many genes are involved in known pathways that lead to gonadal formation

and differentiation. In addition, potentially novel and alternative pathways may play a role (3,4). Therefore, whereas the scope of molecular genetic analysis has markedly expanded, it is still not always possible to identify the molecular genetics causes of these disorders.

This paper describes the various steps in the diagnostic approach of a large cohort of Indonesian patients with DSD. Most of them had never received medical attention relating to DSD. The aim of our study was to determine the etiological spectrum of DSD. In addition, clinical and laboratory data were analyzed to determine which clinical, biochemical and molecular genetics parameters proved most useful to reach a final diagnosis relevant for further management.

SUBJECTS AND METHODS

Patients

For this study we evaluated 286 patients with various DSD phenotypes were evaluated. They were referred for chromosomal analysis by clinicians of the departments of Urology, Pediatrics, Internal Medicine and Obstetrics to the gender team of the Dr. Kariadi Hospital, Semarang, Indonesia. Referral and data collection took place between 2004 and 2010 at the division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University, Semarang, Indonesia. Reasons of referral were the presence of ambiguous genitalia or atypical external or internal genitalia, including severe hypospadias, with or without descended testes. Eighty eight of the patients included in this study have been described previously (6).

Methods

A detailed interview was performed at admission. Data concerning medical history, age of initial presentation, sex of rearing, family history (relatives with a genital disorder) and consanguinity were collected.

The patients were clinically evaluated. A detailed description of the external genitalia was obtained and the genitalia of 46,XY and sex chromosome DSD were staged according to Quigley (7), as well as using the External Masculinization Score (EMS) (8). The Prader stage

was used to determine the degree of virilization in patients with 46, XX DSD (9). Dysmorphic features were recorded.

A peripheral blood sample was obtained from all patients for karyotyping, hormonal analysis and DNA extraction for genetic analysis. Karyotypes were determined using a G-banding technique. In patients with 46, XY DSD or Y chromosome containing aneuploid DSD an additional blood sample was obtained 72 hr after the intramuscular injection of 1500 IU human chorionic gonadotropin (hCG;Pregnyl^R, MSD, Oss, The Netherlands).

The medical ethics committee of Dr Kariadi Hospital approved this study and informed consent was obtained from all participants, their parents or guardians prior to their participation in this study.

Diagnostic criteria

Patients who were identified on the basis of genital ambiguity or atypical external and/or internal male or female genital development were categorized according to the primary root of the recent classification based on karyotype: 46,XX DSD, 46,XY DSD or sex chromosome DSD (10).

Serum hormone measurements

The measurements of inhibin B, AMH, LH and FSH in the basal serum samples were performed as described previously (11). Testosterone concentrations were determined in serum collected before and after injection of hCG using the Coat-a-Count radioimmunoassay (Siemens, Los Angeles, CA) (11). Androstenedione was also measured in these samples using the Immulite 2000 (Siemens). Finally, 17-hydroxyprogesterone and 11-desoxycortisol levels were estimated using in house methods (12). Age and sex dependent hormone levels in the normal population are given in supplementary Tables 1 and 2 (11, 13-15).

Genetic analysis

DNA was extracted from leucocytes of EDTA blood using the salting out method as described earlier (16). Based on the clinical and hormonal information, specific genes were

analysed such as *CYP11B1* (11 β -hydroxylase; P450c11 β reference sequence NM_000497.3 (17, 18), *CYP21A2* (21-hydroxylase; P450c21)(19), the *LHCGR* (LH receptor) (20) and the Glucocorticoid Receptor (*GR*) (21). The genes encoding the androgen receptor (*AR*), *SRY* and *WNT4* were analysed by Sanger sequencing of the coding exons and exon-flanking intronic regions (reference sequence *AR*: NM_000044.2 (22-24), *SRY*: X53772.1 and *WNT4*: NM_030761). Primer sequences and locations are available upon request.

Patient DNAs were also analysed for large genomic re-arrangements and copy number variations (CNVs), using genome wide SNP chip arrays (different versions of Illumina arrays) (25) and/or Multiplex Ligation-dependant Probe Amplification (MLPA) (26). MLPA was also used to confirm CNVs which were identified using arrays.

Pathology

Histo-pathological assessments of gonadal tissue following gonadal excision or biopsy were performed using hematoxylin and eosin staining and immuno-histo-chemistry for various germ cells markers, e.g. OCT3/4, TSPY, VASA, SCF (including double staining for OCT3/4-TSPY or VASA); as well as SOX9 and FOXL2 for supportive cells as described earlier (6, 27).

Flow of the diagnostic process

For the first diagnostic evaluation, patients were grouped based on their karyotype, and further assessment for the second stage of diagnosis was conducted based on clinical data, notably EMS with or without imaging (USG, MRI). The analysis of candidate genes was carried out based on the results of the two previous assessments and the results of the hormonal evaluation.

Statistics

Data was processed using the IBM SPSS statistics 20 software. To calculate the differences between hormonal values for each diagnosis, we used ANOVA followed by post hoc Tukey analysis. Pearson correlation coefficients were used to determine the relationship between the EMS score and the androgen sensitivity index. Mann-Whitney U tests and Independent T

tests were used to determine the difference of various variables among patients with an androgen action disorder with and without *AR* mutations. Data are presented as means \pm s.e.m.

RESULTS

Amongst the 286 patients 46,XY DSD was most common accounting for 68.2% (195 patients), 46,XX DSD accounted for 23.4% (67 cases), and 8.4% (24 cases) had a sex chromosome DSD, as shown in Table 1 and Figure 1.

The age at presentation was 0-0.5years in 41 cases (14.3%), >0.5-12 years in 181 cases (63.3%), and >12 years in 64 cases (22.4%). Familial DSD was reported in eleven cases from five families with 46, XX DSD, and in eleven cases from five families with 46, XY DSD. Consanguinity of parents of any of the patients was not reported.

46, XX DSD

Hormonal evaluation

From a total of 67 patients, 43 presented with varying degrees of virilization of the external genitalia. Forty of them showed hormonal levels characteristic for CAH while three had unclassified hyperandrogenism. 21-hydroxylase deficiency (*CYP21A2*) was suspected in 38 patients based on increased levels of 17-hydroxyprogesterone, androstenedione and testosterone, while in two cases 11 β -hydroxylase dehydrogenase (*CYP11B1*) deficiency was suggested due to increased levels of 17-hydroxyprogesterone (Table 2) and 11-desoxycortisol (177 and 371 nmol/l, respectively). Clinically salt wasting was suspected in at least eleven cases based on intercurrent illnesses with hyponatremic episodes, but no confirmation by laboratory tests was available. Five of these patients died during the time this study was performed. One patient with a sex chromosome mosaic DSD, 46, XX (96%)/46, XY (4%) was also diagnosed with a 21-hydroxylase deficiency. As expected, LH, FSH, and inhibin B levels were normal with low levels of cortisol and AMH for age.

Table 1. Summary of patient diagnosis and characteristics

Diagnosis	Total	median (range) age at presentation (years)	sex of rearing			Prader score	EMS score	number of family
			male	NA	female			
46.XX DSD	67							
Disorders of gonadal development	2		2	0	0			2
Androgen excess						3.7 (2-6)		
Fetal (CAH)								
- mutation CYP21A2	38	6.5 (0.01-33)	11 (6)	4	23 (28)		4 (1-7)	34
- mutation CYP11B1	2	11 (3-12)	0	0	2		(4-7)	1
unclassified androgen excess	3	0.1 (1-29)		2	1		1 (1-4)	3
Other								
Defect of mullerian development (MRKH)	12	21 (2-27)	0	0	12		1	12
Unclassified	10	3 (0.2-15)	2	0	8		1 (1-5)	10
						Quigley stage		
46.XY DSD	195							
Androgen action disorder (AAD)								
AR mutation +	24	9(0.1-26)	20	0	4	3 (2-6)	6(1-8.5)	21
AR mutation -	73	1.95 (0.02-23)	64	2	7	3 (2-6)	6(2-8.5)	72
Disorders of gonadal development	31	14.5(0.1-10.5)	15	2	14	3 (2-7)	5 (1-10)	29
Other								
Unknown male undermasculinisation (UMU)	55	7 (0.1-29)	55	0	0	2 (2-4)	9 (9-11)	54
unclassified	12	1.2 (0.01-12)	10	1	1	4 (1-6)	6 (1-8)	12
							4.5 (1-10)	
Sex Chromosome DSD	24	10 (0.1-39)	13	1	10			24
		KARYOTYPE						
Turner syndrome variants	4	X/XX						
Klinefelter syndrome variants	3	XY/XXY						
Mixed gonadal dysgenesis	8	X/XY						
Chimeric. ovotesticular DSD	6	XX/XY XX/XXq- ;XXq-;						
Other	3	X,idiicY						
Total	286		192	11	83			

NA*: Not available

Figure 1. Stepwise diagnostic evaluation in 286 patients with DSD in Semarang Indonesia

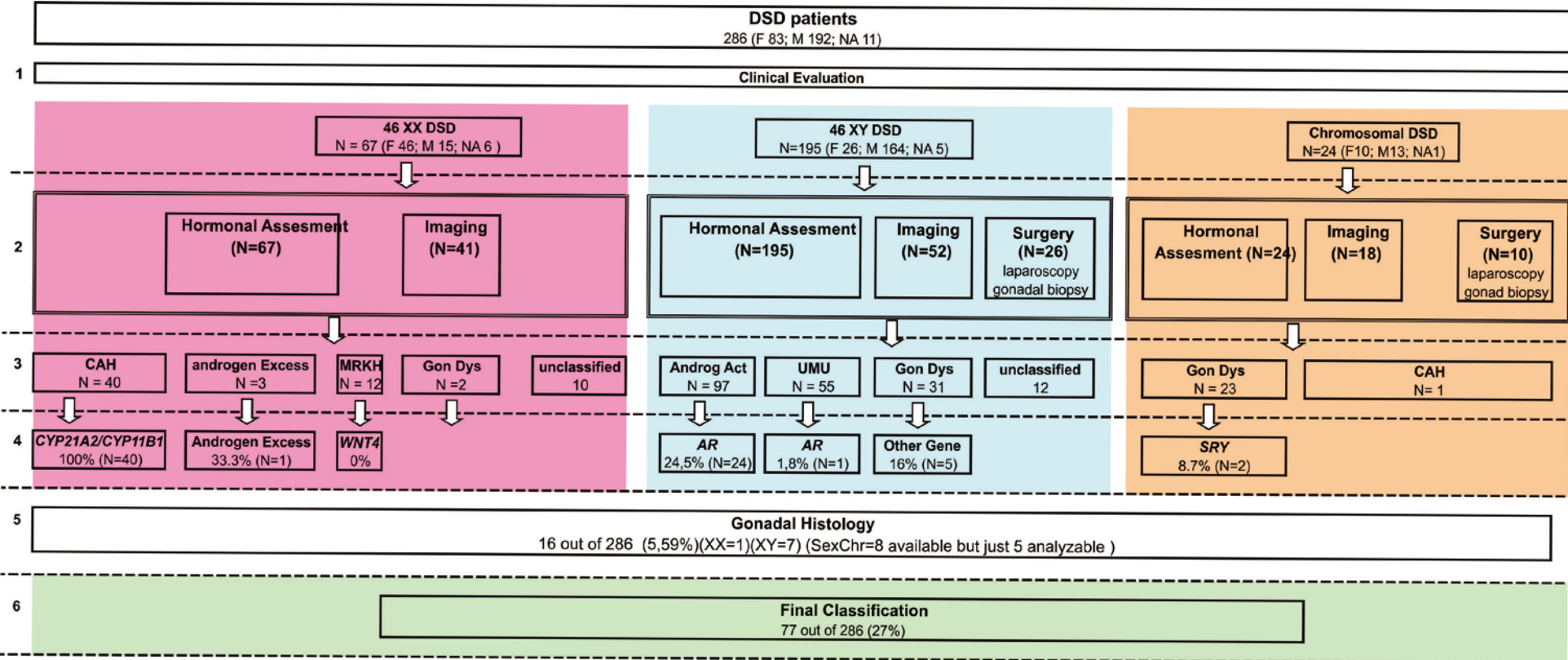


Table 2. Hormonal values for 46,XX DSD

AGE (yrs)	N	LH (IU/l)	FSH (IU/l)	Cortisol (nmol/l)	A'dione (nmol/l)	17-OHP (nmol/l)	Testosterone (nmol/l)	AMH (ug/l)	Inhibin B (ng/l)
CYP 21*									
0-0.5	9	0.49±0.18	2.08±1.06	110±30	44.8±16.6	296±133	8.4±4.4	1.86±0.88	36.3±11.8
>0.5-12	18	0.97±0.50	2.63±0.65	141±20	49.4±9.0	392±46	8.6±1.5	3.92±0.48	28.5±6.9
>12	4	1.96±0.95	5.26±1.99	138±24	111±51.8	484±246	16.7±7.4	3.21±1.27	42
CYP11B1									
>0.5-12	2	<0.1	0.22±0.12	<35	133±2.5	15±5.3	12.0±4.0	2.20±0.60	25.5±7.5
Ovarian Tumor									
>12	1	12.3	8.11	191	18	9.9	59.5	2.76	NA
Cortisol Resistance									
0-0.5	2	0.2±0.06	3.43±2.66	1343±172	63.8±16.9	14.5±0.8	4.05±1.35	0.25±0.19	38±35
Transient virilization									
>0.5-12	2	0.09±0.04	3.87±1.14	229±163	NA	15.2	<0.1	6.23±3.88	52±16
Gonadal Dysgenesis									
0-0.5	1	0.69	18.5	76.7	<1.05	0.7	0.1	0.8	62
>0.5-12	1	0.18	2.55	387	<1.05	4.5	0.3	60	47
Cloacal Malformation									
0-0.5	1	1.22	24.8	83	3.71	0.9	0.2	0.38	NA
>0.5-12	7	1.14±0.48	5.92±1.20	265±75	6.6±4.8	1.29±0.44	0.29±0.11	3.73±1.43	72.7±20.0
MRKH									
>0.5-12	2	0.15±0.01	2.53±0.01	187±27	<1.05	0.3±0.2	<0.1	4.19±2.31	67.5±59.5
>12	10	7.0±1.54	4.71±0.72	292±41	8.7±0.42	4.3±0.66	1.34±0.19	9.67±1.38	96.3±17.0

* we excluded seven patients who had been treated with glucocorticoid

Among the three patients with unclassified androgen excess, one patient was diagnosed with an ovarian Leydig cell tumor confirmed by histo-pathological analysis (28). In two patients high levels of cortisol and androgens were found (Table 2) and glucocorticoid resistance was suspected.

Gonadal dysgenesis was found in two patients, presenting with ambiguous genitalia. In one patient the serum level of FSH was high and of AMH low for age, whereas in the other both levels were normal in the presence of testes.

Mayer-Rokitansky-Kuster-Hauser syndrome (MRKH) was suspected in phenotypic females with normal gonadal function with hypoplasia of the vagina and absence of the uterus.

Eight out of ten patients in the “46,XX DSD other, unclassified” group had cloacal malformations with normal hormonal data for age whereas two showed clinically transient hypervirilization. Follow up revealed normal female genitalia (see Table 2).

Molecular genetics evaluation

The diagnosis of CAH was confirmed in all patients by *CYP11B1* (n=2) or *CYP21A2* (n=38) gene mutation analysis. As reported previously (19), gene sequencing revealed p.R356W, c.IVS2 - 12A>G and p.I172N as the most common *CYP21A2* mutations. In the MRKH patients *WNT4* gene mutation analysis was negative. In the two patients with suspected glucocorticoid resistance gene analysis did not show mutations in the *GR*.

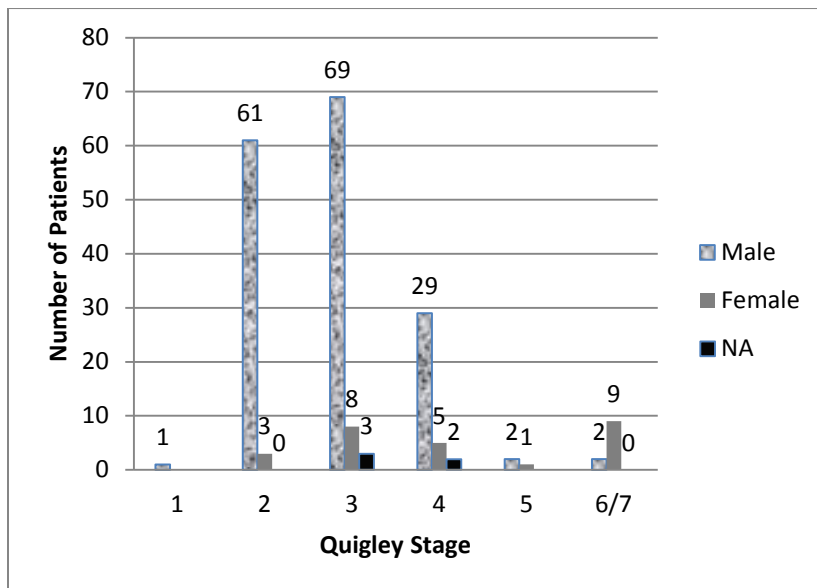
46, XY DSD

We observed a preference of male sex of rearing in these patients: of 180 patients with a severe degree of ambiguity (Quigley stage 2-4), 159 were assigned to the male sex (Figure2).

Hormonal evaluation

On the basis of an EMS score <9, ninety-seven patients were classified as suspected of Androgen Action Disorder (AAD). On the basis of an EMS score ≥ 9 , 55 patients were classified as Unknown Male Undermasculinization (UMU) (29). Comparison between hormone levels in the AAD and UMU groups showed similar concentrations for LH, FSH and testosterone before and after hCG, AMH and inhibin B in all age groups, with the exception of increased levels of LH and basal testosterone in post-pubertal AAD patients (Figure 3). In none of the AAD or UMU cases the measured values of gonadal and adrenal steroids, as well as the T/DHT ratio, revealed an underlying diagnosis of an androgen synthesis defect or 5 α -reductase deficiency (data not shown).

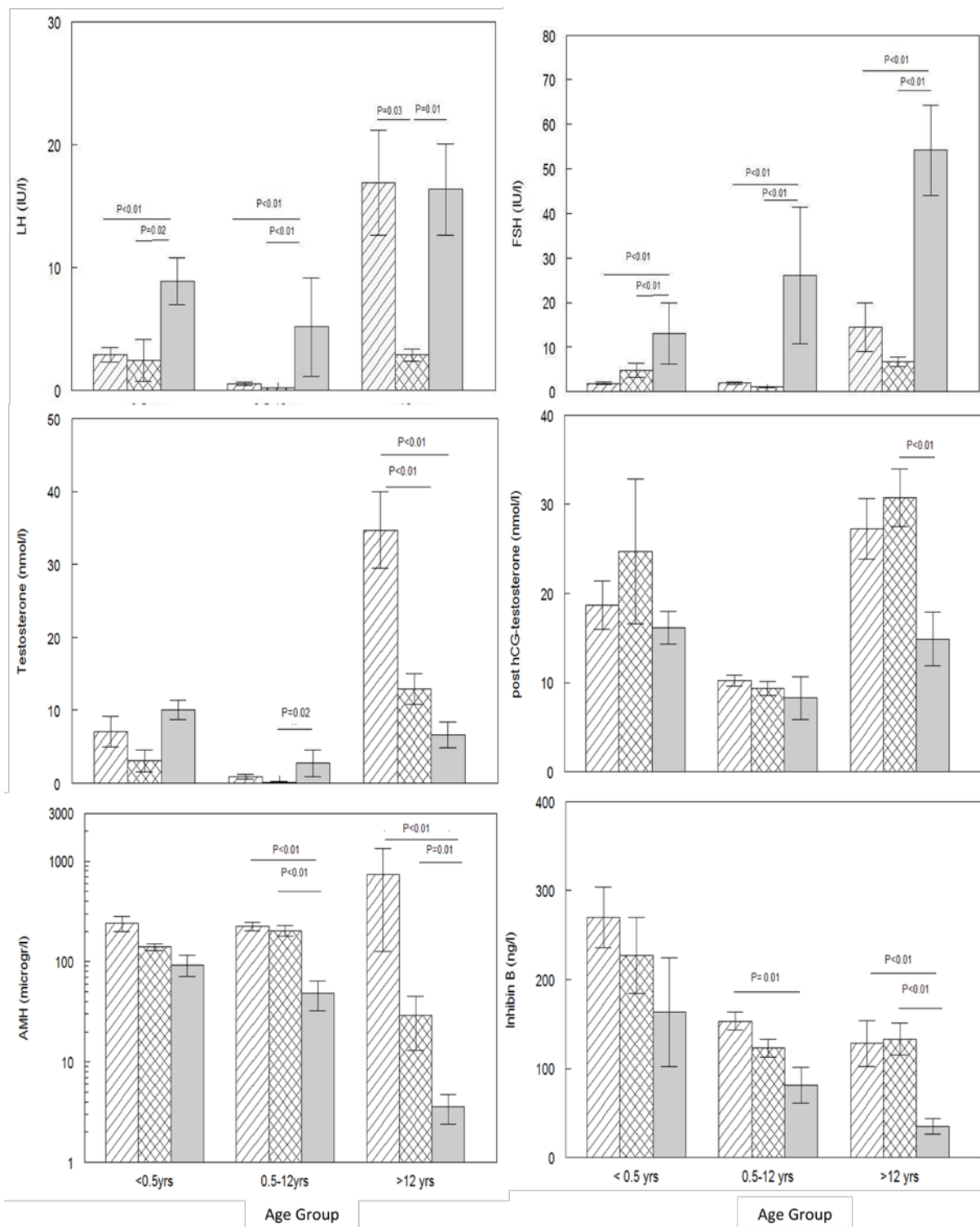
Figure 2. Quigley stage and gender of patient with 46,XY DSD



Gonadal dysgenesis was presumed in 31 patients. In this group of patients, external genitalia were often ambiguous, depending on the degree of impairment of gonadal function. Serum levels of LH and FSH were elevated compared to reference values, whereas AMH and inhibin B levels remained low for age in all age groups (Figure 3).

FSH levels were significantly higher in the group of gonadal dysgenesis compared to the groups of AAD and UMU patients at all ages. The same results were also shown for LH except in the age group over 12 years old, where LH levels were equally high in AAD and gonadal dysgenesis patients. AMH levels in patients with gonadal dysgenesis were decreased compared to the AAD and UMU groups but significant differences were only observed in the age groups >0.5-12 and >12 years. Inhibin B levels were significantly lower in the gonadal dysgenesis group, compared to the AAD group, except in newborn patients. In the post-pubertal age group (>12 years), inhibin B levels were significantly different between the UMU and gonadal dysgenesis patients.

Figure 3. Comparison of hormonal levels based on diagnosis and age



Basal testosterone levels in pre-pubertal boys (>0.5-12 yrs) with gonadal dysgenesis were higher compared to UMU but not different from AAD. These levels slightly increased after puberty but were then substantially lower than those in the other two groups (AAD & UMU). Post-hCG testosterone levels showed significantly different levels only in the age group of over 12 years old, and showed insufficient response to hCG in the group of gonadal dysgenesis (Figure 3).

The ratio of FSH over inhibin B was significantly higher in patients with gonadal dysgenesis (2.14 ± 0.57) compared to UMU (0.02 ± 0.004) and AAD (0.03 ± 0.009). This was already apparent in the age group of >0.5-12 years and became very obvious in patients over 12 years of age.

Statistical analysis of the data on the androgen sensitivity index (ASI, the product of the serum concentrations of LH and testosterone) for post-pubertal patients with AAD and UMU revealed a significant correlation between ASI and EMS score ($r = -0.615$, $p < 0.01$). Mean values of ASI in the AAD group were $516 \pm 91 \text{ U} \times \text{nmol/l}^2$, and $43 \pm 12 \text{ U} \times \text{nmol/l}^2$ in the UMU group (30).

Evaluation of the 12 unclassified cases revealed hormonal values within the normal range for the following cases: aphalia (n=2), cloacal anomalies (n=2), double penis (n=1), and severe hypospadias with multiple malformations (n=2). In the remaining five cases, results were compatible with hypogonadotropic-hypogonadism (n=2, low levels of LH, FSH and testosterone), Leydig Cell Hypoplasia (LCH, n=2, low testosterone after hCG test) and *CYP11A1* deficiency (n=1, high gonadotropins and low levels of testosterone and adrenal steroids) (see supplement Table 3).

Molecular Genetic Evaluation

The *AR* gene was sequenced in all patients with AAD and UMU. We detected *AR* sequence variants in 24 out of 97 patients (24.5%) with AAD (23 pathogenic mutations and 1 unclassified variant). The phenotype was partial androgen insensitivity in 22 patients and complete androgen insensitivity in two. In one out of 55 patients with UMU, a pathogenic *AR* mutation was detected. We identified a total of 19 types of *AR* mutation, none of which was

found to be most prevalent. Four mutations had been unclassified so far, but we showed previously that three of these are pathogenic in nature (31). In the 2 patients with LCH analysis of the *LHCGR* gene was negative.

As a final step, DNA of *AR* mutation negative patients with AAD or UMU (n=73 and 54 respectively), or with gonadal dysgenesis (n= 31), was subjected to further analysis. In the group with *AR* mutation negative AAD and UMU no CNVs were found. However, in five out of 31 gonadal dysgenesis cases CNVs were detected , which include the following DSD candidate genes : *DAX1* duplication (n=2), deletions of *DMRT1* (n=1), of *WT1* (n=1), of *WWOX* (n=1).

AR mutation positive versus AR mutation negative AAD patients

Multiple variables were analyzed to find out whether AAD patients having an *AR* mutation (n= 24) differed from patients without detected *AR* mutation (n= 73) (referred to as AAD (+) and AAD (-), respectively). Results are shown in Table 3.

Table 3. Comparison of AR(+) and AR (-) AAD patients

	Androgen Action Disorder		P value	Method
	Androgen Receptor Mutation (+) N=24	Androgen Receptor Mutation (-) N=73		
EMS*	5.60 ± 0.34	5.34 ± 0.17	0.202	Mann-Whitney U
Scrotal fusion (EMS)	1.50 ± 0.31	0.78 ± 0.16	0.030	Mann-Whitney U
Microphallus (EMS)	2.12 ± 0.28	1.76 ± 0.17	0.299	Mann-Whitney U
Urethral opening (EMS)	0.16 ± 0.08	0.11 ± 0.04	0.374	Mann-Whitney U
Quigley Stage	3.29 ± 0.23	3.15 ± 0.09	0.928	Mann-Whitney U
Delta testosterone**	6.95 ± 1.41	9.58 ± 0.77	0.095	t- test
SDS*** AMH	-0.19 ± 0.65	-0.63 ± 0.12	0.294	t- test
SDS Inhibin B	-0.55 ± 0.35	0.19 ± 0.18	0.046	t- test

* EMS = External Masculinisation Score ** difference between pre and post hCG test *** SDS = standard deviation score

With regard to the clinical appearance such as total EMS, micropenis, location of the urethral opening and Quigley stage, there were no significant differences between AAD (+) and AAD (-) patients except for the scrotal fusion score. There were hardly any differences in hormone levels between the AAD (+) and AAD (-) patients except inhibin B. We did not find a significant difference in ASI between these groups of patients.

Sex Chromosome DSD

The karyogram of 18 out of the 24 patients with sex chromosome DSD contained a Y-chromosome; five of these patients and all patients who did not carry a Y chromosome were raised as females. SRY analysis were performed in 7 patients and 2 patients found to have Y deletion.

All patients without a Y-chromosome containing karyotype had an EMS score 1 and were adults at the time of referral.

Hormonal profiles of the patients with a Y-chromosome containing karyotype showed the same tendencies as found in patients with 46,XY gonadal dysgenesis particularly in the post-pubertal group (>12 years), i.e. high levels of LH and FSH, and low levels of AMH, inhibin B, and testosterone (Table 4).

Histology

In 16 patients, gonadal tissue was available for histology following gonadectomy or biopsy. Due to specimen quality issues, the analysis could be performed on tissue samples of 13 patients. One 46,XX patient had a Leydig cell tumor (28). Three out of seven patients with 46,XY DSD showed Leydig cell hyperplasia while the other four showed the following: carcinoma *in situ* (CIS), CIS with seminoma, streak gonad and gonadoblastoma. Five patients with sex chromosome DSD showed an ovary with multiple cysts (46,XY/46,XX), a normal ovary (46,XY/46,XX), Sertoli cells only phenotype (46,XY/45,X), Leydig cell hyperplasia (46,X,idiY) and seminoma (46,XY/45,X)(27).

Table 4. Sex Chromosome DSD

Karyotype	AGE (years)	Number	TOTAL EMS	LH (IU/l)	FSH (IU/l)	Basal T* (nmol/l)	T*hCG (nmol/l)	AMH (ug/l)	Inhibin B (ng/l)
Y chromosome containing	0-0.5	2	2.66 ± 0.88	3.62 ± 1.66	4.99 ± 2.34	1.67 ± 1.57	11.5	35.8 ± 19.3	98 ± 22
	>0.5-12	12	7.27 ± 0.80	0.51 ± 0.29	9.76 ± 7.93	0.19 ± 0.05	6.38 ± 1.56	109 ± 33	94 ± 16
	>12	4	4.75 ± 1.29	13.1 ± 6.6	33.7 ± 15.6	6.05 ± 2.73	15.7 ± 5.7	9.0 ± 4.0	60 ± 12
Non-Y chromosome containing	>12	6	1 ± 0	10.3 ± 2.9	50.1 ± 18.8	0.65 ± 0.13	NA**	2.29 ± 0.99	47 ± 19

*T=Testosterone. **NA= Not available

DISCUSSION

We studied a large cohort of patients with a wide variety of genital anomalies referred to a single centre in Indonesia. The age distribution differs from what is observed in developed countries (32, 33). Patients referred after the age of one year and adults were dominant among our patients. Warne et al. pointed out that in many underprivileged Asian countries, a child born with ambiguous genitalia will grow up bearing the congenital anatomic sex features, which remain surgically untreated until adolescence or adulthood (33, 34). However, during the study period we noticed a trend in referral at an earlier age (data not shown).

The patients were grouped in accordance with the recommendations following a consensus meeting on DSD in 2005 (10). CAH was most common among 46,XX DSD patients. Following clinical and sonographic evaluation of these patients, the diagnosis of CAH was established with 17-hydroxyprogesterone and androgen measurements in serum and confirmed by gene mutation analysis. It should be noted that in 27/67 (40%) of the 46,XX DSD patients other causes of ambiguity or atypical genital development had to be considered. Among the three patients with unclassified androgen excess, in one patient an ovarian Leydig cell tumor was diagnosed. In two patients glucocorticoid resistance was suspected on the basis of the hormonal data but not confirmed by *GR* gene mutation analysis as described earlier (35).

We conclude that serum determinations of 17-hydroxyprogesterone and androstenedione are the most predictive parameters in determining the underlying cause in 46,XX DSD patients as CAH was the most common diagnosis whereas LH, FSH, testosterone and AMH levels are the subsequent parameters to determine the gonadal function in the non- CAH patients.

46,XY DSD was most prominent (68.2 %) in our patient population. Various studies from non-Western countries have shown different results with respect to the proportion of 46,XY DSD: Joshi *et al* (India), Gollu *et al* (Turkey), Al-Jurayyan (Saudi Arabia) and Nimkarn *et al* (Thailand), showed that 46,XY DSD accounts for 31-52 % of the DSD cases (36-39).

The diagnostic management of 46,XY DSD remains the greatest challenge. We made a distinction between patients with suspected AAD and UMU on the basis of the EMS score as suggested by Rodie *et al* (29). These authors studied 572 Scottish patients with DSD and found an EMS score < 9 in patients with disorders of androgen synthesis or androgen action. However in our AAD patient group we did not find any patients with androgen synthesis disorders and the AAD and UMU groups did not differ with respect to hormone levels before the age of 12 years, whereas in the post-pubertal patients both testosterone and LH levels were significantly higher in the AAD group compared to the UMU patients.

AR gene sequencing was performed in all patients with AAD and UMU and mutations were found in 25 of 152 patients, all but one being in the AAD group. The hormone levels of the 24 patients with androgen insensitivity, in whom indeed an *AR* mutation was found, did not differ from patients in whom no *AR* gene mutation was established. Apparently most of our patients classified as partial androgen insensitive, whereas in the recent study of Rodie *et al*, complete androgen insensitivity was most prominent (29). We could confirm the observation of Zuccarello *et al*. that the product of the levels of LH and testosterone forms an index for mild androgen insensitivity (30). There was a negative correlation between ASI and severity of virilization (EMS) in 46,XY DSD post-pubertal patients with AAD and UMU.

Subsequent CNV array and MLPA analysis in the *AR* mutation negative patients revealed no further diagnostic confirmation

Levels of gonadotropins are clearly increased in patients with gonadal dysgenesis already at a young age. Our results suggest that there is no further discriminatory value of the determinations of AMH and inhibin B. Subsequent gene mutation analysis yielded the confirmation of a mutation of a gene involved in gonadal development and differentiation in 5 out of 31 patients. However, a definite diagnosis for this group is the pathological examination of the gonads, which is cumbersome for logistical, socioeconomic and cultural reasons (6). Importantly, in spite of the significantly lower risk of gonadal germ cell tumors in the general Asian population a preliminary study indicated that this risk is increased in Indonesian patients with DSD (27).

From our study we conclude that examination of LH, FSH and basal testosterone are the most important parameters to distinguish between groups of patients with 46,XY DSD, followed by AMH or inhibin B. The hCG test had limited value as in this large cohort of patients, we confirmed our previous observation that we could not establish the diagnosis of androgen synthesis disorder caused by e.g. deficiencies of 17 β -hydroxysteroid dehydrogenase type 3 or 17-hydroxylase (6). We hypothesize that one of the reasons might be the absence of parents' consanguinity in our population in contrast to other studies (36, 40).

On the basis of these data we conclude that it is not recommended to sequence the AR gene in patients with 46, XY DSD with an EMS score ≥ 9 because of the very low yield. The aetiology of male undervirilization, also confusingly termed PAIS-like phenotype remains unclear, raising questions whether additional factors may play a role such as unknown genetic variations (resulting from DNA methylation, histone modifications or other unknown related genes) or environmental factors (41-45).

Regarding sex chromosome DSD, karyotyping is the most important measure to establish aneuploidy. Hormonal data are largely depending on the degree of gonadal differentiation. Measurement of AMH and inhibin B at a pre-pubertal age and additionally post-pubertal FSH are most indicative for the quality of gonadal function.

Management in DSD varies greatly depending on many factors. Of utmost importance is the determination of an accurate and rapid diagnosis. After a thorough evaluation of clinical, karyotype, biochemical and if possible molecular data, treatments can be applied on basis of the results of these investigations. Evaluation is meant to determine sex of rearing, to detect life-threatening conditions, to plan the long-term management, to explain related issues such as fertility, risk of tumor genesis, sexual function and to counsel the parents and/or patients themselves on genetic aspects (46, 47). The final goal is to reach an optimal long-term outcome.

In conclusion, DSD is not infrequent in the Indonesian community. The etiological spectrum is broad, 46,XY DSD being most common phenotype, with a remarkable absence of androgen synthesis disorders. Among the 46,XY DSD patients the distinction of androgen action disorder and male undervirilization syndrome cannot be made on the basis of hormonal evaluation apart from the difference in LH and testosterone levels after the age of 12 years. The yield of *AR* gene mutation analysis in UMU is very low. In 46,XX DSD, the levels of 17-hydroxy progesterone and androstenedione are the most important hormones that should be examined and the yield of *CYP21A2* or *CYP11B1* mutation analysis are high. Using Sanger sequencing of candidate genes, CNV arrays, MLPA, and histology, a final diagnosis could be made in approximately 30 % of the patients. New Massively Parallel Sequencing techniques, which provide a higher resolution (down to the nucleotide level), compared to CNV arrays and MLPA, and which allow simultaneous sequencing of multiple genes, are expected to improve the percentage of patients who can be given a molecular diagnosis.

References

1. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev.* 2007 Jan;87(1):1-28.
2. Vilain E. Anomalies of human sexual development: clinical aspects and genetic analysis. *Novartis Found Symp.* 2002;244:43-53; discussion -6, 79-85, 253-7.
3. Piprek RP. Molecular and cellular machinery of gonadal differentiation in mammals. *Int J Dev Biol.* 2010;54(5):779-86.
4. Kousta E, Papathanasiou A, Skordis N. Sex determination and disorders of sex development according to the revised nomenclature and classification in 46,XX individuals. *Hormones (Athens).* 2010 Jul-Sep;9(3):218-131.
5. Barsoum IB, Yao HH. Fetal Leydig cells: progenitor cell maintenance and differentiation. *J Androl.* 2010 Jan-Feb;31(1):11-5.
6. Juniarto AZ, van der Zwan YG, Santosa A, Hersmus R, de Jong FH, Olmer R, et al. Application of the new classification on patients with a disorder of sex development in indonesia. *Int J Endocrinol.* 2012;2012:237084.
7. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev.* 1995 Jun;16(3):271-321.
8. Ahmed SF, Khwaja O, Hughes IA. The role of a clinical score in the assessment of ambiguous genitalia. *BJU Int.* 2000 Jan;85(1):120-4.
9. Prader A. Der Genitalbefund beim Pseudohermaphroditismus femininus deskongenitalen Adrenogenitalen syndroms: Morphologie, Häufigkeit, Entwicklung und Vererbung der verschiedenen Genital formen. *Helv Pediatr Acta* 1954;9:231–48.
10. Hughes IA, Houk C, Ahmed SF, Lee PA. Consensus statement on management of intersex disorders. *J Pediatr Urol.* 2006 Jun;2(3):148-62.
11. Pierik FH, Deddens JA, Burdorf A, de Muinck Keizer-Schrama SM, Jong FH, Weber RF. The hypothalamus-pituitary-testis axis in boys during the first six months of life: a comparison of cryptorchidism and hypospadias cases with controls. *Int J Androl.* 2009 Oct;32(5):453-61.
12. Lamberts SW, Bons EG, Bruining HA, de Jong FH. Differential effects of the imidazole derivatives etomidate, ketoconazole and miconazole and of metyrapone on the secretion of cortisol and its precursors by human adrenocortical cells. *J Pharmacol Exp Ther.* 1987 Jan;240(1):259-64.
13. Crofton PM, Evans AE, Groome NP, Taylor MR, Holland CV, Kelnar CJ. Dimeric inhibins in girls from birth to adulthood: relationship with age, pubertal stage, FSH and oestradiol. *Clin Endocrinol (Oxf).* 2002 Feb;56(2):223-30.
14. Crofton PM, Evans AE, Groome NP, Taylor MR, Holland CV, Kelnar CJ. Inhibin B in boys from birth to adulthood: relationship with age, pubertal stage, FSH and testosterone. *Clin Endocrinol (Oxf).* 2002 Feb;56(2):215-21.
15. Boonstra VH, Weber RF, de Jong FH, Hokken-Koelega AC. Testis function in prepubertal boys and young men born small for gestational age. *Horm Res.* 2008;70(6):357-63.
16. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res.* 1988 Feb 11;16(3):1215.
17. Curnow KM, Slutsker L, Vitek J, Cole T, Speiser PW, New MI, et al. Mutations in the CYP11B1 gene causing congenital adrenal hyperplasia and hypertension cluster in exons 6, 7, and 8. *Proc Natl Acad Sci U S A.* 1993 May 15;90(10):4552-6.

18. Geley S, Kapelari K, Johrer K, Peter M, Glatzl J, Vierhapper H, et al. CYP11B1 mutations causing congenital adrenal hyperplasia due to 11 beta-hydroxylase deficiency. *J Clin Endocrinol Metab.* 1996 Aug;81(8):2896-901.
19. Goossens K, Juniarto AZ, Timmerman MA, Faradz SM, Wolffenbuttel KP, Drop SL, et al. Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients. *Clin Endocrinol (Oxf).* 2009 Nov;71(5):628-35.
20. Richter-Unruh A, Martens JW, Verhoef-Post M, Wessels HT, Kors WA, Sinnecker GH, et al. Leydig cell hypoplasia: cases with new mutations, new polymorphisms and cases without mutations in the luteinizing hormone receptor gene. *Clin Endocrinol (Oxf).* 2002 Jan;56(1):103-12.
21. Huizenga NA, de Lange P, Koper JW, de Herder WW, Abs R, Kasteren JH, et al. Five patients with biochemical and/or clinical generalized glucocorticoid resistance without alterations in the glucocorticoid receptor gene. *J Clin Endocrinol Metab.* 2000 May;85(5):2076-81.
22. Gottlieb B, Beitel LK, Trifiro MA. Variable expressivity and mutation databases: The androgen receptor gene mutations database. *Hum Mutat.* 2001 May;17(5):382-8.
23. Androgen Receptor Gene Mutation Database.
24. Gottlieb B, Beitel LK, Nadarajah A, Paliouras M, Trifiro M. The androgen receptor gene mutations database: 2012 update. *Hum Mutat.* 2012 May;33(5):887-94.
25. Hersmus R, Stoop H, White SJ, Drop SL, Oosterhuis JW, Incrocci L, et al. Delayed Recognition of Disorders of Sex Development (DSD): A Missed Opportunity for Early Diagnosis of Malignant Germ Cell Tumors. *Int J Endocrinol.* 2012;2012:671209.
26. White SJ, Breuning MH, den Dunnen JT. Detecting copy number changes in genomic DNA: MAPH and MLPA. *Methods Cell Biol.* 2004;75:751-68.
27. Juniarto AZ, Setyawati BA, Miranti IP, Santosa A, Hersmus R, Stoop H, et al. Gonadal malignancy in 13 consecutive collected patients with disorders of sex development (DSD) from Semarang (Indonesia). *J Clin Pathol.* 2013 Mar;66(3):198-204.
28. Juniarto AZ, Setyawati BA, Ediati A, van der Zwan YG, Looijenga LHJ, de Jong FH, et al. Virilization due to Androgen Hypersecretion in a Patient with an Ovarian Leydig Cell Tumor: Diagnostic and Psychosocial Implications. *Acta Med Indones.* 2013;In Press.
29. Rodie M, McGowan R, Mayo A, Midgley P, Driver CP, Kinney M, et al. Factors that influence the decision to perform a karyotype in suspected disorders of sex development: lessons from the Scottish genital anomaly network register. *Sex Dev.* 2011;5(3):103-8.
30. Zuccarello D, Ferlin A, Vinanzi C, Prana E, Garolla A, Callewaert L, et al. Detailed functional studies on androgen receptor mild mutations demonstrate their association with male infertility. *Clin Endocrinol (Oxf).* 2008 Apr;68(4):580-8.
31. Elfferich P, Juniarto AZ, Dubbink HJ, van Royen ME, Molier M, Hoogerbrugge J, et al. Functional analysis of novel androgen receptor mutations in a unique cohort of Indonesian patients with a disorder of sex development. *Sex Dev.* 2009;3(5):237-44.
32. Sytsma SE, editor. *Ethics and Intersex*: Springer; 2006.
33. Warne GL. Long-term outcome of disorders of sex development. *Sex Dev.* 2008;2(4-5):268-77.
34. Warne GL, Raza J. Disorders of sex development (DSDs), their presentation and management in different cultures. *Rev Endocr Metab Disord.* 2008 Sep;9(3):227-36.
35. Russcher H, Smit P, van Rossum EF, van den Akker EL, Brinkmann AO, de Heide LJ, et al. Strategies for the characterization of disorders in cortisol sensitivity. *J Clin Endocrinol Metab.* 2006 Feb;91(2):694-701.

36. Al-Jurayyan NA. Ambiguous Genitalia, Two Decades of Experience: Clinical Management and Sex Assignment. *J T U Med Sc* 2010;5(1):13 - 20
37. Joshi RR, Rao S, Desai M. Etiology and clinical profile of ambiguous genitalia an overview of 10 years experience. *Indian Pediatr.* 2006 Nov;43(11):974-9.
38. Gollu G, Yildiz RV, Bingol-Kologlu M, Yagmurlu A, Senyuçel MF, Aktug T, et al. Ambiguous genitalia: an overview of 17 years' experience. *J Pediatr Surg.* 2007 May;42(5):840-4.
39. Nimkarn S, Likitmaskul S, Sangacharoenkit P, Pathomvanich A, Sawathiparnich P, Wacharasindhu S, et al. Ambiguous genitalia: an overview of 22 years experience and the diagnostic approach in the Pediatric Department, Siriraj Hospital. *J Med Assoc Thai.* 2002 Aug;85 Suppl 2:S496-505.
40. Ellaihi M KA, Saber. O, Hiort.O. Consanguinity and Disorders of Sexual Developments in the Sudan. *Sudan JMS.* 2011 December 2011;Volume 6(Number 4):267-70.
41. Ahmed SF, Cheng A, Dovey L, Hawkins JR, Martin H, Rowland J, et al. Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. *J Clin Endocrinol Metab.* 2000 Feb;85(2):658-65.
42. Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroyannopoulou K, Macdougall J. Androgen insensitivity syndrome. *Lancet.* 2012 Jun 12.
43. Sultan C, Lumbroso S, Paris F, Jeandel C, Terouanne B, Belon C, et al. Disorders of androgen action. *Semin Reprod Med.* 2002 Aug;20(3):217-28.
44. Jaaskelainen J. Molecular biology of androgen insensitivity. *Mol Cell Endocrinol.* 2012 Apr 16;352(1-2):4-12.
45. Gaspari L, Paris F, Philibert P, Audran F, Orsini M, Servant N, et al. 'Idiopathic' partial androgen insensitivity syndrome in 28 newborn and infant males: impact of prenatal exposure to environmental endocrine disruptor chemicals? *Eur J Endocrinol.* 2011 Oct;165(4):579-87.
46. Ahmed SF, O'Toole S. Management of boys and men with disorders of sex development. *Curr Opin Endocrinol Diabetes Obes.* 2012 Jun;19(3):190-6.
47. Faisal Ahmed S, Achermann JC, Arlt W, Balen A, Conway G, Edwards Z, et al. UK Guidance On The Initial Evaluation Of An Infant Or An Adolescent With A Suspected Disorder Of Sex Development. *Clin Endocrinol (Oxf).* 2011 Apr 16.

Supplement 1 Reference values for males

Hormone	Units	0-6 mo	6mo- 6yrs	6- 10yrs	10- 12yrs	12-18 yrs	adult
LH	IU/l	0.6-6.0	<0.5	<0.5	<2.5	<7.0	1.5-8.0
FSH	IU/l	0.4-2.0	<1.0	<2.0	<6.0	1.0-8.0	2.0-7.0
Cortisol	nmol/l	200-800	200-800	200-800	200-800	200-800	200-800
Progesterone	nmol/l	<0.6	<0.6	<0.6	<0.6-2.0	<0.6-2.0	<0.6-2.0
	before	1	<0.6	<0.6	<0.6	<0.6	<0.6
	after	1	<0.6	<0.6	<0.6	<0.6	<0.6
	hCG	1	3.0	1.0	1.5	5.0	5.0
17OHprogesterone	nmol/l		<1	<1	0-10	0-10	0-10
	before	1					
	after	1					
	hCG	1	2.0-9	0.5-2.0	0.5-7.0	0.5-7.0	3.0-7.0
DHEAS	umol/l	0.3-1.4	0.3-1.4	0.3-1.4	0.8-17	0.8-17	0.8-17
	before	1	1.4	0.3-1.4	0.3-1.4	0.8-17	0.8-17
	after	1	1.4	0.3-1.4	0.3-1.4	0.8-17	0.8-17
	hCG	1	1.4	0.3-1.4	0.3-1.4	0.8-17	0.8-17
Androstenedione	nmol/l	0.35-1.2	0.35-1.2	0.7-3.8	1.4-5.2	2.0-10	2.0-10
	before	1	1.2	0.7-3.8	1.4-5.2	2.0-10	2.0-10
	after	1	<1	1.0-5.0	1.0-5.0	1.5-12	1.5-12
	hCG	1	5.0	<1-5.0	1.0-5.0	1.5-12	1.5-12
Testosterone	nmol/l	2.0-10.0	0.3-0.5	0.3-0.5	3.0-6.5	10-30	10-30
	before	1	10.0	0.3-0.5	3.0-6.5	10-30	10-30
	after	1	10.0	0.3-0.5	3.0-6.5	10-30	10-30
	hCG	1	5-20	5-20	5-20	5-30	10-30
Inhibin B	ng/l	200-800	20-100	20-120	20-300	80-300	150-400
AMH	ug/l	300-900	100-1000	100-400	30-200	10-100	5.0-30

Supplement 2 Reference values females

Hormone	Units	0-6 mo	6mo- 6yrs	6- 10yrs	10- 12yrs	12-18 yrs	adult
LH	IU/l						
FSH	IU/l						
Cortisol	nmol/l	200- 800	200- 800	200- 800	200- 800	200-800	200- 800
Progesterone	nmol/l	<0.6	<0.6	<0.6		women luteal (mid) : 15-70 women follicular : <0.5-3.0	
17OHprogesterone	nmol/l		<1	<1		women first half of cycle :0.5-2.0 women second half of cycle :4.0- 10.0	
DHEAS	umol/l	0.3- 1.4	0.3-1.4	0.3-1.4	1.4- 10.0	1.4- 10.0	1.4- 10.0
Androstenedione	nmol/l	0.35- 1.2	0.35- 1.2	0.7-3.8	1.4-5.2	2.0-10	2.0-10
Testosterone	nmol/l	0.2- 0.5	0.2-0.5	0.2-0.5	0.2-0.5	0.3-0.9	0.5-3.0
Inhibin B	ng/l						<150
AMH	ug/l	1.0- 4.0	1.0-4.0	1.0-4.0	27-42	2-30	0.5-7.0

Chapter 5

Functional analysis of novel Androgen Receptor mutations in a unique cohort of Indonesian DSD patients

Peter Elfferich, Achmad Zulfa Juniarto, Hendrikus Jan Dubbink, Martin E. van Royen, Michel Molier, Jos Hoogerbrugge, Adriaan B. Houtsmuller, Jan Trapman, Ardy Santosa, Frank H. de Jong, Stenvert L.S. Drop, Sultana M.H. Faradz, Hennie Brüggewirth and Albert O. Brinkmann

Sex Dev. 2009;3(5):237-44. doi: 10.1159/000252814. Epub 2009 Oct 23. PMID:19851057

Abstract

Mutations in the androgen receptor (AR) gene, rendering the AR protein partially or completely inactive, cause androgen insensitivity syndrome (AIS), which is a form of 46,XY disorder of sex development (DSD). We present three novel AR variants found in a cohort of Indonesian DSD patients; p.I603N, p.P671S and p.Q738R. The aim of this study was to determine the possible pathogenic nature of these newly found unclassified variants. To investigate the effect of these variants on AR function, we studied their impact on: transcription activation, AR-LBD interaction with an FxxLF motif containing peptide, AR sub cellular localization and on AR nuclear dynamics and DNA binding. AR-I603N had completely lost its transcriptional activity due to disturbed DNA binding capacity and did not show the 114 kDa hyperphosphorylated AR protein band, normally detectable after hormone binding. The patient with AR-I603N displays a PAIS phenotype, which is explained by somatic mosaicism. A strongly reduced transcriptional activity was observed for AR-Q738R together with diminished interaction with an FxxLF motif containing peptide. AR-P671S also showed reduced transactivation ability, but no change in DNA or FXXLF binding capacity. AR-P671S interferes with transcriptional activity for as yet unclear reasons.

Introduction

Disorders of sex development (DSD) are defined as any congenital condition in which development of chromosomal, gonadal or anatomical sex is atypical (1). One of these disorders is caused by defects in androgen action. The androgen insensitivity syndrome (AIS) results from mutations in the X-linked androgen receptor (AR) gene. A great variety of mutations in the AR gene has been reported (www.mcgill.ca/androgendb) and resulted in a wide spectrum of clinical phenotypes (2).

The AR is a transcription factor that belongs to the super family of nuclear receptors and is composed of distinct domains characteristic for steroid hormone receptors. The variable NH₂-terminal domain (NTD) is mainly involved in transcription activation (3). The DNA-binding domain (DBD) of the AR contains two zinc-clusters that specifically bind regulatory sequences in promoter/enhancer regions of androgen-regulated genes (4). A short flexible hinge region, containing highly positively charged amino acid residues links the DBD domain to the ligand-binding domain (LBD). The AR LBD consists of 10-12 α helices. Upon hormone binding, helix 12 functions as a lid and fixates the hormone in the ligand-binding pocket. As a result a hydrophobic coactivator-binding groove on the LBD-surface is created (5). The coactivator-binding groove functions as an interaction surface for LxxLL- and FxxLF-like motif containing coactivators (5-7) (where L is a leucine, F is a phenylalanine and X represents any amino acid) as well as for the FxxLF motif in the AR-NTD, important in the NH₂/COOH terminal domain interaction (N/C- interaction) (8, 9).

Here we report three novel AR missense mutations found in a unique cohort of 101 Indonesian DSD patients. The aim of this study was to determine the possible pathogenic nature of these newly found unclassified variants. To investigate the effect of these mutations on AR function, we studied their impact on transcription activation, on AR-LBD interaction with an FxxLF motif containing peptide, on AR subcellular localization and on AR nuclear dynamics and DNA binding. Together these data provide a molecular explanation for the clinical presentation of these PAIS patients.

Patients, Materials and Methods

Patients

Patient A was referred to the Dr. Kariadi Hospital, Semarang, Indonesia at the age of 12 years because of genital ambiguity. He had a male gender and physical examination showed a bifid scrotum with palpable testes and perineal hypospadias, Quigley stage 5 (10) and breast development Tanner stage 3-4. His serum concentrations of LH (2.1 IU/l) and FSH (5.3 IU/l) were in the normal range for early puberty, and serum testosterone (basal 1.6 nmol/l) rose to 20.4 nmol/l at 3 days after the injection of 1500 IU of human chorionic gonadotrophin (hCG), indicating absence of disorders in testosterone biosynthesis. The serum level of AMH, which should decline under the influence of testosterone in early puberty, was relatively high (21 µg/l). He underwent surgical correction for his hypospadias and breast development. Sequencing analysis revealed a mosaic missense mutation in exon 3 of the AR gene (c.2170 T→A) at amino acid residue 603 (according to <http://www.mcgill.ca/androgendb>) leading to a substitution of isoleucine by asparagine. Both parents tested negative for this mutation.

Patient B was referred to the clinic at the age of 2.5 years because of hypospadias. He had a male gender. Testes were palpable in a bifid scrotum and there was scrotal hypospadias, Quigley stage 2. Serum LH (<0.1 IU/l) and FSH (0.55 IU/l) were normal for his age, as was serum testosterone (<0.1 nmol/l). The level of testosterone increased to 16.9 nmol/l after administration of hCG. Serum AMH was in the normal prepubertal range (68 µg/l). Hypospadias correction was performed at the age of 3.5 years. Using sequencing analysis a missense mutation was identified in exon 4 of the AR gene (c.2373 C→T) leading to a substitution of proline 671 by serine. Sequencing analysis of the parents revealed that the mother was a carrier of the P671S mutation.

Patient C was seen at the clinic at the age of 4.5 years because of penoscrotal hypospadias. He presented with a bifid scrotum and a micropenis (2 cm), Quigley stage 4. Serum LH (0.26 IU/l) and FSH (0.68 IU/l) were normal for his age, as was serum testosterone (0.1 nmol/l). The level of testosterone increased to 20.5 nmol/l after administration of hCG. Serum AMH was in the normal prepubertal range (158 µg/l). He underwent a hypospadias correction.

Topical DHT treatment with DHT cream during 3 months resulted in an increase of penis length of 1.6 cm. Using sequencing analysis a missense mutation was identified in exon 5 of the AR gene (c.2575 A→G) leading to a substitution of glutamine 738 by arginine. Sequencing analysis of the parents DNA revealed that the mother was carrier of the Q738R mutation.

All these patients were of Javanese Indonesian origin, had a 46, XY karyotype and were diagnosed with partial androgen insensitivity syndrome (PAIS).

Mutation analysis, site-directed mutagenesis and construction of AR expression vectors

Numbering of the amino acid residues is according to the National Center for Biotechnology Information (NCBI) accession number AAA51729, which refers to the AR consisting of 919 amino acid residues (11). Extraction of DNA of peripheral blood cells was performed according to standard techniques. The coding exons and exon/intron boundaries of the AR gene were analysed by direct sequencing on a ABI3730XL automated sequencer. All variations except known neutral variants were confirmed by a second sequencing experiment on DNA of the patients.

The human wild type AR cDNA expression plasmid pSG5AR, a gift from Dr Andrew Cato, was used to generate constructs encoding the mutant AR's using Quick Change site-directed mutagenesis (Stratagene, La Jolla CA). The following sense and antisense primers containing the mutated sequence (depicted in lowercase lettering) were used: for preparation of pSG5AR-I603N sense primer 5'-GTGCGCCAGCAGAAATGATTGCACTAaTGATAAATTCC-3' and antisense primer 5'-GGAATTTATCAtTAGTGCAATCATTCTGCTGGCGCAC-3' ; for pSG5AR-P671S sense primer 5'-CTATGAATGTCAGtCCATCTTTCTGAATGTCCTGGAAGC-3' and antisense primer 5'-GCTTCCAGGACATTCAGAAAGATGGaCTGACATTCATAG-3' and for pSG5AR-Q738R sense primer 5'-CCAGATGGCTGTCATTCgGTACTCCTGGATG-3' and antisense primer 5'-CATCCAGGAGTACcGAATGACAGCCATCTGG-3'. The introduction of the mutations was confirmed by sequence analysis. By digestion with restriction enzymes and subcloning, the mutant fragments were exchanged with wild type fragments of vector pSG5AR. For exchange of the I603N encoding fragment digestions with *Asp718* and *AspI* were used. Digestions with *AspI* and *BamHI* were applied for the P671S and Q738R encoding fragments.

GFP-tagged mutant AR expression constructs were generated by subcloning of the mutated fragments into pEGFP-AR0 (Farla et al, 2004) by *EcoRI* and *HindIII* digestions. The cloning sites of all constructs were screened by direct sequencing. The Gal4-DBD-AR FxxLF expression construct has been described previously (5).

Measurements of hormone levels

Hormone levels were determined using a chemo luminescence-based immunometric method (Immulite 2000, Siemens-DPC, Los Angeles, CA) for LH and FSH, a coated tube radioimmunoassay (Coat-a-Count, Siemens-DPC) for testosterone and an enzyme immunometric assay (DPC, Webster, TN) for AMH.

Western blot analysis

For Western blot analysis COS-1 cells were seeded in 6-well plates (diameter 35 mm) at a density of 0.15×10^6 cells per well in DMEM:F12 supplemented with 100 U/ml penicillin, and 100 µg/ml streptomycin + Glutamax + 5% fetal calf serum (FCS) treated with dextran coated charcoal. The next day the cells were transfected with either pSG5AR, pSG5AR-I603N, pSG5AR-P671S and pSG5AR-Q738R or empty vector pSG5, respectively. For each well 0.25 µg DNA was mixed with 0.6 µl FuGENE reagent in MEM medium without FCS. After 24 hours 50 nM R1881 or vehicle (0.1% ethanol) was added to the wells. The next day the cells were washed with PBS. Per well 200 µl ice-cold SDS Laemmli sample buffer containing 10 mM dithiotreitol (DTT) was added and the cells were scraped with a policeman. The cell lysates were transferred to 1.5 ml eppendorf tubes and boiled for 2 min. After a short sonication step 7 µl of the lysate was loaded onto a 7% SDS-polyacrylamide gel. Proteins were separated and blotted to a nitrocellulose membrane (Schleicher & Schuell Dassel Germany). Immunoblotting was performed using polyclonal antibody SP197 (12) and proteins were visualized via the ECL method (Perkin Elmer NEL101 Shelton USA).

Luciferase (LUC) assays

For transcription activation studies Hep3B cells were cultured in α MEM medium (Lonza BioWhittaker) supplemented with 5% FCS (PerBio), 100 U/ml penicillin, and 100 μ g/ml streptomycin. One day before transfection Hep3B cells were plated in 24-well plates (Greiner Bio) at a density of 5×10^4 cells per well. After 24 hours α MEM medium supplemented with 5% FCS was replaced by α MEM medium with 5% charcoal treated FCS supplemented with either vehicle (0.1% ethanol) or a range of 10 pM – 10 nM synthetic androgen R1881. Four hours after addition of R1881 the cells were transfected with AR expression constructs using FuGENE reagent (Roche Diagnostics, Basel, Switzerland). The DNA mixture was composed of 50 ng AR expression construct, 100 ng ARE₂-TATA-Luc or MMTV-Luc reporter plasmid and 1 μ l FuGENE reagent per well in 25 μ l α MEM. The DNA mixture was pre-incubated for two hours at room temperature before addition to the cells.

For interaction assays the same procedure of cell culture was used as described above. All the interaction assays were performed with or without the addition of 1 nM R1881. A mixture of 50 ng AR expression construct, 50 ng Gal4-DBD-AR FxxLF expression construct and 150 ng UAS4-TATA-luc reporter plasmid was applied per well. Twenty-four hours after DNA transfection the cells were lysed and Luciferase activity was measured in a Fluorescent Ascent FL (Labsystems Oy, Helsinki, Finland).

Subcellular localization and nuclear mobility of GFP tagged AR mutants

Two days before microscopic analysis, Hep3B cells were grown on glass cover slips in 6-well plates in α -MEM (Cambrex) supplemented with 5% FCS (Perbio), 2 mM Glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. At least 4 hours before transfection, the medium was substituted by medium containing 5% dextran charcoal stripped FCS. Transfections were performed with 1 μ g GFP-AR expression construct in FuGENE6 transfection medium (Roche). Four hours after transfection, the medium was replaced by medium with 5% dextran charcoal stripped FCS when indicated with 1 nM R1881.

Live-cell imaging and strip-FRAP analysis were performed using a confocal laser-scanning microscope (LSM510; Carl Zeiss MicroImaging, Inc.) equipped with a Plan-Neofluar

40X/1.3 NA oil objective (Carl Zeiss MicroImaging, Inc). Enhanced green fluorescent protein (EGFP) was excited using a 488 nm laser line of an argon laser at moderate laser power to obtain images. EGFP emission was detected using a 505-530 bandpass emission filter.

Strip-FRAP analysis was performed as described by (13). In short, fluorescence in a narrow strip (~700nm – corresponding to 10 pixels at zoom 6) spanning the entire nucleus is bleached and the recovery of fluorescence inside this strip is monitored in time with a 21 ms interval using a low laser power. Fluorescence intensity in the strip is expressed relative to prebleach intensities and the intensity directly after bleaching.

Results

To find a possible pathogenic effect for the clinical phenotypes of PAIS patients harbouring AR mutations p. I603N, p. P671S and p. Q738R, the corresponding mutant AR expression constructs were generated and the effect of the amino acid substitutions on AR function were studied *in vitro*. We investigated hyperphosphorylation, transcription activation, AR-LBD interaction with an FxxLF peptide motif, AR subcellular localization and nuclear mobility and DNA binding capacity of the distinct mutants.

AR isoform pattern

Previous experiments have shown that in the absence of hormone the AR protein displays a protein doublet of 110-112 kDa and an extra hyperphosphorylated 114-kDa band upon hormone binding during SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Part of the hormone-induced phosphorylation occurs following DNA binding and during or following transcription regulation (14). To study the effect of the PAIS-associated AR mutations on phosphorylation, wild type AR and AR mutants I603N, P671S and Q738 were expressed in COS-1 cells by transient transfection of the distinct AR constructs and Western blot analysis was performed after SDS-PAGE. Figure 1 shows expression of the 110-112 kDa protein doublet by wild type AR and all mutant ARs. However, incubation with the synthetic androgen R1881

induced expression of the hyperphosphorylated 114-kDa protein band in case of wtAR, AR mutants P671S and Q738R, but not of the AR mutant I603N (Figure 1, lane 5).

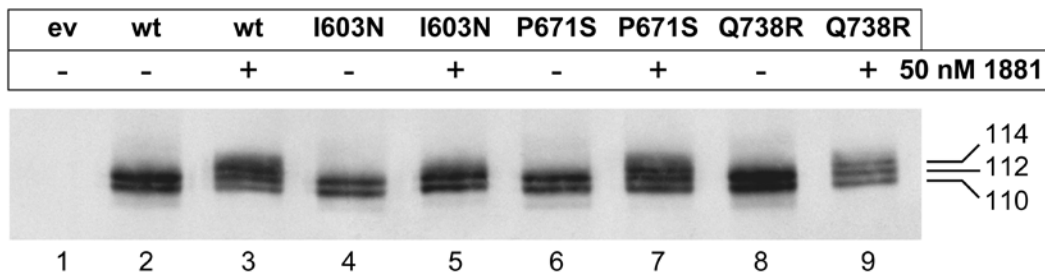


Fig 1: Western Blot analysis of PAIS-associated AR mutants. Wild type AR (wt) and AR mutants I603N, P671S and Q738R were expressed in COS-1 cells in the absence (-) or presence (+) of 50nM R1881. After SDS-PAGE and immunoblotting AR protein was visualized by polyclonal antibody Sp197. ev= empty vector.

Transcription activation

Next we studied transcriptional activation by the different mutant AR's. Hep3B cells were transiently transfected with one of the AR expression constructs described above and an ARE₂-TATA-Luc luciferase reporter construct. Cells were incubated in the presence of increasing concentrations of R1881. The AR-I603N mutant completely lost its transactivation potential even at the highest hormone concentration tested (Figure 2). Both the Q738R and P671S mutant showed reduced maximal transactivation capacity of 56% and 78% of wild type AR activity, respectively, in the presence of 10⁻⁸ M R1881. In addition, higher R1881 concentrations were required for androgen induction as at 10⁻¹⁰ M R1881 their activities were only 10 % and 32 % of wild type AR, respectively. In CHO cells and with a MMTV-Luc reporter construct similarly reduced transcriptional activities were observed for the distinct mutants (data not shown).

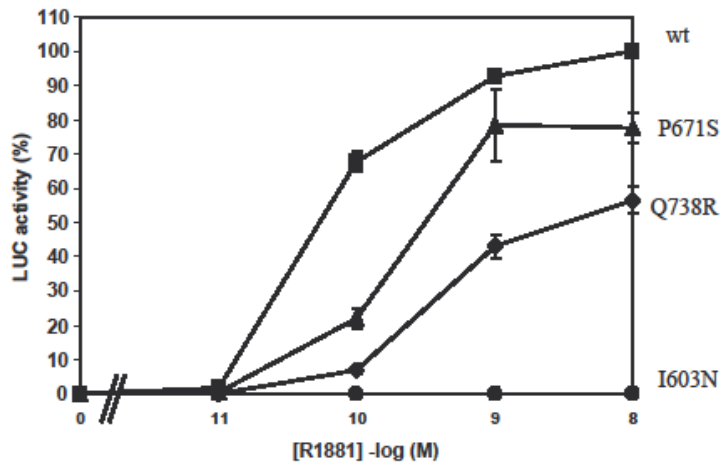


Fig 2: Transcription activation assay in Hep3B cells. Dose-response curves of wild type AR, AR-P671S, AR-Q738R and AR-I603N, in the presence of increasing amounts of R1881. The activity of wild type AR at 10 nM R1881 was set at 100% and the other data points were calculated relative to that. Values represent the mean \pm SEM of two independent experiments, performed in triplicate.

Intracellular distribution in living cells

To study the distribution of AR in living cells, wild type AR, a non-DNA binding AR mutant, AR-A573D as a positive control and the three AR mutants, were N-terminally tagged with green fluorescent protein (GFP). In the absence of hormone the proteins were mainly cytoplasmic (left panels in fig 3 a, b, c, e and g). After addition of 1 nM R1881 the GFP-AR translocated to the nucleus (right panels in fig 3 a, b, c, e and g). In the nucleus AR-P671S and AR-Q738R displayed a typical punctate distribution pattern similar to wild type AR (right panels in fig 3 a, e and g) (15, 16). Nuclei of cells transfected with AR-I603N however lacked this punctate pattern and showed a more homogeneous distribution (right panel in fig 3 c), similar to the non-DNA binding mutant AR-A573D (right panel in fig 3 b) (15).

FRAP was used to study the intranuclear mobility of the AR mutants. In concordance with their distribution pattern AR-P671S and AR-Q738R showed a transient immobilisation similar to wild type AR (fig 3 f and h), whereas the redistribution of AR-I603N was as fast as the non-DNA binding mutant AR-A573D (fig 3 d).

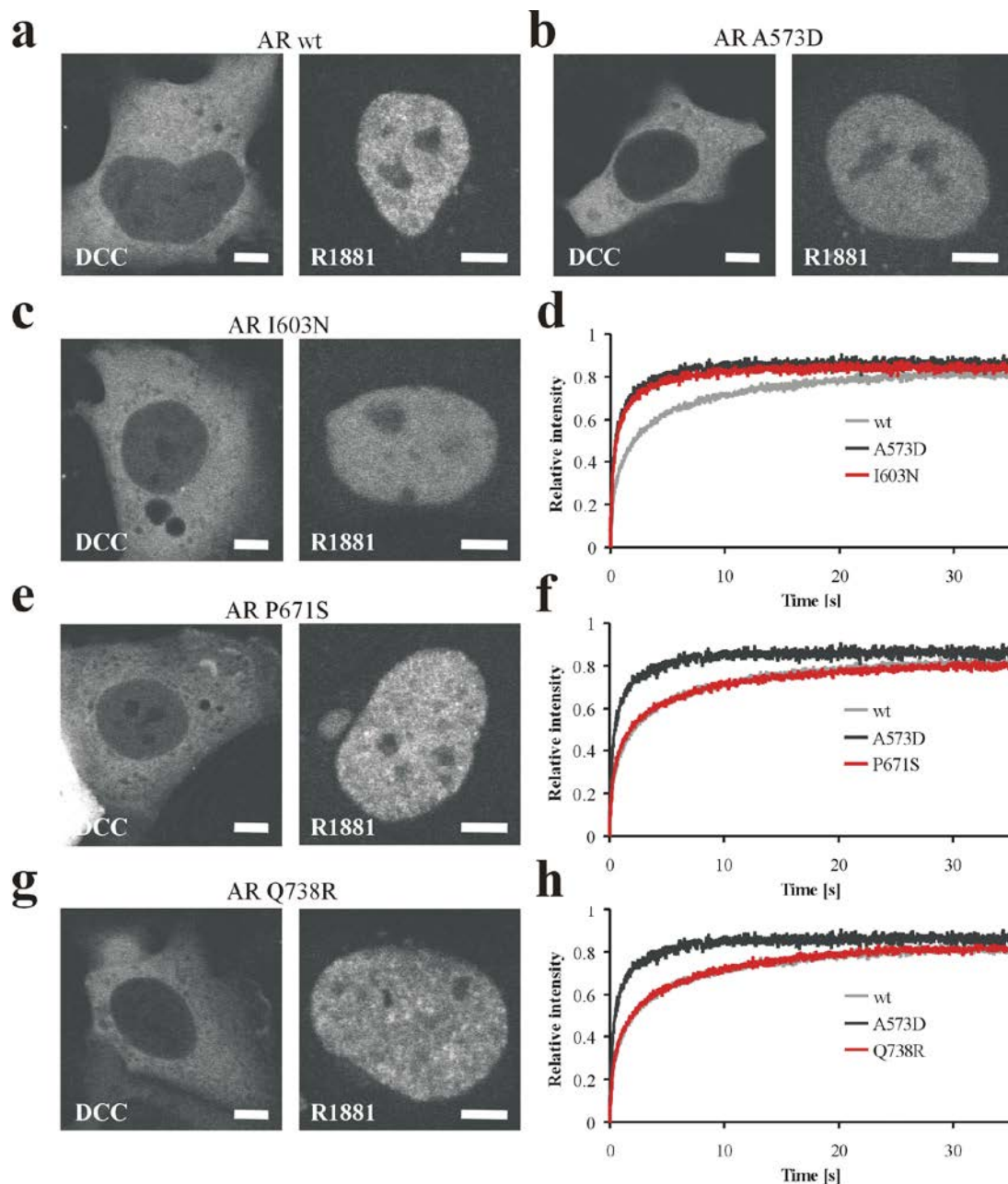


Fig 3: High-resolution confocal images and strip-FRAP analysis of cells expressing GFP-tagged AR mutants. (a, b, c, e and g) Confocal images of Hep3B cells expressing GFP tagged wild type AR (a) and mutant GFP constructs (b, c, e and g) in the absence (left panels) and presence (right panels) of 1 nM R1881. The nuclei in the hormone treated cells were represented at 1.6 x higher magnification as compared to the cells in the “minus hormone” situation in order to emphasize the differences in the speckled patterns in the nuclei. Bars represent 5 μm. (d, f and h) Strip-FRAP analysis of the indicated AR mutants in the presence of 1 nM R1881 (red curves). Redistribuition of wild type AR (grey curve) and a non-DNA binding mutant (AR-A573D) (black curve) were plotted as references.

Interaction with an α -helical FxxLF peptide motif

Upon hormone binding a hydrophobic groove is formed in the AR LBD surface. This coactivator-binding groove is lined by 13 amino acid residues, which reside in helices H3, H4, H5 and H12 (Fig. 4), one of which is Q738 (7). The coactivator groove interacts with LxxLL- and FxxLF-like motifs present in AR cofactors and with the FxxLF motif in the AR-NTD. To investigate the effect of the AR mutations on LxxLL and FxxLF motif interaction, binding to the AR FxxLF motif was studied. For this purpose, Hep3B cells were transiently transfected with constructs expressing a Gal4-DBD-FxxLF fusion protein and full length ARs together with an UAS4-TATA luciferase reporter construct.

Experiments were done in the absence and presence of 1 nM R1881. Interaction of the AR FxxLF motif with AR-I603N and AR-P671S was strongly induced by hormone to similar levels as shown for wild type AR (Figure 5). However, AR FxxLF interaction with AR-Q738R was five-fold decreased compared to wild type AR. These data indicate that cofactor binding to the coactivator groove is hindered by Q738R substitution, but not by I603N and P671S substitutions.

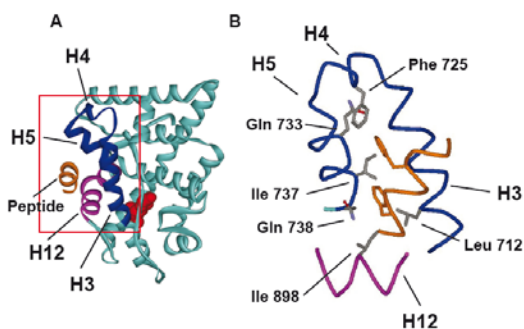


Fig 4: Structure of the androgen receptor ligand-binding domain (LBD). (A) Ribbon representation of the androgen receptor LBD. In red the ligand is indicated positioned in the ligand-binding pocket. In dark blue helices 3, 4 and 5 are indicated, helix 12 is purple. In brown an FxxLF peptide motif is indicated interacting with the cofactor-binding groove in the LBD surface. The region in the red square is enlarged and rotated in (B). (B) Position of an FxxLF peptide (in brown) in the cofactor-binding groove of the LBD. Important amino acid residues are indicated in the LBD lining the cofactor-binding groove, in which missense mutations have a proven correlation with AIS. Colours are as shown in (A). The figures are generated in ViewerLite 5.0 (Accelrys) using the coordinates of Protein Data Bank entry 1XOW.

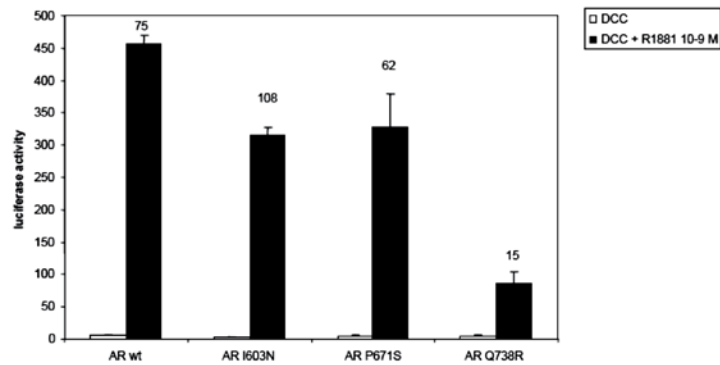


Fig 5: Interaction assay as measured in Hep3B cells transfected with wt AR and AR mutant constructs. The AR FxxLF-motif peptide construct and the UAS4-TATA-Luc reporter plasmid were used in this interaction assay. Interaction was measured in the absence (open bars) and presence of 1nM R1881 (closed bars). On top of the bars standard deviation is displayed plus the fold induction. DCC = dextran coated charcoal.

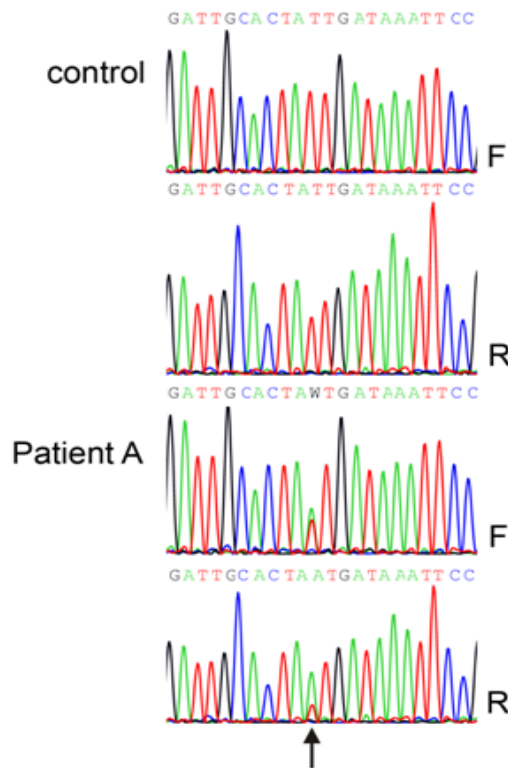


Fig 6: Sequencing analysis pattern of a part of exon 3 of the AR gene in control DNA and DNA of patient A (Sequencing analysis version 5.3.1). The arrow indicates base pair position 2170 of the Androgen receptor gene. F = Forward strand; R = Reversed strand. In the basecalling panel W represents an A or T nucleic acid. In DNA from patient A a mosaic pattern is seen at position 2170.

Discussion

In this study we report three novel mutations in the AR gene; p. I603N, p. P671S and p. Q738R, found in a cohort of 101 Indonesian DSD patients. All three patients presented with a PAIS phenotype. We performed extensive functional analyses of the mutant ARs to understand how these missense mutations correlate with the clinical phenotype of these patients. The results of these experiments are summarized in Table 1.

Table 1. Summary of functional studies of AR mutants

Mutant	Hyperphosphorylation (114-kDa isoform)	Transactivation	Co-activator binding	Transient immobilization
I603N	Absent	-	+	-
P671S	Present	±	+	+
Q738R	Present	±	-	+

- : diminished, no activity; +: wild type activity; ± : 50-75% wild type activity

The AR-I603N mutation is located in the DBD region of the AR, close to the D box in the second zinc cluster. The D box is involved in AR dimerization and Hormone Responsive Element (17) half site recognition. Amino acid substitutions in the D box normally result in a PAIS phenotype (18). Our results show that the AR-I603N mutation leads to a defective hormone-induced hyperphosphorylation of the protein and absence of the 114 kDa phosphoprotein band probably due to a disturbed DNA binding. AR I603N displays a normal translocation to the nucleus upon hormone stimulation, but has completely lost its DNA binding capacity as shown by live cell imaging, which explains its total lack of transcriptional activity from two distinct promoters. Previously, we have shown that disrupted DNA binding underlies a complete androgen insensitivity syndrome (CAIS) phenotype of a patient harboring an A573D mutation in the first zinc cluster (19, 20). However, despite the presence of an inactivating AR I603N mutation the patient presented with a PAIS phenotype and partly suppressed AMH levels. Examination of the DNA sequence of exon 3 showed besides the mutant also a wild type nucleotide at position 2170 in DNA from blood cells of the patient (Fig. 6), indicative for

somatic mosaicism. A similar occurrence of a somatic mosaicism has previously been reported in a PAIS patient harboring a wild type AR and an AR with a premature stopcodon in the NTD (21). Variable tissue distribution of wild type and mutant AR alleles may also underlie the PAIS phenotype of the AR-I603N patient.

The AR-P671S mutation is located in helix 1 of the LBD and encoded by exon 4 of the AR gene. This proline residue is highly conserved among human steroid receptors, underscoring an important role for this residue. AR P671S substitution results in a reduced transcription activity to 78% at physiological hormone concentrations, which could be partly overcome by increasing the level of androgen. The reason for this decreased activity is not yet understood, because in all other functional assays the AR-P671S mutation behaved like wild type AR. Hiort and colleagues reported a different mutation of the same amino acid residue, P671H, in a PAIS patient, although its effect on AR function has not been tested (22). Our data indicate that suboptimal AR functioning due to P671S mutation may be the underlying cause of the PAIS phenotype in this patient.

AR-Q738 is located in the hydrophobic coactivator groove in the surface of the AR LBD (see Fig.4) and (5, 17, 23). The coactivator groove is essential for both cofactor binding via LxxLL and FxxLF-like motifs and interaction with the FxxLF motif in the NTD. We have shown that AR-Q738R substitution does not affect the AR isoform pattern, intranuclear distribution and mobility, and DNA binding, but results in a two-fold decreased transcriptional activity at high hormone concentrations. The effect on transcription is even more pronounced at lower, more physiological hormone levels yielding only 10% of wild type activity. Most likely the underlying reason for loss of activity is the strongly diminished interaction with the AR FxxLF motif. Our data are in line with previous results showing that abrogation of N/C interaction via the coactivator groove reduced transcriptional activity (5, 8). According to crystal structures, residue Q738 makes hydrophobic contacts with the N-terminal F (F+1) of the AR FxxLF motif (Fig 4B) (17, 23). Substitution of the polar but uncharged glutamine residue by a charged arginine residue is probably not allowed because of interference with hydrophobic interactions with F+1, thus destabilizing FxxLF motif interaction. It is likely that binding of AR cofactors that largely depend on AR interaction via the coactivator groove in the LBD, is also negatively influenced by

the Q738R mutation (5, 6, 24). Altogether these data underscore the essential physiological role of AR-Q738 in FxxLF motif binding, including binding to the NTD, i.e. N/C interaction.

Several other amino acid substitutions in residues located in the hydrophobic coactivator groove are PAIS-associated. The F725L and I737T mutations disrupt the N/C terminal interaction (25). The L712F was found several times in PAIS patients and the Q733H mutation displayed a mosaic pattern (26).

In conclusion, we describe three novel PAIS-associated AR mutations present in the DBD (I603N), helix 1 of the LBD (P671S) and coactivator groove (Q738R) and provide insights in the clinical phenotypes of the patients harboring these mutations. AR-I603N entirely lost DNA binding capacity and transcriptional activity. Probably because of somatic mosaicism the patient displays a PAIS phenotype; AR-P671S mutation interferes with transcriptional activity for as yet unclear reasons; and AR-Q738R replacement strongly reduced transcriptional activity because of abrogated capability to perform N/C interaction and cofactor binding. Altogether, our functional analysis of these mutants further increases the molecular understanding of AR functioning.

Acknowledgements

We thank Dr. Andrew Cato for supplying the pSG5AR expression plasmid, Dr R. Dijkema for providing MMTV-luc, Dr G. Jenster for providing ARE₂- TATA- LUC and Renske Olmer for excellent technical assistance.

References

1. Hughes IA. Disorders of sex development: a new definition and classification. *Best Pract Res Clin Endocrinol Metab.* 2008 Feb;22(1):119-34.
2. Gottlieb B, Trifiro M, Lumbroso R, Vasiliou DM, Pinsky L. The androgen receptor gene mutations database. *Nucleic Acids Res.* 1996 Jan 1;24(1):151-4.
3. Jenster G, van der Korput HA, van Vroonhoven C, van der Kwast TH, Trapman J, Brinkmann AO. Domains of the human androgen receptor involved in steroid binding, transcriptional activation, and subcellular localization. *Mol Endocrinol.* 1991 Oct;5(10):1396-404.
4. Claessens F, Verrijdt G, Schoenmakers E, Haelens A, Peeters B, Verhoeven G, et al. Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J Steroid Biochem Mol Biol.* 2001 Jan-Mar;76(1-5):23-30.
5. Dubbink HJ, Hersmus R, Verma CS, van der Korput HA, Berrevoets CA, van Tol J, et al. Distinct recognition modes of FXXLF and LXXLL motifs by the androgen receptor. *Mol Endocrinol.* 2004 Sep;18(9):2132-50.
6. van de Wijngaart DJ, van Royen ME, Hersmus R, Pike AC, Houtsmuller AB, Jenster G, et al. Novel FXXFF and FXXMF motifs in androgen receptor cofactors mediate high affinity and specific interactions with the ligand-binding domain. *J Biol Chem.* 2006 Jul 14;281(28):19407-16.
7. Trapman J, Dubbink HJ. The role of cofactors in sex steroid action. *Best Pract Res Clin Endocrinol Metab.* 2007 Sep;21(3):403-14.
8. He B, Kempainen JA, Wilson EM. FXXLF and WXXLF sequences mediate the NH₂-terminal interaction with the ligand binding domain of the androgen receptor. *J Biol Chem.* 2000 Jul 28;275(30):22986-94.
9. Steketee K, Berrevoets CA, Dubbink HJ, Doesburg P, Hersmus R, Brinkmann AO, et al. Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor. *Eur J Biochem.* 2002 Dec;269(23):5780-91.
10. Quigley CA, De Bellis A, Marschke KB, el-Awady MK, Wilson EM, French FS. Androgen receptor defects: historical, clinical, and molecular perspectives. *Endocr Rev.* 1995 Jun;16(3):271-321.
11. Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, et al. The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis and gene expression in prostate. *Mol Endocrinol.* 1988 Dec;2(12):1265-75.
12. Kuiper GG, de Ruiter PE, Trapman J, Jenster G, Brinkmann AO. In vitro translation of androgen receptor cRNA results in an activated androgen receptor protein. *Biochem J.* 1993 Nov 15;296 (Pt 1):161-7.
13. van Royen ME, Farla P, Mattern KA, Geverts B, Trapman J, Houtsmuller AB. Fluorescence recovery after photobleaching (FRAP) to study nuclear protein dynamics in living cells. *Methods Mol Biol.* 2009;464:363-85.
14. Jenster G, de Ruiter PE, van der Korput HA, Kuiper GG, Trapman J, Brinkmann AO. Changes in the abundance of androgen receptor isoforms: effects of ligand treatment, glutamine-stretch variation, and mutation of putative phosphorylation sites. *Biochemistry.* 1994 Nov 29;33(47):14064-72.
15. Farla P, Hersmus R, Geverts B, Mari PO, Nigg AL, Dubbink HJ, et al. The androgen receptor ligand-binding domain stabilizes DNA binding in living cells. *J Struct Biol.* 2004 Jul;147(1):50-61.

16. van Royen ME, Cunha SM, Brink MC, Mattern KA, Nigg AL, Dubbink HJ, et al. Compartmentalization of androgen receptor protein-protein interactions in living cells. *J Cell Biol.* 2007 Apr 9;177(1):63-72.
17. Hur E, Pfaff SJ, Payne ES, Gron H, Buehrer BM, Fletterick RJ. Recognition and accommodation at the androgen receptor coactivator binding interface. *PLoS Biol.* 2004 Sep;2(9):E274.
18. Giwercman YL, Ivarsson SA, Richthoff J, Lundin KB, Giwercman A. A novel mutation in the D-box of the androgen receptor gene (S597R) in two unrelated individuals is associated with both normal phenotype and severe PAIS. *Horm Res.* 2004;61(2):58-62.
19. Bruggenwirth HT, Boehmer AL, Lobaccaro JM, Chiche L, Sultan C, Trapman J, et al. Substitution of Ala564 in the first zinc cluster of the deoxyribonucleic acid (DNA)-binding domain of the androgen receptor by Asp, Asn, or Leu exerts differential effects on DNA binding. *Endocrinology.* 1998 Jan;139(1):103-10.
20. Farla P, Hersmus R, Trapman J, Houtsmuller AB. Antiandrogens prevent stable DNA-binding of the androgen receptor. *J Cell Sci.* 2005 Sep 15;118(Pt 18):4187-98.
21. Holterhus PM, Bruggenwirth HT, Hiort O, Kleinkauf-Houcken A, Kruse K, Sinnecker GH, et al. Mosaicism due to a somatic mutation of the androgen receptor gene determines phenotype in androgen insensitivity syndrome. *J Clin Endocrinol Metab.* 1997 Nov;82(11):3584-9.
22. Hiort O, Sinnecker GH, Holterhus PM, Nitsche EM, Kruse K. The clinical and molecular spectrum of androgen insensitivity syndromes. *Am J Med Genet.* 1996 May 3;63(1):218-22.
23. He B, Gampe RT, Jr., Kole AJ, Hnat AT, Stanley TB, An G, et al. Structural basis for androgen receptor interdomain and coactivator interactions suggests a transition in nuclear receptor activation function dominance. *Mol Cell.* 2004 Nov 5;16(3):425-38.
24. Dubbink HJ, Hersmus R, Pike AC, Molier M, Brinkmann AO, Jenster G, et al. Androgen receptor ligand-binding domain interaction and nuclear receptor specificity of FXXLF and LXXLL motifs as determined by L/F swapping. *Mol Endocrinol.* 2006 Aug;20(8):1742-55.
25. Quigley CA, Tan JA, He B, Zhou ZX, Mebarki F, Morel Y, et al. Partial androgen insensitivity with phenotypic variation caused by androgen receptor mutations that disrupt activation function 2 and the NH(2)- and carboxyl-terminal interaction. *Mech Ageing Dev.* 2004 Oct-Nov;125(10-11):683-95.
26. Hiort O, Sinnecker GH, Holterhus PM, Nitsche EM, Kruse K. Inherited and de novo androgen receptor gene mutations: investigation of single-case families. *J Pediatr.* 1998 Jun;132(6):939-43.

Chapter 6

Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients

Kristel Goossens, Achmad Zulfa Juniarto, Marianna A. Timmerman, Sultana M. H. Faradz,
Katja P. Wolffenbuttel, Stenvert L. S. Drop and Frank H. de Jong

**ClinEndocrinol (Oxf). 2009 Nov;71(5):628-35. doi: 10.1111/j.1365-2265.2009.03550.x. Epub
2009 Feb 18. PMID:19226270**

Abstract

Background: Mutations in CYP21A2 lead to deficiency of 21-hydroxylase and have a severe effect on phenotype, which can be partly prevented by early treatment. We studied long-term effects of this deficiency on phenotype in patients who had not been treated for prolonged periods and correlated these phenotypes with the mutations found in our patients.

Objective: To assess the correlation between genotype and phenotype in untreated patients with 21-hydroxylase deficiency.

Design: Subjects with 21-hydroxylase deficiency were selected from a large population of Indonesian patients with disorders of sexual differentiation (DSD). CYP21A2 mutations in these patients were correlated with their phenotype in terms of genital development and steroid hormone levels.

Patients: Fifteen 46,XX patients with ages between 1 and 33 years, of whom 12 had never been treated before.

Measurements: Mutations in CYP21A2, genital phenotype according to Prader and steroid hormone levels.

Results: We found in all patients CYP21A2 mutations which affect enzyme activity, with a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2-1A>G (13%). The phenotype was not always concordant with the genotype: different phenotypes (mild to severe virilization) were found in sibling pairs with the mutations IVS2-13A>G or I172N. The high frequency of homozygous mutants for R356W in patients aged from 1 to 11 years old is remarkable, since this mutation has been described only in salt wasting patients. In our study this mutation caused a Prader genital stage of III-IV with a urogenital sinus in 3 out of 7 cases, whereas in the remaining cases the labia were at least partially fused. This mutation caused severe virilization with remarkably high serum levels of renin. We found one novel mutation in intron 2 (IVS2-37A>G), containing the branch site, which is likely to affect the CYP21-enzyme. Two additional intron 2 mutations were discovered which are supposed to affect the 21-hydroxylase (i.e. IVS2+33A>C and IVS2+67C>T).

Conclusion: We conclude that a correlation exist between the concentration of androgens and the extent of virilization. However, there was no clear correlation between genotype and phenotype, except for the mutation R356W.

INTRODUCTION

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder with impaired cortisol secretion (1,2). More than 90% of cases of CAH are caused by deficiency in the enzyme 21-hydroxylase (3).

21-hydroxylase deficiency impairs production of aldosterone and cortisol and as a result the secretion of the stimulating hormones renin and adrenocorticotrophic hormone (ACTH) is enhanced. The adrenal glands become hyperplastic and produce excess sex hormone precursors that do not require 21-hydroxylation for their synthesis. Therefore, levels of aldosterone and cortisol are decreased and production of adrenal androgens is increased, leading to sodium loss, Addisonian crisis and virilization (2,4).

Based on severity of symptoms, patients with 21-hydroxylase deficiency may belong to the classical or the non-classical form of the disease (1,2,5). The classical form encompasses the complete 21-hydroxylase deficiency, which leads to life-threatening salt-wasting crises during the neonatal phase in both sexes and prenatal virilization in females. Partial 21-hydroxylase deficiency leads to the simple virilizing form of CAH, and is characterized by prenatal virilization in females and pseudoprecocious puberty in males. An additional form is due to mild 21-hydroxylase deficiency. This so-called non-classical form is mostly asymptomatic or may be associated with signs of postnatal androgen excess consisting of pseudoprecocious puberty, acne, hirsutism and ovarian dysfunction (5). This makes it difficult to recognize the disease during the neonatal period and therefore these patients are mostly diagnosed in a later stadium of childhood (4).

In the Western world, a patient with CAH is most often diagnosed early in life on the basis of screening programs. Subsequent determination of serum hormone levels and investigation of gene mutations lead to a lifelong therapy with corticosteroids starting at a very

early age. Thus salt loss and Addisonian crises are prevented and postnatal progression of virilization will not occur. This situation differs from that in developing countries, where early diagnosis (new born screening) and treatment are not routinely available.

Within a large and heterogeneous group of patients with ambiguous genitalia followed by one of the authors (SMHF) 15 patients suspected of having CAH were identified.

This group of patients offers a unique opportunity to use modern methods to investigate the correlation between phenotype and genotype in untreated CAH patients.

PATIENTS AND METHODS

Patients

The local Medical Ethics Review Committee approved of this study, and informed consent was obtained from all participants, their parents or guardians. Out of a group of 130 patients with disorders of sexual differentiation (accumulated from 1991 until 2004) from the Dr. Kariadi University Hospital in Semarang, Central Java, Indonesia, 15 patients were provisionally diagnosed as suffering of CAH, based on the 46, XX karyotype in combination with hypervirilization. The ages of these patients varied from 1 to 33 years (Table 1). There were two pairs of siblings (pt 7-10 and pt 5-11). Twelve of the patients had never been treated; three of them (pt 13, 14 and 15) received glucocorticosteroids during a short period before the study.

Methods

Each patient underwent a physical examination and photographs were taken of the genital area. Blood was obtained for determination of hormone concentrations and isolation of DNA.

Phenotype

Most important characteristics were the length of the clitoris, presentation of a urogenital sinus and labial fusion (6). The photographs of the genital area were evaluated by an independent paediatric urologist (KPW).

Serum hormones

Levels of the following hormones were analysed in serum: 17-hydroxyprogesterone (17OHP), progesterone, androstenedione, dehydroepiandrosterone sulphate (DHEAS), testosterone, cortisol and renin. Cortisol, progesterone, androstenedione and DHEAS were analysed using luminescence-based immunoassays on the Immulite 2000 (Diagnostic Products Corporation, Los Angeles, CA). 17OHP was analysed using the ³H-radioimmuno-assay as described earlier (7). Testosterone was determined using the Coat-a-Count radioimmunoassay purchased from Diagnostic Products Corporation. Renin levels were estimated using an immunoradiometric assay purchased from CIS Bio international (Gif sur Yvette, France). Interassay coefficients of variation of those assays were all below 12%.

CYP21A2 mutation analysis

The CYP21A2 gene was amplified from DNA using the polymerase chain reaction and subsequently sequenced for detection of mutations. The CYP21A2 gene of each individual patient was sequenced from position -23 up to 90 bases downstream of the stop codon.

PCR amplification of CYP21A2 gene fragments

CYP21A2 gene specific amplification was performed using four sets of primers (Table 2) (8-10). Thirty cycles of PCR were carried out in a final volume of 50 µl containing 5 µl (10ng/µl) genomic DNA of each individual, PCR-buffer containing 1.5 mM MgCl₂ (Applied Biosystems, Foster City, USA), 250 µM dNTPs, 3.125 U AmpliTaq DNA Polymerase (Applied Biosystems), 400 nM forward-primer, 400 nM reverse-primer and 5% DMSO. Amplifications were performed using the GeneAmp PCR System 9700 (Applied Biosystems). The PCR-products were purified using the GFX-96 PCR Purification Kit (Amersham Biosciences, Little

Chalfont, UK) or the High Pure PCR Product Purification Kit (Roche Applied Science, Mannheim, Germany).

Sequencing the CYP21A2 PCR-products

The primers used for sequencing were described earlier (8-10). Sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) to perform the sequence-reactions, a DyeEx 96 Purification Kit (Qiagen, Venlo, the Netherlands) or the Micro-Bio-Spin purification columns (Biorad, Veenendaal, the Netherlands) to purify the fragments, and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) to read the sequences. To analyse the sequencing outcome the computer programme Sequencher 4.2 Ink for Windows (Gene Codes Corporation, Ann Arbor, USA) was used.

Restriction fragment length polymorphism (RFLP) and Deletion detection PCR (DDP)

DDP was used to detect whether apparent homozygous mutations were due the large deletions in one of the alleles, using DNA of homozygous patients and their parents. We used the techniques described by Asanuma et al (11) to detect hemizyosity in the 5'UTR and that of l'Allemand et al (12) for exon 3. To detect the CYP21-R356W mutation in the parents' DNA, we used the RFLP method (13).

RESULTS

Phenotype

Clinical data of the patients are provided in Table 1. All patients had the female gender, except patient 11 who changed her female gender to male at the age of 20 years. Phenotype was based on genital characteristics. Most of the patients had no urogenital sinus and the labia were not or only partially fused. However, most patients had a large clitoris. This made it difficult to assign a Prader stage, because the Prader staging is based on the length of the clitoris (phallus) as well as on labial fusion and presentation of a urogenital sinus. We decided the clitoris length to be the decisive marker of the severity of virilization. As is indicated in Table 1, only one patient (pt 11) had completely fused labia.

Hormone levels

Hormone levels are also summarized in Table 1. In all patients levels of progesterone and 17OHP were elevated with the exception of patients 13, 14 and 15, who were treated irregularly with glucocorticosteroids. Cortisol levels were low to low-normal in all patients. Similarly, in all patients (except in the treated patients) levels of progesterone, androstenedione and testosterone were elevated.

In 10 out of the 15 patients renin levels were elevated, in some of them to more than 100 fold the upper level of normal. Only in 2 untreated patients (pt 7 and 12) renin levels were within the normal range. As expected in the 3 corticosteroid treated patients renin levels were normal.

Table 1. Overview of features of the population (age-dependent reference ranges for hormone levels are shown under each age group in *italics*)

Subject-number	Age (years)	Gender	Karyotype	Phenotype				Serum hormones							
				Clitoris length (cm)	Urogenital sinus	Labia fusion (f)/partial fusion (pf)	Cortisol (nmol/l)	17OHP (nmol/l)	Prog (nmol/l)	Andren (nmol/l)	DHEAS (µmol/l)	T (nmol/l)	Renin (µU/ml)		
1	<7 years	0/2	Female	46,XX	2/5	Yes	pf	302	<i>1169-7</i>	87.2	135	<0.41	7.4	6855.7	
2		1	Female	46,XX	3	Yes	pf	117	524.4	29.6	42.2	<0.41	4.4	1838.4	
3		2	Female	46,XX	2/5	No	pf	119	273.3	14	>35	<0.41	4.2	141.4	
4		4	Female	46,XX	4/1	No	pf	129	428.0	52.2	23.3	<0.41	2.7	224.6	
5		3	Female	46,XX	2/8	No	pf	143	664.2	105	15.6	<0.41	2.4	261.4	
6	7-12 years	7	Female	46,XX	5/8	Yes	pf	<i>200-800</i>	<i><5.0</i>	<i><2.0</i>	<i><2.6</i>	<i><1.4</i>	<i><0.3</i>	<i><60</i>	
7		7	Female	46,XX	5	No	pf	67.4	551.4	107	138	8.84	16.6	152.5	
8		10	Female	46,XX	4/5	No	pf	172	392.4	35.4	56.6	5.08	6.7	49.5	
9		11	Female	46,XX	3/2	No	pf	147	548.1	70.8	130	8.05	18.4	1148.4	
10		11	Female	46,XX	3/6	No	no	310	137.3	34.4	13.6	<0.41	2.2	910.8	
11	>12 years	17	Male	46,XX	7	Yes	f	213	357.8	42.4	74.8	11.4	12.9	172.7	
12		33	Female	46,XX	3/6	No	pf	<i>200-800</i>	<i><5.0</i>	<i><2.0</i>	<i><3.8</i>	<i><5.0</i>	<i><0.6</i>	<i><60</i>	
13	Treated	6	Female	46,XX	4/5	No	pf	205	771.4	192	245.0	16.0	35.7	190.3	
14	Treated	7	Female	46,XX	7	No	pf	122	140.2	21.3	67.4	11.4	11.4	30.5	
15	Treated	21	Female	46,XX	normal	No	no	89.6	<i>140-2</i>	<i><3.0</i>	<i>2-15</i>	<i>1-10</i>	<i>1-3</i>	<i><60</i>	
									<i>Interd 4-10</i>						
									12.7	3.34	4.68	<0.41	0.8	57	
									0.2	<0.64	<1.05	<0.41	<0.1	20.8	
									1.0	7.52	1.41	<0.41	0.3	40.6	

Table 2. PCR-primers and conditions used in primary amplification

The levels of androstenedione and testosterone correlated significantly with the extent of

	annealing			
primer set	temperature	PCR primer*	position (bp)	sequence**
A	69°C	1	-57 to -31	5'-ATGGCTGGGGCTCTTGAGCTATAAGT-3'
		2	1380 to 1405	5'-CCTCAGCTGC <u>AT</u> CTCC <u>ACG</u> ATGTGA-3'
B	68°C	3	-265 to -245	5'-AGCTGACTCTGGATGCAGGA-3'
		4	706 to 727	5'-AGCAGG <u>GAGTAGTCT</u> CCCAAG-3'
C	65°C	5	695 to 719	5'-CCGGACCTGTCC <u>TTGGGAGACTAC</u> -3'
		6	2630 to 2656	5'-GAAAGGCTGCATCTTGAGGATGACAC-3'
D	61°C	7	700 to 720	5'-CCTGTCC <u>TTGGGAGACTACT</u> -3'
		8	2892 to 2912	5'-TCTCGCACCCCAAGTATGACT-3'

*reference NC_000006, A from ATG startsite is 1

**specific nucleotides for the CYP21B gene are shown by bold letters and are underlined

virilization and clitoris length (see Fig. 1 for the correlation between testosterone and phallus length in non-treated patients), with one exception: patient 10 had high levels of androstenedione and testosterone but suffered from only slightly virilized features. Her younger sister (pt 7), was much more virilized with less elevated levels of androgens.

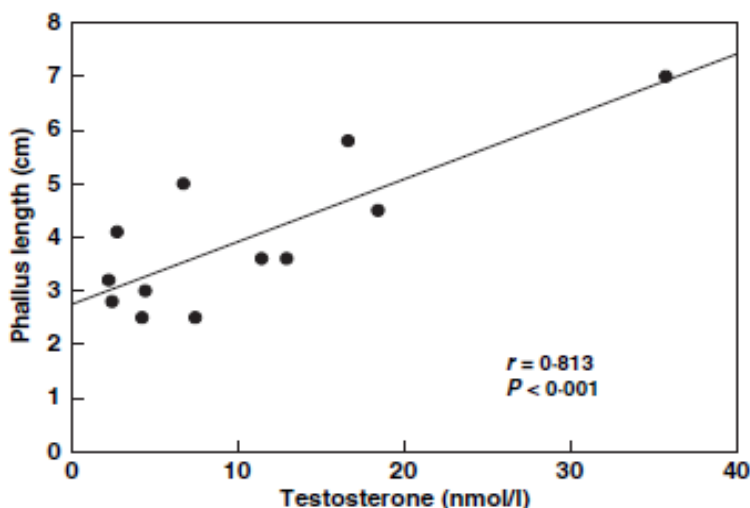


Fig. 1 Relationship between serum testosterone levels and clitoris length in 12 non-treated patients with mutation of CYP21A2

It is well known that the conversion of 17-hydroxypregnenolone to dehydroepiandrosterone (DHEA) and 17OHP to androstenedione by the enzyme 17,20-lyase is facilitated by cytochromeB5 (CyB5) (14). Between the age of 1 and 7 years, CyB5 is hardly expressed in the adrenal glands (14) and therefore levels of DHEAS are low. Levels of this adrenal steroid increased after the age of 7 years, as did the ratios androstenedione - 17OHP (Fig. 2A), and testosterone - 17OHP (Fig. 2B). These ratios were significantly correlated with age.

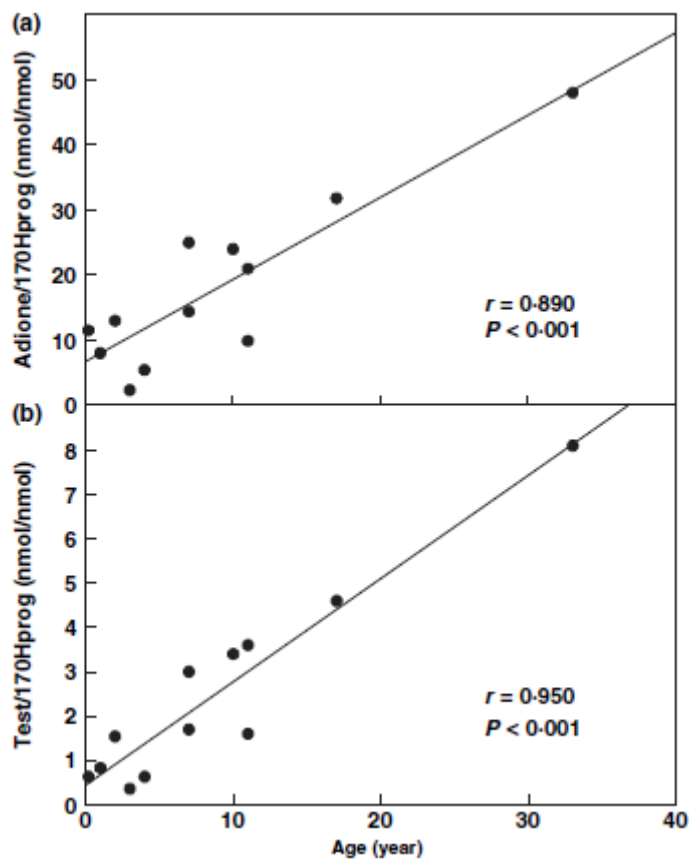


Fig. 2 Age dependence of the ratio between serum androstenedione/17OHP concentrations (a) and serum testosterone/17 OHP concentrations (b) in 21-hydroxylase deficient patients (N= 12)

Levels of renin correlated with increased levels of 17OHP as explained by the severity of the 21-hydroxylase enzyme deficiency (Fig. 3).

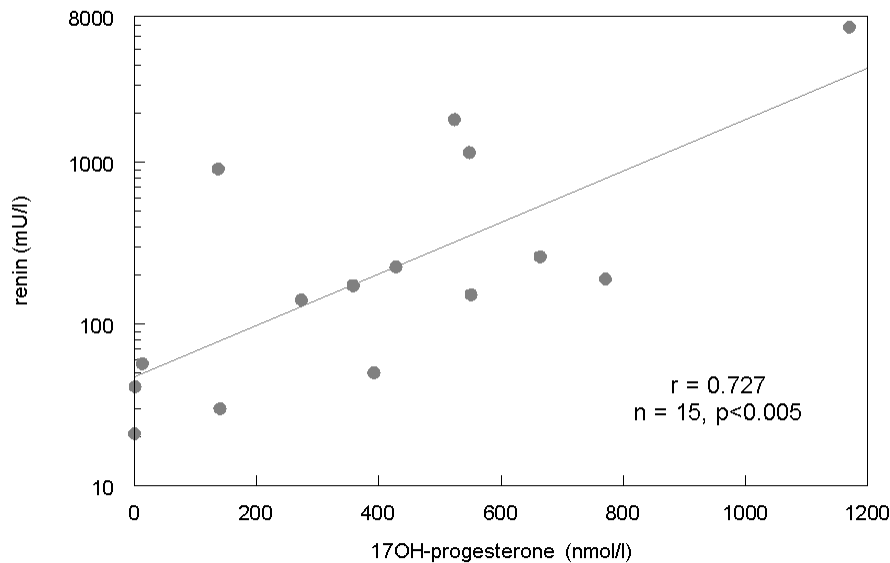


Fig.3 Relationship between serum levels of renin and 17 OHP in 21-hydroxylase-deficient patients

CYP21A2 gene mutation analysis

Table 3 summarizes the mutations in the CYP21A2 gene detected in each patient. Only mutations that are expected to affect the 21-hydroxylase activity are shown.

Missense and nonsense mutations in exons

In 13 patients a total of 4 missense mutations were discovered in exons, which resulted in an amino acid change. These mutations are known to affect the activity of the 21-hydroxylase enzyme and have been described in the literature: P30L, I172N, V281L, and R356W (see Table 3). No novel missense mutations were found. In addition, the nonsense mutation Q318X was detected. Patients 2 and 6 were hemizygous for the R356W mutation; both inherited the deleted gene from their mothers and the mutated gene from their fathers. Patients 7 and 10, who showed homozygosity for the I172N mutation, showed additional heterozygous single nucleotide polymorphisms close to the site of the mutation and were therefore assumed to be homozygous for this mutation.

Table 3. CYP21A2 mutations found in 15 Indonesian patients

Patient	CYP21-mutation	Known mutations which cause a phenotype if homozygous	References	Cortisol/17OHP*
7	I172N/I172N	Simple virilizing	20–22	0.438
10	I172N/I172N	Simple virilizing	20–22	0.595
5	IVS2 - 13A > G/IVS2 - 13A > G	Salt-wasting/simple virilizing	17,18	0.215
11	IVS2 - 13A > G/IVS2 - 13A > G	Salt-wasting/simple virilizing	17,18	0.266
1	R356W/R356W	Salt-wasting	1,13,16,24	0.258
2	R356W/-	Salt-wasting	1,13,16,24	0.223
3	R356W/R356W	Salt-wasting	1,13,16,24	0.435
6	R356W/-	Salt-wasting	1,13,16,24	0.122
8	R356W/R356W	Salt-wasting	1,13,16,24	0.268
9	R356W/R356W	Salt-wasting	1,13,16,24	2.258
15	I172N	Simple virilizing	20–22	Received therapy
	R356W	Salt-wasting	1,13,16,24	
12	I172N	Simple virilizing	20–22	0.870
	R356W	Salt-wasting	1,13,16,24	
4	-126C > T -113G > A -110T > C -103A > G -4C > T P30L	Salt-wasting/simple virilizing	12,29,30	0.301
	Q318X	Salt-wasting	16,25,26	
13	Q318X	Salt-wasting	16,25,26	Received therapy
	IVS2-37A > G/IVS2-37A > G			
14	V281L			
	F306 + Int	Salt-wasting	11	Received therapy
	Q318X	Salt-wasting	16, 25, 26	
	IVS2 + 33A > C			
	IVS2 + 67C > T			

*Reference value >40, see also Table 1 for age-dependent changes.

Intron and 3'UTR mutations

In all patients a total of 18 intron mutations were found: 12 mutations in intron 2, 1 mutation in intron 3, 3 mutations in intron 6 and 2 mutations in the 3'UTR. One of these (IVS2-13A>G) has been described in the literature as having an effect on enzyme activity. Both affected patients had 2 mutated alleles. The mutation IVS2-37A>G, which has not been described previously, contains the branch site in intron 2 and is likely to affect the formation of the lariat, which is involved in the RNA-splicing mechanism.

5'UTR mutations due to gene-conversion

In patient 4 we found 5 mutations in the 5'UTR region as a result of a small gene-conversion between the CYP21A2 gene and the CYP21A1 pseudogene.

DISCUSSION

In view of the age of the patients at the time of diagnosis, we assumed that the salt wasting type of CAH would not occur in our study population, since this is considered a life-threatening situation. For this reason, we were surprised to find only 2 patients without biochemical evidence of aldosterone deficiency. Some of the patients had marked elevation of renin levels and apparently have been able to compensate salt wasting by dietary means.

We could not determine the degree of virilization at birth because the patients did not visit a medical doctor shortly after birth, except for patient 1. This means that all patients have been exposed to high levels of androgens post nately resulting in progressive virilization. Fusion of the labia and the presence of a urogenital sinus are prenatally determined and postnatal exposure to androgens will have no further effect. Remarkably all patients received the female gender at birth, suggesting that the patients had only slightly virilized genitals at that time. 17OHP levels were more elevated than progesterone in all untreated patients because of conversion of progesterone into 17OHP. The ratios androstenedione/17OHP and testosterone/17OHP were markedly age correlated, probably because of the age dependent expression of CyB5 (14).

We could not predict the severity of the 21-hydroxylase deficiency based on the renin values, since the values differed in equal genotypes even in siblings. This could be due to 'mature onset', which means that the adrenal glands fail during aging. Alternatively, this could be explained by variations in genotype / phenotype relationships. However, as expected, the renin levels rise with the increasing levels of 17OHP (Fig. 3).

Most frequent CYP21A2 gene mutations in the Indonesian patients

We have analysed the CYP21A2 gene of 15 Indonesian CAH patients. To our knowledge, there have been only few reports on CYP21A2 gene mutations in Asian patients. In 65 CAH families in Taiwan (15) and 28 families in Singapore (16) the most common mutation (40%) was the intron 2 mutation (IVS2-13A/C>G). I172N mutations occurred in ~22% and the R356W mutation in ~16%. In a study of 6 Japanese patients, IVS2-13A/C>G and I172N were responsible for 58% of the cases of 21-hydroxylase deficiency (8). Loke et al (16) suggested that the R356W mutation occurs more commonly in the Asian patients both in Singapore and in Taiwan (15-17%) and less in Caucasians (3-9%). The frequency of these mutations described in Caucasian patients is 11 to 30% for IVS2-13A/C>G, 1 to 30% for I172N and 2 to 10% for R365W (3). The mutation IVS2-13A>G in the splicing donor site is reported to be the most prevalent mutation appearing in the CYP21A2 gene among all ethnic groups (2). In our Indonesian patients there was a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2-13A>G (13%).

CYP21A2 gene mutation analysis; correlation with phenotype

Two patients (a sibling pair) are homozygous for the intron 2 mutation IVS2-13A>G (pt 5 and 11). This mutation activates a cryptic splice site that results in aberrant splicing of the pre-mRNA with retention of 19 nucleotides normally spliced out of the pre-mRNA. As a result a shift in the reading frame occurs which generates a frame shift in the third exon, resulting in a stop codon and the production of a truncated protein (17,18). In cultured cells a small amount of normally spliced mRNA is detected. Therefore, a small amount of normal enzyme might be synthesized. Since this mutation is associated with the simple virilizing / salt wasting form we

can explain the phenotype of the two patients (19).

Four patients had the mutation I172N, of whom 2 were homozygous (pt 7 and 10, a sibling pair) and 2 were heterozygous (pt 12 and 15). The I172N mutation is associated with the simple virilizing form of CAH (20,21). In a study of Tusie-Luna et al (22), the mutation I172N showed 2% of wild-type activity for 17OHP and progesterone substrates. K_m and V_{max} values for both 17OHP and progesterone were determined in cellular lysates. When activities were expressed as first-order rate constants V_{max}/K_m , the activity of the I172N mutant was decreased 200-fold (0.5% activity). Therefore, the phenotype of the sibling pair, patients 7 and 10 can be fully explained. Nevertheless, patient 7 was more virilized than her sister.

Patients 12 and 15 have an additional heterozygous R356W mutation. Chiou et al (23) described a patient who was compound heterozygous for I172N/R356W, having the simple virilizing form of CAH. With the techniques we used, we were not able to determine whether the heterozygous mutations are located on the same allele. However, if the two mutations would have been present on the same allele, it is likely that the other, intact allele would yield sufficient enzyme to prevent the abnormalities detected in these patients.

Four patients were homozygous for the mutation R356W (pt 1, 3, 8 and 9) and two patients were hemizygous for this mutation (pt 2 and 6). This mutation has a dramatic effect on enzyme functioning (1,23), associated with the salt-wasting form. In 1990 it was assumed that the protein region involving R356W, constitutes a steroid-binding site (23). It was speculated that the replacement of Arg356 by Trp might be drastic enough to unfold the protein, which might destroy the steroid binding capacity of the enzyme leading to a 50-fold lower activity of the enzyme.

Recently it was discovered that in P450c21, Arg356 is probably located in a region that is involved in redox partner interaction (2). Stickelbroeck et al (3) as well as Loke et al (16) found that all CAH patients carrying the homozygous mutation R356W were salt wasters. However, all our six patients have survived 1 to 11 years in spite of the mutation. In all patients the Prader stage was III-IV. Furthermore, patients 1, 2 and 6 had a urogenital sinus. If we take the renin values into account, the four highest levels of renin (indicating a severe deficiency of 21-hydroxylase) are detected in patients with the homozygous R356W mutation. We can conclude

that the mutation R356W in our patients caused a severe 21-hydroxylase deficiency resulting in the severe virilizing form of CAH.

Patients 4, 13 and 14 are heterozygous for the mutation Q318X. This mutation is predicted to result in a completely non-functional enzyme due to premature termination of translation of the mRNA before the conserved heme-binding region of the P450 polypeptide (24). When the mutated sequence was transfected into mouse Y1 adrenal cells, the resulting mRNA levels were decreased. In the studies of Kharrat et al (25) and Loke et al (16) all patients who were homozygous for the Q318X mutation had the salt wasting form. However Kharrat et al also studied 2 patients with the simple virilizing form who were compound heterozygous for this mutation, having the mutation R356W or I172N on the other allele.

Patient 14 is compound heterozygous for F306+1nt, V281L and Q318X. Wu et al (26) demonstrated a fivefold reduced enzyme activity for the mutant V281L and Stikkelbroeck et al (3) associated this homozygous mutation with the non-classical form of CAH. Besides that, Stikkelbroeck et al (3) and Asanuma et al (11) reported a cluster mutation in exons 7 and 8 of V281L -F306+1nt-Q318X: an extra T nucleotide is inserted in the allele which results in a 100% reduced enzyme activity and consequently in a salt wasting phenotype. However, since this patient is heterozygous for this cluster mutation, two additional intron 2 mutations in this patient (IVS2+33A>C and IVS2+67C>T) could influence the enzyme activity. To prove this, these mutations should be investigated in more detail.

Patient 4 had five additional mutations in the promoter region, 5'UTR and exon 1, which are compatible with the sequence of the pseudogene CYP21A1 and can decrease the transcriptional activity with 20% (12). It seems likely that a hybrid was formed between CYP21A1 and CYP21A2, with a junction site just before intron 2. This hybrid would be formed by unequal crossover, resulting in a deletion of the 5'end of the CYP21A2 gene, the C4B gene and the 3'end of the CYP21A1 pseudogene (11,27). In addition, the mutation P30L causes an enzymatic activity of 60% (28).

The heterozygous combination of the mutations together with Q318X in patient 4 explains the phenotype.

Besides the heterozygous Q318X mutation, we found in patient 13 the homozygous

mutation IVS2-37A>G, which contains the branch site in intron 2. This mutation has not been described previously and may affect the formation of the lariat during RNA splicing (29) which results in intron 2 retention or exon 2 skipping. This may have an important affect on the phenotype.

In conclusion, we found mutations in the CYP21A2 gene in all patients with a relatively high allele frequency of R356W (40%), I172N (20%) and IVS2-13A>G (13%). Most remarkable in this study is the high frequency of the mutation R356W, which is very often described as causing the life threatening salt-wasting form of CAH. Apparently patients having the R356W mutation were able to compensate salt waste by dietary means. The 21-hydroxylase deficiency resulted in postnatal progressive virilization and almost all patients had the female gender and a strong desire to have a female appearance. Therefore, a project has been initiated to offer medical and surgical treatment.

ACKNOWLEDGEMENTS

We thank the Gender team of the Medical Faculty and the Molecular and Cytogenetic Laboratory of the Diponegoro University / Dr. Kariadi Hospital for help in research of the patients and the karyotyping.

References

1. **Speiser PW, White PC** 2003 Congenital adrenal hyperplasia. *New England Journal of Medicine*, 349, 776-788
2. **White PC, Speiser PW** 2000 Congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Endocrine Reviews*, 21, 245-291
3. **Stickelbroeck M** 2003 Congenital Adrenal Hyperplasia From childhood into adulthood. Thesis, UMC St Radboud, Nijmegen, The Netherlands
4. **Behrman RE KR** 2002 congenital adrenal disorders. In: Nelson Essentials of Pediatrics. W.B. Saunders Company, Philadelphia, pp. 744-750
5. **New MI** 2006 Extensive clinical experience: nonclassical 21-hydroxylase deficiency. *Journal of Clinical Endocrinology and Metabolism*, 91, 4205-4214
6. **Prader A** 1954 Der Genitalbefund beim Pseudohermaphroditismus femininus deskongenitalen adrenogenitalen Syndroms; Morphologie, Haufigkeit, Entwicklung und Vererbung der verschiedenen Genitalformen. *Helvetica Paediatrica Acta*, 9, 231-248

7. **Lamberts SWJ, Bons EG, Bruining HA, de Jong FH** 1987 Differential effects of the imidazole derivatives etomidate, ketoconazole and miconazole and of metyrapone on the secretion of cortisol and its precursors by human adrenocortical cells. *Journal of Pharmacology and Experimental Therapeutics*, 240, 259-264
8. **Usui T, Nishisho K, Kaji M, Ikuno N, Yorifuji T, Yasuda T, Kuzuya H, Shimatsu A** 2004 Three novel mutations in Japanese patients with 21-hydroxylase deficiency. *Hormone Research*, 61, 126-132
9. **Dolzan V, Prezelj J, Vidan-Jeras B, Breskvar K** 1999 Adrenal 21-hydroxylase gene mutations in Slovenian hyperandrogenic women: evaluation of corticotrophin stimulation and HLA polymorphisms in screening for carrier status. *European Journal of Endocrinology*, 141, 132-139
10. **Day DJ, Speiser PW, White PC, Barany F** 1995 Detection of steroid 21-hydroxylase alleles using gene-specific PCR and a multiplexed ligation detection reaction. *Genomics*, 29, 152-162
11. **Asanuma A, Ohura T, Ogawa E, Sato S, Igarashi Y, Matsubara Y, Iinuma K** 1999 Molecular analysis of Japanese patients with steroid 21-hydroxylase deficiency. *Journal of Human Genetics*, 44, 312-317
12. **L'Allemand D, Tardy V, Gruters A, Schnabel D, Krude H, Morel Y** 2000 How a patient homozygous for a 30-kb deletion of the C4-CYP 21 genomic region can have a nonclassic form of 21-hydroxylase deficiency. *Journal of Clinical Endocrinology and Metabolism*, 85, 4562-4567
13. **Stikkelbroeck NM, Hoefsloot LH, de Wijs IJ, Otten BJ, Hermus AR, Siskens EA** 2003 CYP21 gene mutation analysis in 198 patients with 21-hydroxylase deficiency in The Netherlands: six novel mutations and a specific cluster of four mutations. *Journal of Clinical Endocrinology and Metabolism*, 88, 3852-3859.
14. **Rainey WE, Carr BR, Sasano H, Suzuki T, Mason JI** 2002 Dissecting human adrenal androgen production. *Trends in Endocrinology and Metabolism*, 13, 234-239
15. **Lee HH, Chao HT, Lee YJ, Shu SG, Chao MC, Kuo JM, Chung BC** 1998 Identification of four novel mutations in the CYP21 gene in congenital adrenal hyperplasia in the Chinese. *Human Genetics*, 103, 304-310
16. **Loke KY, Lee YS, Lee WW, Poh LK** 2001 Molecular analysis of CYP-21 mutations for congenital adrenal hyperplasia in Singapore. *Hormone Research*, 55, 179-184
17. **Higashi Y, Tanae A, Inoue H, Hiromasa T, Fujii-Kuriyama Y** 1988 Aberrant splicing and missense mutations cause steroid 21-hydroxylase [P-450(C21)] deficiency in humans: possible gene conversion products. *Proceedings of the National Academy of Science of the U S A*, 85, 7486-7490
18. **Higashi Y, Hiromasa T, Tanae A, Miki T, Nakura J, Kondo T, Ohura T, Ogawa E, Nakayama K, Fujii-Kuriyama Y** 1991 Effects of individual mutations in the P-450(C21) pseudogene on the P-450(C21) activity and their distribution in the patient genomes of congenital steroid 21-hydroxylase deficiency. *Journal of Biochemistry(Tokyo)*, 109, 638-644

19. **Day DJ, Speiser PW, Schulze E, Bettendorf M, Fitness J, Barany F, White PC** 1996 Identification of non-amplifying CYP21 genes when using PCR-based diagnosis of 21-hydroxylase deficiency in congenital adrenal hyperplasia (CAH) affected pedigrees. *Human Molecular Genetics*, 5, 2039-2048
20. **Speiser PW, Dupont J, Zhu D, Serrat J, Buegeleisen M, Tusie-Luna MT, Lesser M, New MI, White PC** 1992 Disease expression and molecular genotype in congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Journal of Clinical Investigation*, 90, 584-595
21. **Wedell A, Thilen A, Ritzen EM, Stengler B, Luthman H** 1994 Mutational spectrum of the steroid 21-hydroxylase gene in Sweden: implications for genetic diagnosis and association with disease manifestation. *Journal of Clinical Endocrinology and Metabolism*, 78, 1145-1152
22. **Tusie-Luna MT, Traktman P, White PC** 1990 Determination of functional effects of mutations in the steroid 21-hydroxylase gene (CYP21) using recombinant vaccinia virus. *Journal of Biological Chemistry*, 265, 20916-20922
23. **Chiou SH, Hu MC, Chung BC** 1990 A missense mutation at Ile172----Asn or Arg356---Trp causes steroid 21-hydroxylase deficiency. *Journal of Biological Chemistry*, 265, 3549-3552
24. **Globerman H, Amor M, Parker KL, New MI, White PC** 1988 Nonsense mutation causing steroid 21-hydroxylase deficiency. *Journal of Clinical Investigation*, 82, 139-144
25. **Kharrat M, Tardy V, M'Rad R, Maazoul F, Jemaa LB, Refai M, Morel Y, Chaabouni H** 2004 Molecular genetic analysis of Tunisian patients with a classic form of 21-hydroxylase deficiency: identification of four novel mutations and high prevalence of Q318X mutation. *Journal of Clinical Endocrinology and Metabolism*, 89, 368-374
26. **Wu DA, Chung BC** 1991 Mutations of P450c21 (steroid 21-hydroxylase) at Cys428, Val281, and Ser268 result in complete, partial, or no loss of enzymatic activity, respectively. *Journal of Clinical Investigation*, 88, 519-523
27. **Lee HH, Chang JG, Tsai CH, Tsai FJ, Chao HT, Chung B** 2000 Analysis of the chimeric CYP21P/CYP21 gene in steroid 21-hydroxylase deficiency. *Clinical Chemistry*, 46, 606-611
28. **Tusie-Luna MT, Speiser PW, Dumic M, New MI, White PC** 1991 A mutation (Pro-30 to Leu) in CYP21 represents a potential nonclassic steroid 21-hydroxylase deficiency allele. *Molecular Endocrinology*, 5, 685-692
29. **Strachan T, Read AP** 2004 Pathogenic mutations. In: *Human molecular Genetics*, 3rd edition, Taylor and Francis, London, pp 333-340

Chapter 7
Correlation between Androstenedione and 17-
hydroxyprogesterone Levels in Saliva and Plasma in Treated
Patients with Congenital Adrenal Hyperplasia.

Achmad Zulfa Juniarto, Kristel Goossens, Bestari A Setyawati, SLS Drop, FH de Jong, Sultana MH Faradz.

Singapore Med J. 2011 Nov;52(11):810-3.PMID:22173250

Abstract

Background: Congenital adrenal hyperplasia (CAH) or adrenogenital syndrome (AGS) is the most common cause of female ambiguous genitalia. Management of these patients involves medical treatment using glucocorticoids such as hydrocortisone, prednisone or dexamethasone. Monitoring is done by measurement of 17-hydroxyprogesterone or androstenedione in serum, plasma or saliva.

Objective: To develop a monitoring system of steroid treatment in CAH patients using solely saliva.

Methods: We studied saliva of 24 CAH patients who received glucocorticoid replacement therapy. Patients were asked to collect saliva after awakening, and in the afternoon and evening. Levels of 17-hydroxyprogesterone and androstenedione were measured both in saliva and serum by immunoassay.

Results: There was a significant positive correlation between 17-OHP in serum and 17-OHP in saliva ($R=0.929$, $p<0.01$). A significant positive correlation between androstenedione level in saliva and serum was also found ($R=0.611$, $P<0.01$). This study also revealed a significant positive correlation between androstenedione and 17-OHP in serum ($R=0.647$; $p<0.01$) and in saliva ($R=0.799$; $p<0.01$). All patients showed increased level of 17-hydroxyprogesterone and androstenedione in the sample collected at awakening time.

Conclusions: Determination of salivary androstenedione and 17-hydroxyprogesterone in CAH patients can be a useful alternative to measurement of these hormones in serum.

Introduction

Sexual differentiation follows a specific complex sequence of events. The start of this process takes place between 6-14 weeks of gestation (1). During this period, developmental errors may occur and lead to sexual ambiguity or discordance between chromosomal sex and the appearance of external genitalia. Disorders of sexual development (DSDs) are now classified as 46,XY DSD, 46,XX DSD, and chromosomal DSD (2). Congenital adrenal hyperplasia (CAH), or adrenogenital syndrome (AGS) in older literature, is the most common cause of ambiguous genitalia in females (46,XX DSD) (2). This syndrome is due to a mutation in one of the genes encoding enzymes needed for the production of gluco- and/or mineralocorticoids in the adrenal cortex. About 90 % of CAH is caused by 21-hydroxylase deficiency leading to a block in both cortisol and aldosterone production and resulting in an excessive adrenal androgen production causing masculinization of the genital and urinary structures. Lack of aldosterone and cortisol will lead to adrenal crisis which can cause severe illness and even lead to death. A milder, non-life-threatening form of CAH (non-classic form) in which salt loss does not occur becomes manifest in later childhood or even young adult life as a result of progressive virilization (3).

Management of CAH patients involves medical treatment in addition to surgery and psychological counselling. Glucocorticoids such as hydrocortisone, prednisone or dexamethasone are the drugs of choice in CAH patients (4). The synthetic mineralocorticoid fludrocortisone is needed for salt wasters to retain salt. Follow up of the treatment is important in order to prevent overtreatment causing growth inhibition and under treatment resulting in virilisation and increased height velocity (5). The effectiveness of treatment is traditionally monitored by measurements of 17-hydroxyprogesterone, androstenedione and testosterone in serum (6). This means the invasive procedure of phlebotomy every 3 to 6 months in patients, some of whom are babies or young children. Therefore we compared steroid hormone levels obtained simultaneously in plasma and saliva, with the purpose to develop a monitoring system of steroid treatment in CAH patients using solely saliva.

This paper aims to make a contribution in the area of CAH treatment by measuring the diurnal rhythm of salivary steroids during treatment with 3 daily doses of cortisol.

Materials and methods

Samples

For analysis of steroids in saliva we collected saliva of 24 patients with CAH with relatively high levels of 17-hydroxyprogesterone (17-OHP) in serum, which likely will result in high levels of salivary 17-OHP. The age range of the patients was from 1.4 to 16.3 years old. All of them received Hydrocortisone 12 mg/ metre body square /day in three divided doses.

Patients with CAH were willing to participate in the study and consented to the procedures of saliva and blood collection. The diagnoses varied from the severe form of classical CAH to the non-classical form of CAH. All samples of saliva and blood were taken prior the glucocorticoid treatment. After a clear explanation of the procedure, saliva was collected with the Salivette of Sarstedt®, Nümbrecht, Germany (figure 1) by chewing on the swab. Patients were asked to collect saliva at awakening, 30 minutes after awakening in the afternoon and in the evening before eating or brushing their teeth to prevent blood contamination. Patients were also instructed to keep the saliva in the refrigerator and to immediately hand the saliva during hospital visit at scheduled time. A duplicate saliva collection was performed during the patient's hospital visit together with peripheral blood collection. After collection of saliva, the samples were centrifuged and stored frozen at -20°C until analysis.

This study has been approved by the Local Medical Ethics Committee.

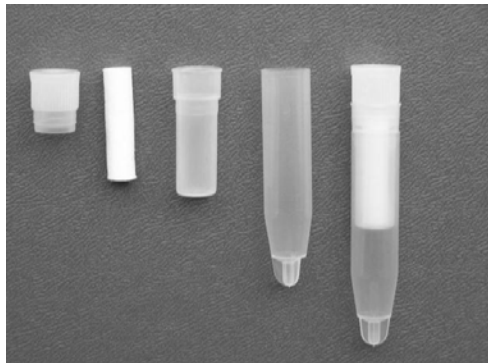


Figure 1. Salivette device for the collection of saliva from left to right: stopper, cotton swab, cotton swab holder with hole in bottom, collection tube and complete device.

Measurement of 17-hydroxyprogesterone and androstenedione

Serum concentrations of 17-OHP were determined using a RIA technique as described before (7). Androstenedione levels in serum were determined using a commercially obtained chemoluminescence-based immunoassay (Immulite 2000, Siemens, Los Angeles, CA).

Salivary concentrations of 17-OHP and androstenedione were determined using salivary ELISA Kits (DRG, Marburg, Germany). The procedure was as follows: 25 μ l of saliva was pipetted into duplicate wells of a 96-well plate, coated with a specific antibody. 250 μ l of enzyme-conjugate (17-OHP or androstenedione conjugated to horseradish peroxidase) was added. After 1-hour incubation at room temperature, the wells were washed 3 times with diluted wash solution and subsequently 200 μ l substrate solution (tetramethylbenzidine) was added. After 15 minutes at room temperature the reaction was stopped with 100 μ l stop solution (0.5 M H_2SO_4). The wells were read within 10 minutes in the Spectra Count (Packard Bioscience, Boston, USA) at 450 nm. Inter- and intra- coefficients of variation for salivary androstenedione; salivary 17 OHP; serum androstenedione and serum 17-OHP were <13.6% and <7.9%; 12.3% and <7.9%; <11% and <8%; and <10% and 6% respectively.

Statistics

Results are given as means \pm s.e.m. Differences between hormone levels in samples collected at different times of the day were compared by paired Student's t-test. Logarithmically transformed concentrations of saliva 17-OHP and androstenedione were correlated with serum values and the Pearson correlation coefficients were calculated using SPSS 11.0 for Windows. P-values < 0.05 were considered significant.

Results

1. Correlation between 17-OHP in serum and saliva

There was a significant positive correlation between 17-OHP in serum and 17-OHP in saliva collected from the same subject at one moment in time ($R=0.929$, $p<0.01$, figure 2).

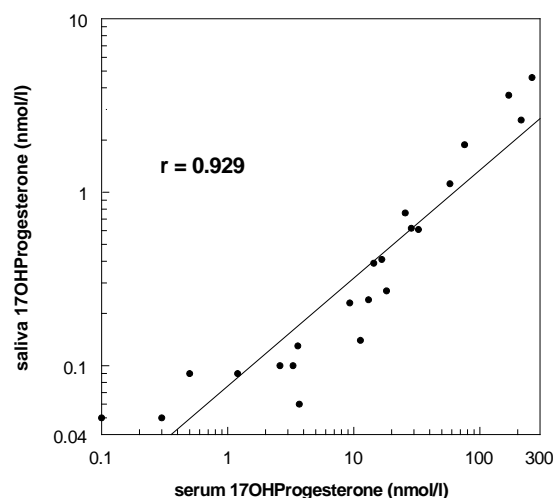


Figure 2. Relationship between saliva and serum levels of 17-OH progesterone collected at the same time.

2. Correlation between Androstenedione in serum and saliva

As we can see in figure 3, there was also a significant positive correlation between androstenedione levels in saliva and serum ($R=0.611$, $P<0.01$).

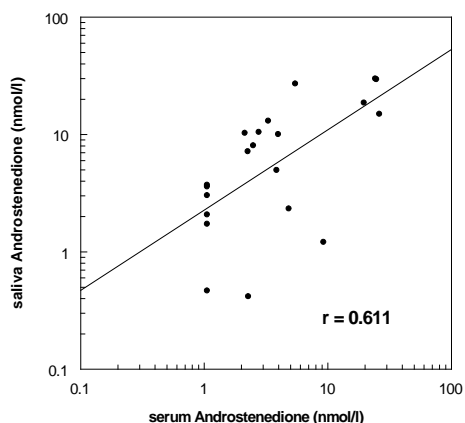


Figure 3. Relationship between saliva and serum levels of Androstenedione collected at the same time

3. Correlation Androstenedione and 17-OHP in saliva and serum

Androstenedione levels in saliva showed a significant positive correlation with 17-OH level in saliva ($R=0.641$; $p<0.01$, figure 4). This study also revealed a significant positive correlation between androstenedione and 17-OHP in serum ($R=0.647$; $p<0.01$, data not shown).

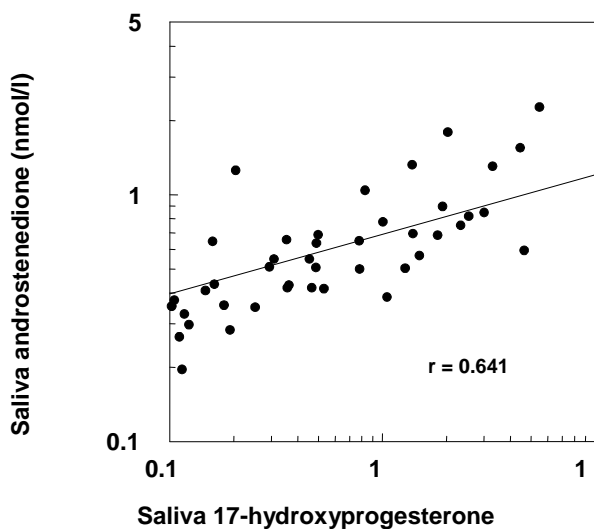


Figure 4. Relationship between saliva levels of 17-OH progesterone and Androstenedione

4. Diurnal rhythms of salivary 17-OHP and androstenedione

A circadian variation of salivary 17OHP and androstenedione levels was found in this study (figure 5). Morning values of 17-OHP showed significantly higher levels than during the afternoon and evening. Furthermore, 17OHP levels in samples collected 30 minutes after awakening (3.00 ± 0.75 pmol/l) were significantly higher than those at the time of awakening (3.55 ± 0.87 pmol/l, $P < 0.05$). Concentrations of androstenedione at awakening were greater than at other times during the day, but significance was only reached when morning and evening levels were compared.

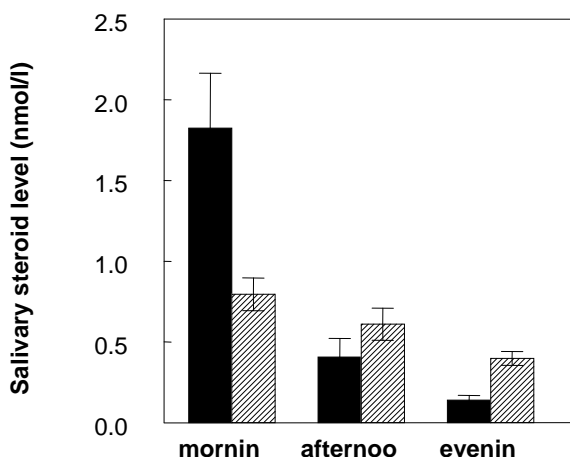


Figure 5. Diurnal rhythm of salivary 17-OH progesterone (black bar) and Androstenedione (heded bar) . means +/- SEM

Discussion

Hormone levels are often measured in serum with the use of immunoassays. The result is most of the time based on the interpretation of hormone concentrations in a single sample. Single serum analysis is not ideal for the study of hormones that undergo circadian rhythms of secretion. Hormones such as cortisol and 17-hydroxyprogesterone can be quite different in concentration throughout the day depending on time of day and physical condition (8). Measurements of salivary 17OHP in healthy children revealed that values tended to be higher in the morning than during the afternoon and evening (9). Within the first minutes after

awakening, salivary cortisol levels rise by 50-60%. This response was not dependent on the time of awakening, sleep quality, physical activity. After the morning peak, cortisol values will decline during the day (10,11). A similar rise of 17OHP was found in treated CAH patients in this study. Furthermore, all patients showed relatively high levels of 17OHP in samples collected at awakening time probably because of the fact that the saliva was collected before taking the morning dose of glucocorticoid. This observation suggests insufficient suppression of adrenal steroid production during the early morning hours.

Salivary androstenedione values showed lower values than 17OHP and gradually fell during the day to lowest values at evening time. There was a circadian rhythm in the concentration of androstenedione in treated patients with CAH, but the level was lower than for 17OHP (12).

It was noted that 17 OHP is a better marker to make a treatment evaluation especially in young patients because the level of androstenedione in these patients was rather low due to the relatively inefficient adrenal conversion of 17OHP to androstenedione at this age.

We found a good correlation between serum and saliva levels of both 17-OHP and androstenedione. These results indicate that the steroid levels in saliva describe steroid levels in serum. Levels of androstenedione were also correlated with levels of 17-OHP. These findings correlate well with earlier results of Young et al. (1988) who also found that there were high correlations between plasma concentrations of androstenedione and testosterone as well as with 17OHP (12). A strong correlation between the salivary androstenedione profiles and plasma testosterone concentrations also found in the study (12). The same correlation was also found between concentration of androstenedione in saliva and the unbound concentration in plasma in the study of Baxendale et al. ⁽¹³⁾ Otten et al found significantly positive correlations between salivary and plasma values of both Androstenedione and 17-OHP (14). Measurement of 17-OHP concentrations was also performed by Hughes et al (15) in 19 patients with CAH who received hydrocortisone treatment. This study found that serial measurement of 17-OHP in saliva provided valuable information in the line of treatment. Arisaka et al (16) found good correlation between 17-OHP concentrations in paired saliva and serum samples from the patients and the control subjects. Salivary steroid profiles are now widely accepted and used to assess the quality

of substitution therapy, providing information on the efficacy of suppressive regimens in the treatment of congenital adrenal hyperplasia patients. In addition to 17 OHP, salivary androstenedione is also a direct marker of adrenal androgen secretion.

The advantages of the use of salivary steroid profiles include easy collection of sample at home and dispatch to the laboratory between regular medical check-up for outpatient monitoring (17-19).

Conclusion

As the measurements of levels of steroid hormone in saliva and serum showed good correlations, we can conclude that determination of salivary androstenedione and 17-OHP are a useful alternative to measurements of those hormones in serum for monitoring of CAH treatment.

Acknowledgments

We thank the members of Gender team of the Faculty of Medicine Erasmus MC and Diponogoro University / Dr. Kariadi Hospital for their help in providing patients for this research.

References

1. Committee on genetics, section on endocrinology and section on urology. Evaluation of the newborn with developmental anomalies of the external genitalia. *Pediatrics* 2000; 106: 138-142
2. Hughes IA, Houk C, Ahmed SF, Lee PA, LWPES/ESPE Consensus Group. Consensus Statement on Management of Intersex Disorders. *Arch Dis Child* 2006; 91:554-563
3. Migeon CJ, Wisniewski AB. Congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. Growth, development, and therapeutic considerations. *EndocrinolMetabClin North Am.* 2001; 30: 193-206.
4. Merke DP, Cutler GB. New ideas for medical treatment of congenital adrenal hyperplasia. *EndocrinolMetabClin North Am.* 2001; 30: 121-33
5. Speiser PW. Congenital adrenal hyperplasia owing to 21-hydroxylase deficiency. *EndocrinolMetabClin North Am.* 2001; 30: 31-59
6. **Joint LWPES/ESPE CAH Working Group.** Consensus statement on 21-hydroxylase deficiency from the Lawson Wilkins pediatric endocrine society and the European society for pediatric endocrinology. *J ClinEndocrinolMetab.* 2002; 87: 4048-4053
7. Lamberts, SWJ, Bons, EG, Bruining HA, de Jong FH. Differential effects of the imidazole derivatives etomidate, ketoconazole and miconazole and of metyrapone on the secretion of cortisol and its precursors by human adrenocortical cells. *J PharmacolExpTher.* 1987; 240: 259-264.
8. Federenko I, Wüst S, Hellhammer DH, Dechoux R, Kumsta R, Kirschbaum C Free cortisol awakening responses are influenced by awakening time. *Psychoneuroendocrinology* 2004; 29: 174-184
9. Gröschl M, Rauh M, Dörr HG. Circadian rhythm of salivary cortisol, 17-hydroxyprogesterone, and progesterone in healthy children. *Clin Chem.* 2003; 49: 1688-1691.

10. Hampl R, Foretová L, Sulcová J, Stárka L. Daily profiles of salivary cortisol in hydrocortisone treated children with congenital adrenal hyperplasia. *Eur J Pediatr.* 1990; 149: 232-234
11. Hammond GL, Langley MS. Identification and measurement of sex hormone binding globulin (SHBG) and corticosteroid binding globulin (CBG) in human saliva. *ActaEndocrinol (Copenh).* 1986; 112: 603-608
12. Young MC, Walker RF, Riad-Fahmy D, Hughes IA. Androstenedione rhythms in saliva in congenital adrenal hyperplasia. *Arch Dis Child.* 1988; 63: 624-628.
13. Baxendale PM, Jacobs HS, James VHT. Plasma and salivary androstenedione and dihydrotestosterone in women with hyperandrogenism. *ClinEndocrinol (Oxf).* 1983; 18: 447-457.
14. Otten BJ, Wellen JJ, Rijken JC, Stoelinga GB, Benraad TJ. Salivary and plasma androstenedione and 17-hydroxyprogesterone levels in congenital adrenal hyperplasia. *J ClinEndocrinolMetab* 1983; 57: 1150-1154
15. Hughes IA, Read GF. Control in congenital adrenal hyperplasia monitored by frequent saliva 17OH-progesterone measurements. *Horm Res.* 1984; 19: 77-85.
16. Arisaka O, Shimura N, Nakayama Y, Arisaka M, Yabuta K. [Salivary 17-hydroxyprogesterone concentration in monitoring of the treatment of congenital adrenal hyperplasia]. *Dtsch Med Wochenschr.* 1988; 113: 1913-1915.
17. Read GF, Walker RF, Wilson DW, Griffiths K. Steroid analysis in saliva for the assessment of endocrine function. *Ann N Y AcadSci*1990. 595:260-274
18. Lewis JG. Steroid analysis in saliva: an overview. *ClinBiochem Rev.* 2006; 27: 139-146.
Gröschl M. Current status of salivary hormone analysis. *Clin Chem.* 2008; 54: 1759-1769.

Chapter 8

Salivary 17-hydroxyprogesterone (17-OHP) and Androstenedione in Monitoring Efficacy of Treatment Among Indonesian Congenital Adrenal Hyperplasia Patients

Achmad Zulfa Juniarto, Gerard Noppe, Nani Maharani, Erica van den Akker , Rudy Susanto, Sultana MH Faradz, Frank H. de Jong, Stenvert L.S. Drop

Journal of the ASEAN Federation of Endocrine Societies Vol. 27 No. 2 November 2012

Abstract

Objective: Early diagnosis of congenital adrenal hyperplasia (CAH) and the need for long term treatment are of great concern to the medical community. The aim of this study was to evaluate the effectiveness of glucocorticoid treatment-monitoring based on salivary 17-hydroxyprogesterone (17-OHP) and androstenedione measurements in CAH patients in Semarang, Indonesia in comparison to patients treated in Rotterdam, the Netherlands.

Methodology: 25 out of 43 patients with CAH from Semarang, Indonesia were included in the study. For comparison, twenty CAH patients from the Sophia Children's Hospital/ Erasmus Medical Center Rotterdam, the Netherlands were included. The effects of treatment were monitored by estimations of the steroids 17-OHP and androstenedione in saliva. Auxology and bone age determination were recorded.

Result: 17-OHP and androstenedione levels were high in a substantial number of Indonesian patients under treatment but decreased after adjustment of dosage and timing. The steroid concentrations obtained after adjustment were similar to those found under comparable circumstances in patients in Rotterdam.

Conclusion: Optimal treatment of CAH patients in Semarang Indonesia can be reached by introducing hydrocortisone treatment and adjusting dosage and timing on the basis of salivary steroid monitoring. However, in Indonesia the management of these patients is still constrained by the lack of diagnostic and therapeutic means.

Introduction

Congenital Adrenal Hyperplasia (CAH) is classified as a Disorder of Sex Development (DSD) which is usually diagnosed on the basis of ambiguous genitalia in the female newborn or a salt losing crisis which may occur in both males and females (1,2). CAH infants with ambiguous genitalia are genetically female (46,XX) whereas 46,XY infants do not have a significant phenotype except for hyperpigmentation of the scrotum. This disorder occurs when one of the enzymes required for synthesis of cortisol and aldosterone in the adrenal glands is deficient. The condition results in hyperplasia of the adrenal glands and in increased secretion of steroid precursors, such as 17-hydroxyprogesterone (17-OHP) and androstenedione, leading also to elevated testosterone levels (3).

Based on its clinical appearance, CAH patients can be divided into two groups, those with the classical and those with the non-classical form. The classical form is characterized by congenital insufficiency of cortisol and aldosterone synthesis with a marked increase of androgen levels. This condition will lead to salt losing and virilisation of the female. The non-classical form is characterized by partial deficiency of cortisol and aldosterone synthesis resulting in late onset of mild clinical symptoms (4). The incidence of the classical form of CAH is approximately 1 in 14,000 births worldwide whereas that of the non-classical form is predicted to be much higher (5).

Early diagnosis and treatment of CAH patients is essential. The need for long term treatment especially of salt losing patient is of great concern to the medical community in view of the high risk of hyponatremia, hyperkalemia, dehydration and shock with a possibly fatal result.

Treatment of CAH patients aims to provide adequate adrenal hormone substitution in order to prevent adrenal crises and to suppress excess testosterone production. The goal of therapy is to optimize growth and pubertal development (6). Hormone replacement therapy using hydrocortisone (HC) is an option that allows control of the adrenal hormone balance. The effectiveness of therapy is measured by monitoring the adrenal precursor steroids (7). In a

previous study we confirmed that determination of salivary androstenedione and 17-OHP in CAH patients is a useful alternative to the measurement of these hormones in serum (8).

This is particularly relevant in Indonesia as the availability of treatment and monitoring of CAH is limited due to the scarcity of medicine, geographical distance and scattered area of patients' dwelling, and financial issues.

Therefore, the aim of this study was to evaluate the effectiveness of glucocorticoid treatment-monitoring based on salivary 17-OHP and androstenedione measurements among Indonesian CAH patients in Semarang in comparison to CAH patients treated in Rotterdam, the Netherlands as reference. The Netherlands is a West-European country counting around 17 million inhabitants where newborns are screened within the first week of life for elevated 17-OHP levels to identify congenital adrenal hyperplasia. The prevalence of classic CAH in the Netherlands is about one in 10.000 to 18.000 newborns, of which 95% is caused by 21-hydroxylase deficiency.

Patients & Methods

Patients Semarang

This study is part of a stepwise diagnostic evaluation of a cohort of patients with DSD in Semarang Indonesia. Out of 286 patients diagnosed with disorders of sexual development between 2004 and 2010 from the Dr.Kariadi University Hospital in Semarang, Central Java, Indonesia, 43 patients were diagnosed as CAH based on phenotype and hormonal analysis. This diagnosis was confirmed by chromosomal and gene mutation analysis. Patients' age at the time of referral ranged from 3 days to 33 years with a median of 6 years. Among the 43 CAH patients, 40 had 46,XX karyotype; two had 46,XY and one had 46, XX (99%) / 46, XY (1%) karyotype.

The latter patient presented at age 0.2 years with undescended testis and severe hypospadias. All patients with a Y chromosome were raised as males, while of the 40 CAH patients with 46, XX karyotype, 5 were raised as males and 35 as females. Thirteen patients were diagnosed at an early age (<2 months), 23 with ages between 1.5 and 13 years while 7 were detected after 12 years of age. Five patients died during the study period because of a salt losing

crisis, 10 were not treated for the following reasons: 5 patients were living as males and another five refused the treatment.

Twenty-eight out of 43 patients were treated and 25 patients were regularly followed up in our outpatient clinic, while 3 patients were followed up in another centre.

The effect of glucocorticoid treatment was studied in these 25 CAH patients: 2 patients received prednisone while 23 patients received HC. Patients age at treatment ranged between 0.7 - 18.7 years (see Table 1).

Patients were treated with HC at a dose of 12-15 mg/m²/day given in 3 divided doses. In 2 adult patients age 18.7 and 17.5 years old, prednisone was given at a dose of 5 mg twice a day.

Table 1. Characteristics of patients

No	Age on Study (year)	Type of CAH	Prader Stage	Puberty Status	Type of Medication	Duration of Treatment (months)	Origin
1	0.7	Suspected Salt Losing	4	Prepuberty	HC**+FC**	8	Central Java
2	1.3	Suspected Salt Losing	4	Prepuberty	HC+FC	15	Central Java
3	1.5	Suspected Salt Losing	4	Prepuberty	HC+FC	17	Central Java
4	1.8***	Suspected Salt Losing	6	Prepuberty	HC+FC	21	Central Java
5	2.1	Suspected Salt Losing	3	Prepuberty	HC+FC	25	Central Java
6	2.5	Suspected Salt Losing	4	Prepuberty	HC+FC	23	Central Java
7	6.3	Classic	4	Prepuberty	HC	45	Central Java
8	6.4	Classic	4	Prepuberty	HC	27	Lampung, Sumatra
9	7.1	Classic	4	Prepuberty	HC	36	Central Java
10	8.2	Classic	3	Prepuberty	HC	45	Central Java
11	9.2	Classic	4	Puberty	HC	25	East Java
12	9.2	Classic	4	Prepuberty	HC	35	Central Java
13	9.9	Classic	4	Prepuberty	HC	1	Central Java
14	10.8	Classic	4	Prepuberty	HC	34	East Java
15	11.0	Classic	4	Prepuberty	HC	45	Central Java
16	12.8	Classic	3	Puberty	HC	23	West Java
17	13.4	Classic	3	Puberty	HC	46	Central Java
18	13.5	Classic	4	Puberty	HC	36	Central Java
19	13.8	Classic	3	Puberty	HC	34	East Java
20	14.2	Classic	4	Puberty	HC	38	West Java
21	14.4	Classic	2	Puberty	HC	34	East Java
22	16.0	Classic	4	Puberty	HC	38	Central Java
23	17.5	Classic	3	Puberty	prednisone	45	Central Java
24	18.4	Classic	2	Puberty	HC	34	Central Java
25	18.7	Classic	3	Puberty	prednisone	38	East Java

* HC: Hydrocortisone; ** FC : Fludrocortisone
 *** Only patient 4 was XY and had male gender.

Six patients were suspected of the salt wasting type of CAH. This diagnosis was based on a history of intercurrent illnesses during therapy. We could not establish the diagnosis as no data on urinary salt loss and on renin levels were available. These patients were treated additionally with 0.05-0.2 mg/day of 9 α -fludrocortisone, a synthetic mineralocorticoid.

The regular follow up every 3 months included physical examinations and measurements of blood pressure, height and weight. Instructions were given to parents and patients which included dosing schedules for medication and check of compliance. Auxological parameters, such as growth velocity and weight were also recorded. The effect of treatment was regularly monitored by assessment of salivary levels of 17-OHP and androstenedione. As much as

possible an X-ray of the left hand for bone age determination was obtained once a year in prepubertal and pubertal children. In addition, we obtained auxological data in prepubertal children

Patients Rotterdam

The Dutch patients were included in the study to provide a reference of salivary steroid precursor concentrations in well-controlled congenital adrenal hyperplasia patients treated in a tertiary reference outpatient-clinic in a country with one of the best health-care systems worldwide. As there are no reference values available for salivary adrenal steroid precursor concentrations, this population provides the next best reference.

Twenty CAH patients from the outpatient clinic of the Sophia Children's Hospital/Erasmus MC, Rotterdam, the Netherlands were included in this study. The age of the patients ranged between 5-16 years, 13 were males and 7 females; 12 patients had a salt wasting type of CAH. All patients in this study were treated with HC, 8.8 to 17.5 mg/24h per square meter body surface area, divided in three doses per day. An additional treatment with 9 α -fludrocortisone at a dose of 0.05-0.2 mg/day was given to the salt wasting patients.

Methodology

Measurement of salivary 17-hydroxyprogesterone and androstenedione

Salivary concentrations of 17-OHP and androstenedione were determined using salivary ELISA Kits (DRG, Amersfoort, the Netherlands).

Saliva Sample collection for day profile investigation

After a clear explanation of the procedure, the patients were asked to collect saliva by passive drooling into Sarstedt® (Nümbrecht, Germany) polypropylene tubes or, for babies, by pipetting with a minimum volume of 1 ml before eating or tooth brushing in order to prevent blood contamination. Contamination with food debris was avoided by rinsing the mouth with water. Collection times were as follows: the first sample before the morning tablet of medication at 06.00-07.00 h, the next three samples at 12.00-13.00, 16.00-17.00 and 21.00-22.00 for two

successive days. Saliva was stored frozen at -20°C until measurement in order to avoid microbial decomposition of the steroids.

Auxology measurements

Height standard deviation scores (SDS) were calculated with a growth calculator using Growth Analyser version 2 (www.growthanalyser.org). Growth data of the Chinese population in the programme were used as reference as no data are available for the Indonesian population. Body mass index (BMI) was calculated as weight (kilograms)/height (meters)².

Bone age was assessed by x-ray of the left hand using the Greulich and Pyle method (9). The patients or their parents gave informed consent to participate in this study, which was approved by the ethics committee of both institutions.

Results

After a period of steroid treatment varying between 1 - 46 months (median of 34 months), 17-OHP and androstenedione levels in saliva were measured four times a day prior to HC administration for two consecutive days for each patient to obtain the diurnal rhythm. Results are shown in figure 1, indicating high levels in a substantial number of patients. Therefore the dosage of HC and particularly the timing were adjusted. Based on these results, additional saliva samples were obtained after 1 month of adjusted treatment and just prior to the administration of HC for one day. Saliva steroid precursor concentrations decreased after treatment was adjusted (Figure 1). A clear correlation between saliva levels of 17-OHP and androstenedione was confirmed ($r = 0.88$). The morning saliva steroid concentrations of the patients in Semarang obtained after adjustment of dosage and timing were compared to steroid concentrations in saliva of CAH patients in Rotterdam obtained under comparable circumstances. As shown in Figure 2, the concentrations were comparable for most patients.

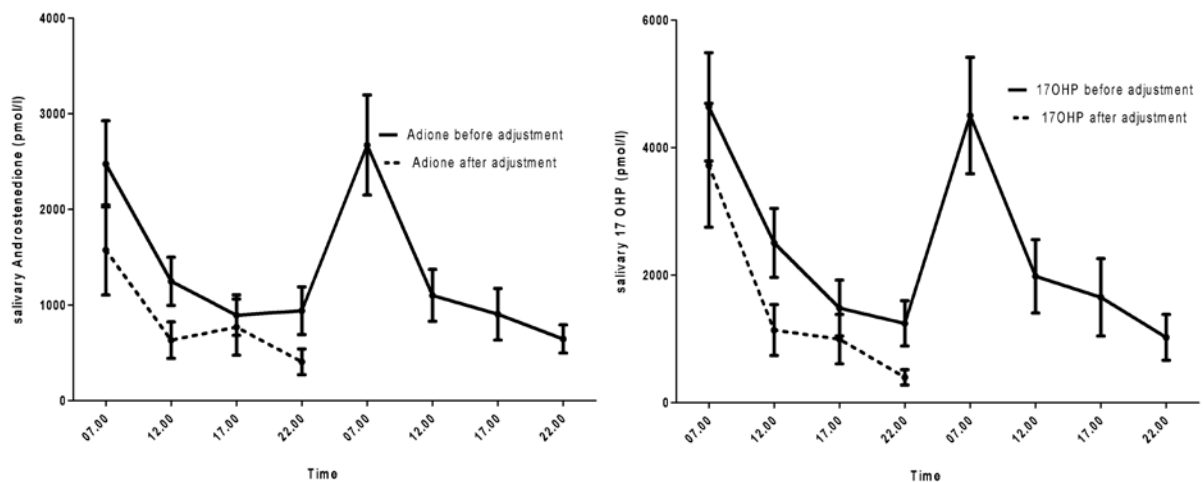


Figure 1: Salivary 17-hydroxyprogesterone and androstenedione diurnal rhythms

Circadian rhythms of 17-hydroxyprogesterone and androstenedione of 25 CAH patients on two subsequent days (full lines). Their levels were significantly correlated. In the morning, the concentrations of these steroids were high and then decreased gradually until the lowest values were found in the evening. A significant decline of hormone levels occurred after adjustment of dosage and time of administration of the medication ($P = 0.01$ and 0.04 respectively for 17-OHP and androstenedione) (broken lines). Figure shows means and SEM. Reference ranges for 17-hydroxyprogesterone and androstenedione are below 225 and 250 pmol/l, respectively.

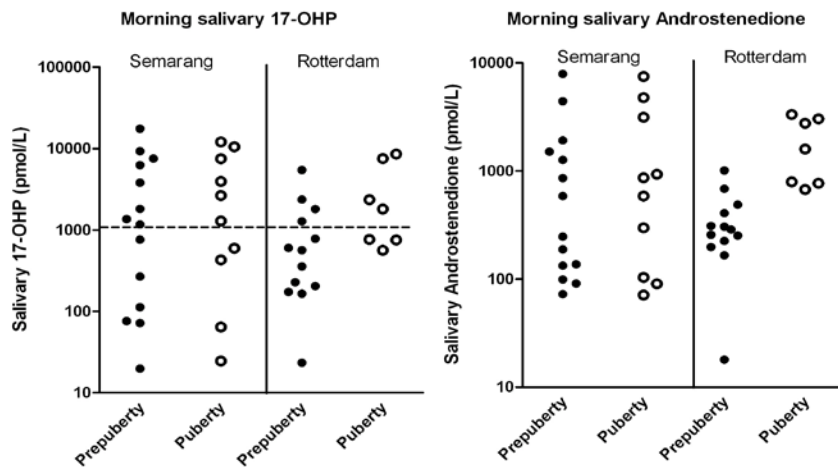


Figure 2: Salivary steroid levels in CAH patients in Semarang and Rotterdam

Comparison of the levels of 17-hydroxyprogesterone and androstenedione in saliva samples collected from CAH patient in Semarang (after treatment adjustment) and Rotterdam showing similar concentrations both in prepubertal and pubertal patients. (Pre-pubertal age: <12 years, pubertal age : ≥ 12 years) Reference ranges for 17-hydroxyprogesterone and androstenedione are below 225 and 250 pmol/l, respectively.

Auxological data

With regard to bone age examinations, 11 patients already stopped growing before start of therapy, 5 patients were examined only once, and in five patients no data were available. In 4 patients, the progression of bone maturation could be analyzed and all were found to have advanced bone age; The progression of maturation decreased in 3 patients after 45, 45, 35 months of therapy respectively.

Of the 16 patients who were still experiencing growth, 8 had improvement in height SDS during therapy, 3 experienced a decrease, 2 were unchanged and the other 3 could not be assessed because no data were available (Table 2).

Table 2: Development of height SDS during period of HC treatment of CAH patients

Number	Age on study (year)	Gender	Height-SDS			Duration of treatment (month)	Compliance
			1 year ago	6 months ago	dos		
1	0.7	Female	NA	-0.49	-1.8	8.0	bad
2	1.3	Female	-1.53	-2.57	-0.5	15.0	good
3	1.5	Female	-1.58	-1.78	-2.86	17.0	bad
4	1.8	Male	-3.21	-3.48	-3.32	21.0	good
5	2.1	Female	NA	NA	-3.87	25.0	bad
6	2.5	Female	-1.59	-1.12	-0.8	23.0	good
7	6.3	Female	0.65	0.26	0.22	45.0	good
8	6.4	Female	0.52	0.96	1.85	27.0	bad
9	7.1	Female	3.91	3.82	3.53	36.0	good
10	8.2	Female	NA	NA	2.63	45.0	bad
11	9.2	Female	0.63	0.54	0.51	35.0	good
12	9.2	Female	2.41	2.24	1.55	25.0	good
13	9.9	Female	NA	NA	0.92	1.0	NA
14	10.8	Female	0.02	0.61	0.16	34.0	good
15	11	Female	0.69	0.57	0.27	45.0	good
16	12.8	Female	-2.43	-2.52	-1.08	23.0	good

*dos: date of study; **NA: Not available

Discussion

Lifelong use of glucocorticoids is the therapy of choice in patients with CAH. Dose adjustments to avoid the adverse consequences of inadequate treatment and overtreatment are based on rigorous assessment of biochemical parameters.

This study shows that the result of treatment of CAH patients in Semarang, Indonesia is comparable to that in a Dutch endocrine centre. Both centres utilized salivary steroid measurements to monitor and adjust HC treatment.

The purpose of early treatment of CAH is to suppress the excessive adrenal androgen secretion without disrupting growth and the development of puberty. To achieve this, the glucocorticoid dosage should not exceed the physiological secretion of cortisol (6 mg/m²/day) which is equivalent to a HC intake of 8-10 mg/m²/day.

The deficiency of the adrenal enzyme 21-hydroxylase leads to markedly increased concentrations of steroid precursors from the adrenal gland. Thus dosing glucocorticoids at slightly supra-physiological levels will suppress the increased adrenal androgen production (10). This can be achieved with an intake of HC 10-15 mg/m²/day.

While our results indicate that suppression can be achieved, adjustment of dosage as well as time of dosing was necessary to reach an acceptable degree of suppression that was comparable to results in another clinic. When the medication was taken at defined hours (07-13-18 hr), the profile of hormones showed a steep increase in the morning and a gradual decline in the evening. This is because intake of HC in the early evening (18 hr) is insufficient to cover the corticosteroid requirement until the next morning. Therefore, we advised to administer the medication regularly every 8 hours at 06-14-22 hr to improve the hormonal profile.

HC is preferred over prednisone or dexamethasone in infants and children for the treatment of CAH because it is well tolerated, leads to less side-effects because of its shorter half-life and it is inexpensive (6). Most importantly, it will not inhibit linear growth provided it is given in the correct dosage. In pre-pubertal and pubertal children, treatment with long acting glucocorticoids such as prednisone or dexamethasone is no longer used because of the side effects of suppressing linear growth. Slow release preparations of HC accommodating the circadian rhythm of cortisol in the body are not yet available (11). Thus, specifically in infants and young children, HC in a dosage adapted to body surface is the drug of choice.

Whereas the short half-life of about 8 hours may allow a dosing schedule of 3 times a day, this dosing schedule may be insufficient especially in infants and young children to cover their overnight requirement. As a result, the HC does not last until the morning explaining high levels of 17-OHP and androstenedione in the morning samples of our patients, whereas the levels of both hormones decline appropriately during the day following morning and noon

administration of HC. In contrast, in patients with compliance problems levels remain high throughout the day.

Monitoring HC therapy of patients with CAH, using saliva to measure steroid hormones is used extensively (7,10). The main advantage of using saliva is the non-invasive way of collecting samples. In fact, steroid levels in saliva reflect the physiologically active fraction of steroid hormones (6,12). Moreover, saliva collection is very patient friendly as it may be done at home at designated times. Steroids in saliva are stable at room temperature allowing to send samples by regular mail to the laboratory for measurement.

Because single sample monitoring provides a picture of adrenal hormonal production at only one point in time and does not take into account the circadian rhythm of steroid secretion, some experts favour checking the hormonal profile during 24 hours by collecting blood or saliva samples several times during the day. Drawing a blood sample is bothersome to the patient, and is often logistically not feasible (10). In several studies, steroid levels in saliva and serum have been found to have a high correlation (10,13).

During periods of rapid growth such as during infancy and puberty the extent to which one should relax standards for 17-OHP, androstenedione and testosterone levels are not fully determined (14). Many of our patients (12 out of 25) were in the age range of 8-15 yr and were in (early) puberty. During this age period, it is difficult to maintain androgen suppression with HC treatment because of compliance problems as a result of pubertal behaviour and the changing endocrine milieu during puberty (15). Growth hormone and IGF 1 levels which are elevated during this period stimulate the activity of 17-hydroxylase/17,20-lyase resulting in increased adrenal androgen biosynthesis (16). Similarly in 7 out of 25 patients who started therapy during infancy HC dosing was notably difficult because of the rapid change of bodily proportions. Therapeutic efficacy was difficult to evaluate because most of our Indonesian patients were diagnosed and started therapy at a late age with advanced bone age which was not compatible with their chronological age. Due to premature epiphyseal closure, many of them did not achieve the optimal height: their average height amounted to $-2.42 (\pm 1.39)$ SDS.

Among the patients who were still experiencing growth, data are insufficient to draw firm conclusions. Education of patients and parents and regular monitoring of auxological parameters

and saliva steroid levels will lead to further improvement of height potential. It is well recognized that the age of diagnosis, the type of CYP21 mutation and the response to a certain dose of HC in view of individual variation of drug metabolism all are relevant factors contributing to the final outcome of HC treatment.

Many patients on treatment live in remote areas. To anticipate the situation when a patient is suffering from inter-current illness during which there is a need to increase HC dose, we offer them a reference letter that serves as information for the local doctor.

Availability of HC is very urgent because it is life-saving and it reduces morbidity not only in CAH patients but also in patients with congenital or acquired ACTH deficiency. It is believed that there are many under reported or unrecognized cases in Indonesia.

Conclusion

The diagnosis of CAH can be established in most of the patients with great certainty. However in Indonesia the management is still constrained by the lack of diagnostic and therapeutic means. The drug of choice in infants, young children and adolescents is HC, which is lifesaving. HC, declared an essential drug by the WHO, should be made widely available throughout Indonesia. With regular follow up HC treatment will enable a favourable outcome of patients with CAH. As saliva can be collected at home and sent by regular mail to the laboratory a major practical problem can be overcome. Collaboration with the local health provider for auxology measurements and monitoring of the health status of patients should be well promoted and will be a major step forward to optimize treatment.

Acknowledgements

This study was supported in part by a research grant from Diponegoro University (PNBP). We gratefully acknowledge the collaboration of the staff of Center for Biomedical Research (Cebior) of the Kariadi Hospital and the assistance of Ronald van der Wal, Endocrine Laboratory of the Department of Internal Medicine, ErasmusMC Rotterdam, the Netherlands.

References

1. Lee PA, Houk CP, Ahmed SF, Hughes IA. Consensus statement on management of intersex disorders. *International Consensus Conference on Intersex. Pediatrics.* 2006;118(2):e488-500.
2. Hindmarsh PC. Management of the child with congenital adrenal hyperplasia. *Best Pract Res ClinEndocrinolMetab.* 2009;23(2):193-208.
3. Stikkelbroeck MM, Otten BJ. [Congenital adrenal hyperplasia: clinical aspects and neonatal screening]. *Ned TijdschrGeneesk.* 2002;146(6):246-9.
4. Riepe FG, Sippell WG. Recent advances in diagnosis, treatment, and outcome of congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *Rev EndocrMetabDisord.* 2007;8(4):349-63.
5. Bonfig W, Pozza SB, Schmidt H, Pagel P, Knorr D, Schwarz HP. Hydrocortisone dosing during puberty in patients with classical congenital adrenal hyperplasia: an evidence-based recommendation. *J ClinEndocrinolMetab.* 2009;94(10):3882-8.
6. Groschl M, Rauh M, Dorr HG. Cortisol and 17-hydroxyprogesterone kinetics in saliva after oral administration of hydrocortisone in children and young adolescents with congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *J ClinEndocrinolMetab.* 2002;87(3):1200-4.
7. Deutschbein T, Unger N, Hauffa BP, Schaaf K, Mann K, Petersenn S. Monitoring medical treatment in adolescents and young adults with congenital adrenal hyperplasia: utility of salivary 17alpha-hydroxyprogesterone day profiles. *ExpClinEndocrinol Diabetes.* 2011;119(3):131-8.
8. Juniarto AZ, Goossens K, Setyawati BA, Drop SL, de Jong FH, Faradz SM. Correlation between androstenedione and 17-hydroxyprogesterone levels in the saliva and plasma of patients with congenital adrenal hyperplasia. *Singapore Med J.* 2011;52(11):810-3.
9. Buken B, Safak AA, Yazici B, Buken E, Mayda AS. Is the assessment of bone age by the Greulich-Pyle method reliable at forensic age estimation for Turkish children? *Forensic Sci Int.* 2007;173(2-3):146-53.
10. Dauber A, Kellogg M, Majzoub JA. Monitoring of therapy in congenital adrenal hyperplasia. *Clin Chem.* 2010;56(8):1245-51.
11. Loechner KJ, McLaughlin JT, Calikoglu AS. Alternative strategies for the treatment of classical congenital adrenal hyperplasia: pitfalls and promises. *Int J PediatrEndocrinol.* 2010;2010:670960.
12. Woodside DB, Winter K, Fisman S. Salivary cortisol in children: correlations with serum values and effect of psychotropic drug administration. *Can J Psychiatry.* 1991;36(10):746-8.
13. Shimon I, Kaiserman I, Sack J. Home monitoring of 17 alpha-hydroxyprogesterone levels by filter paper blood spots in patients with 21-hydroxylase deficiency. *Horm Res.* 1995;44(6):247-52.
14. Speiser PW. Growth and development: congenital adrenal hyperplasia-glucocorticoids and height. *Nat Rev Endocrinol.* 2010;6(1):14-5.

15. Charmandari E, Hindmarsh PC, Johnston A, Brook CG. Congenital adrenal hyperplasia due to 21-hydroxylase deficiency: alterations in cortisol pharmacokinetics at puberty. *J Clin Endocrinol Metab.* 2001;86(6):2701-8.
16. Balducci R, Toscano V, Larizza D, Mangiantini A, Galasso C, Municchi G, et al. Effects of long-term growth hormone therapy on adrenal steroidogenesis in Turner syndrome. *HormRes.* 1998;49(5):210-5.

Chapter 9
Virilization due to Androgen Hypersecretion in a Patient with an
Ovarian Leydig Cell Tumor: Diagnostic and Psychosocial
Implications

Achmad Zulfa Juniarto, Bestari A. Setyawati, Annastasia Ediati, Yvonne G. van der Zwan, Leendert H.J. Looijenga, Frank H. de Jong, Arianne Dessens, Stenvert L.S. Drop, Sultana M.H. Faradz.

Acta Med Indones. 2013 Apr;45(2):130-5. PubMed PMID: 23770793

Abstract

Background: Virilization due to hyperandrogenism in women causes male signs and symptoms such as swelling of the clitoris, deepening of the voice, facial hair and increase in body hair. Virilization is caused by less than 0.5% of all ovarian tumors. Here we report a case of virilizing Leydig cell tumor of the left ovary in a 36 years old woman. Misinterpretation of symptoms, conflicting medical information and advices from previous doctors had confused of the patient.

Design: We performed a diagnostic evaluation including clinical, hormonal parameters, imaging, surgery, histological parameters and psychological assessment.

Results: Blood analysis showed a testosterone level in the high male range. The presence of an ovarian tumor was confirmed by laparoscopy. Since the patient refused ovariectomy, a biopsy of the left ovary was performed. Pathology showed a Leydig cell tumor without histological signs of malignancy. In spite of extensive explanation and psychological counseling, cultural barriers prevented appropriate treatment.

Conclusion: An ovarian Leydig cell tumor should always be considered in a woman in the reproductive age with symptoms of virilization. The diagnosis is suspected on the basis of an ovarian mass on examination and further investigation and should be proven by biopsy. This is important before giving advice and carrying out treatment to the patient.

INTRODUCTION

Androgen excess is the most common endocrine disorder in reproductive-aged women, affecting approximately 7% of this population (1). This results in ovulatory dysfunction and the development of androgenic features such as hirsutism, androgenic alopecia, acne, and, if extreme and prolonged, virilization of the genitalia.

Hyperandrogenism comprises a heterogeneous group of disorders that exhibit a common phenotype. The most common causes include Poly Cystic Ovary Syndrome (PCOS), defects of adrenal or ovarian steroidogenic enzymes, ovarian hyperthecosis and theca cell hyperplasia. Another reason is the presence of an androgen producing ovarian neoplasm, although these are rare and account for less than 0.5% of all ovarian tumors. These tumors are composed entirely or predominantly of Leydig cells. A previous investigation showed that SRY-independent SOX9 expression can be found in ovarian Sertoli-Leydig cell tumors although the exact mechanisms still remain obscure (2). Finally, spontaneous testosterone production might be caused by an activating mutation in the LH receptor (LHR).

Here we report a case illustrating an androgen producing ovarian tumor in an adult woman. In addition, we describe the psychosocial and cultural barriers that prevented appropriate treatment.

CASE ILLUSTRATION

History of patient

A 36-year-old unmarried woman presented at our outpatient clinic with complaints of fatigue, hirsutism and signs of marked virilization (Figure 1). Menarche occurred at the age of 12 and she had regular cycles until the age of 28. After a period of 5 months of amenorrhea, she visited a gynecologist. Presuming the clinical diagnosis of PCOS, progestagen treatment was prescribed, which induced withdrawal bleedings. Since then, her periods under progestagen therapy. She reported that during the following 3 years there she experienced progressive masculinization such as virilization, facial hair growth, deepening of her voice, and a prominent Adam's apple. Her family history was negative for endocrinopathies.

Subsequently, she was referred to our center. Upon physical examination she was 152 cm tall and weighed 56 kg (BMI: 24.2). She had excessive pubic and abdominal hair (Ferriman-Gallwey score 15). A male type beard was present; there was no acne. The breasts were atrophied (Tanner stage 3). The blood pressure varied between 110/70 and 200/110 mm Hg and pulse rate was 80 per minute. External genital examination showed an enlarged clitoris (2.5 cm) and a normal female opening of the urethra, absent of fusion of labia majora and minora (Figure 1).



Figure 1. Enlargement clitoris and excessive pubic hair

Hormonal analyses were performed, of which the results are shown in Table 1. Pelvic and abdominal ultrasonographic examination showed normal ovaries on both sides and a normally sized uterus. The adrenal glands and kidneys were also normal.

Hormonal results suggested an androgen producing tumor originating from the ovary although this was not confirmed by ultrasound. Therefore, abdominal laparoscopy was undertaken. A biopsy was taken of the left ovary, which contained a structure with the

appearance of a polycystic tumor (lesion size 4x3.2x3.4cm). The right ovary did not show macroscopic abnormalities (size:3.3x2.8x1.5 cm).

Table 1. Results of hormonal evaluation of the patient

Hormone*	unit	patient	Reference ranges women**
LH	IU/l	12.3	1.5 - 8.0
FSH	IU/l	8.1	1.0 - 8.0
Progesterone	nmol/l	2.0	<0.5 - 3.0
17-hydroxyprogesterone	nmol/l	9.9	<0.5 - 2.0
Androstenedione	nmol/l	18.0	2.0 - 10.0
Testosterone	nmol/l	59.5	0.5 - 3.0
DHEA sulfate	μmol/l	3.8	1.0 - 10.0
Cortisol	nmol/l	191	200 - 800
AMH	μg/l	0.9	0.5 - 7.0

*DHEA: dehydroepiandrosterone; AMH: anti-Müllerian Hormone; LH: Luteinizing Hormone; FSH: Follicle Stimulating Hormone

** Values during the first week of the menstrual cycle

Histopathological examination

Histopathological examination demonstrated the presence of an ovarian tumor. The tissue consisted of small epithelioid cells with a pale eosinophilic, well-demarcated cytoplasm and small nuclei with dark chromatin. No atypical cells or mitotic figures were noticed. Reinke crystals were not found in the specimen. The tumor cells stained positive for the inhibin α -subunit and LHR, markers for Leydig cells, and negative for SOX9, a marker for Sertoli cells (Figure 2). These findings are consistent with a gonadal stromal tumor, and specifically for a

Leydig cell tumor. No histological signs of malignancy were identified. Analysis of the hot spot region in exon 11 of the LHR gene, mutations of which can lead to constitutive activation of the LHR, did not show a mutation. In conclusion the left ovary contained a gonadal stromal tumor compatible with a Leydig cell tumor lacking histological signs of malignancy.

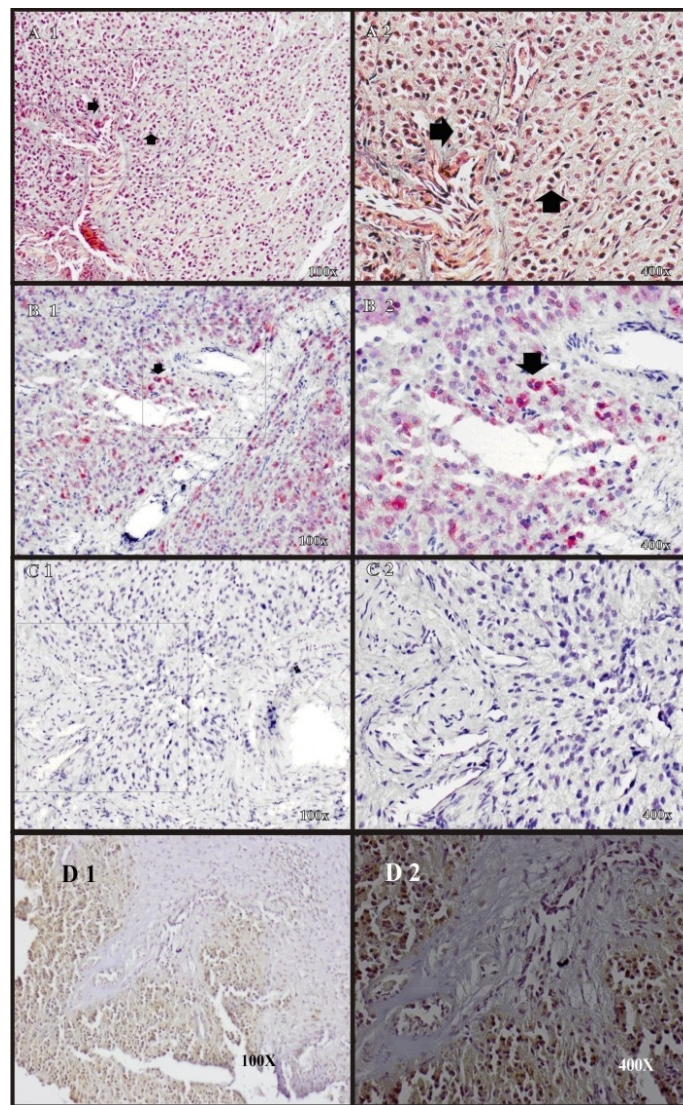


Figure 2.Ovary with a Leydig cell tumor. H&E staining revealed ovarian structures with gonadal stromal tumor compatible with Leydig cells (A1-2) (black arrow). Positive inhibin α -subunit was staining (B1-2); Negative for SOX 9 staining (C1-2). Magnification: left panel x 100, right panel x 400. Luteinizing hormone receptor (LHR) presence was detected by immunohistochemistry. Brown staining in cytoplasm shown in cord-stromal of the ovary. Magnifications were x 100 (D1) and x 400 (D2)

Luteinizing hormone receptor analysis

Analysis of the hot spot region in exon 11 of the LHR gene, mutations of which can lead to constitutive activation of the LHR, did not show a mutation (Figure 3)

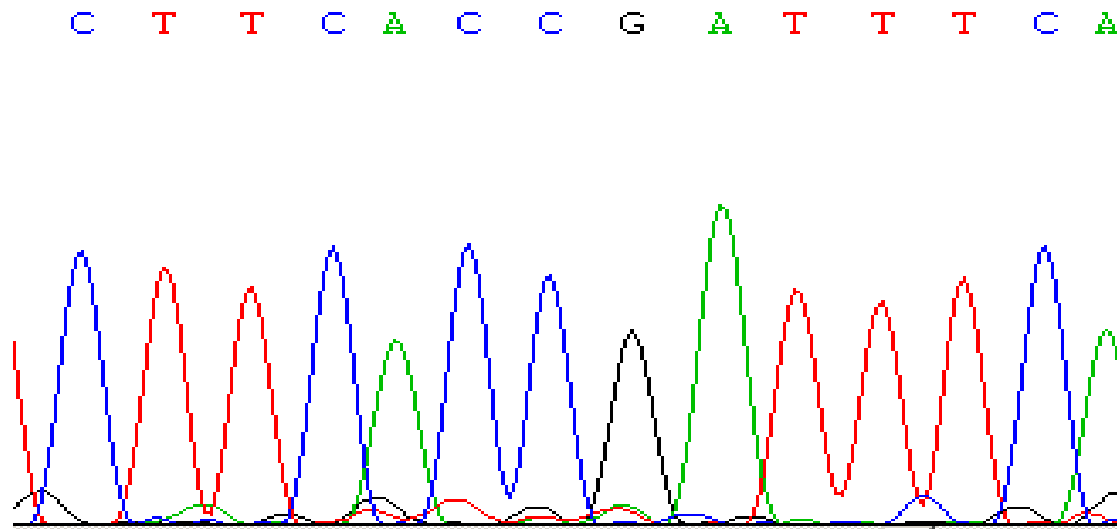


Figure3.Sequence of Luteinizing Hormone Receptor gene on exon 11 from Leydig cell tumor coding for Phe576, Thr577, Asp578 and Phe 579 showing a wild type receptor.

Psychosocial aspects

The patient came from a middle class Javanese family and obtained a university degree. The virilization of her body caused her a lot of distress. She consulted several medical doctors and beauticians who gave her conflicting advice. All treatments she underwent had been unsuccessful as she virilized progressively. After we found the cause of her virilization, we informed her about her condition and the nature of the ovarian tumor and proposed ovariectomy. She refused treatment, as she feared to become infertile after treatment. She particularly feared the social consequences of being an infertile woman. As an alternative, hormonal replacement therapy was proposed, but she refused. In the past she had already experienced that progestagen therapy could not stop virilisation. One of the doctors she had consulted before had informed her that a pregnancy would cure her disease and she preferred to follow this advice. She kept looking for a partner, but remained unsuccessful. Psychological counselling was offered to allow her to

express her worries and to help her understand her condition, its consequences, the necessity for treatment and the rationale behind the proposed treatments. She discussed the pressure she felt to marry and have children. She had already turned 36, whereas most Javanese women will marry and have their first child between age 20 and 30. It was expected that family support could take away some of the experienced shame and distress and proposed to establish such support by informing the family about her condition. However, this was unsuccessful. She told that she had already informed her family and disliked subsequent discussions. No signs of mental disorder and symptoms of depression were present. Her openness to discuss the impact of her condition on her life and her maintained confidence in cure by getting pregnant clarified her resistant attitude.

DISCUSSION

Systematic investigations in adult women with virilization are necessary to get to the right diagnosis. According to the literature, clinical signs of virilization should be confirmed by hormonal and ultrasound examination (3). Laparoscopy followed by biopsy is necessary to obtain a histopathological description and diagnosis

In this patient, symptoms of hyperandrogenism were the main initial clinical features. The signs of hirsutism and amenorrhea appeared at the age of 28 years within a 5 months period. Before she had regular menses and pubertal development had also been normal. Initially a diagnosis of PCOS was considered and indeed hormonal treatment resulted in regular bleedings, which may have convinced the patient that she was fertile. However the progressive virilization even under hormonal treatment had raised suspicion for an androgen-secreting tumor. The hormonal data suggested an androgen-producing tumor of the ovary since serum hormone assay showed increased levels of 17-hydroxyprogesterone and androstenedione and extremely high levels of testosterone with normal levels of the adrenal steroids cortisol, and dehydroepiandrosterone sulfate. Her bodily changes such as signs of virilization, including severe hirsutism, frontal balding, clitoromegaly, increased libido, altered body fat, increased muscle mass, breast atrophy, deepening of voice, and pustular acne were in accordance with

hormonal data are common symptoms in Leydig cell ovarian tumors (4). Estrogenic manifestations, such as irregular menses have also been reported. Symptoms and signs may present gradually with the onset of symptoms ranging from 5 to 7 years prior the diagnosis (5). Leydig cell tumors are usually benign histo-pathologically, these tumors can behave in a clinically malignant fashion (6). About 20% of patients develop metastatic lesions usually within the peritoneal cavity, and rarely at distant sites (7). Therefore, early treatment is necessary to prevent tumor to get worse.

The appearance of a virilizing tumor on radiological imaging depends on the type of tumor. Characteristic of steroid cell tumors are typically small nodules less than 3 cm and unilateral (8). These tumors show a heterogeneous solid mass with internal areas of intracellular lipid and may be difficult to identify on radiological imaging, in part because they are isoechoic to the uterus on ultrasound. This may be the reason why no abnormality of the ovaries was found during ultrasound examination of our patient. CT or MRI was not done because of financial limitations.

In view of patient's age and nulliparity, unilateral oophorectomy was offered as the first therapeutic option. Her refusal worsened her medical condition. Her decision to decline the given advice was related to the psychological mechanisms that will be triggered in case the offered cure is not congruent with the patient's beliefs and also includes unfavorable social consequences (9,10). For young adult females, loss of fertility involves a social disadvantage. It may become difficult to get married, but if she is married, she may get divorced or may have to accept that her husband will seek a second spouse. The patient made clear that she considered her ovaries as the only female part of her body that had been left and removal of even one ovary would mean taking away her femininity and everything that made her life worth to live. Her refusal for further discussion about treatment created a dilemma based on the respect for the patient's right on the one hand, but the risk of further progression of the tumor on the other. In spite of efforts for additional appointments to discuss the possibility of ovariectomy, no changes in her opinion occurred. Therefore it is expected that over the years, virilization and weight gain will become more prominent. Androgen excess in women may lead to shame and social

withdrawal or even social phobia which will normalize after treatment (5,11). However, in our patient, no signs of anxiety or depression were found.

This case report confirms that virilization in women should be investigated systematically and comprehensively. The diagnosis of Leydig cell tumor is made using clinical parameters such as amenorrhea, signs of virilization, and high serum testosterone levels and subsequently the finding of an ovarian mass on imaging. A diagnostic laparoscopy is indicated to confirm the diagnosis. Androgen secreting tumors, especially Leydig cell tumors of the ovary, should be considered amongst disorders causing virilization in women at reproductive age. What can be learned from this case? Even after extensive explanation and counseling psychosocial and cultural barriers prevented appropriate treatment. Social and community support is essential to help individuals to cope with shame and the prospect of infertility.

References

1. Azziz R, Sanchez LA, Knochenhauer ES, Moran C, Lazenby J, Stephens KC, et al. Androgen excess in women: experience with over 1000 consecutive patients. *J ClinEndocrinolMetab*. 2004 Feb;89(2):453-62.
2. Kato N, Fukase M, Motoyama T. Expression of a transcription factor, SOX9, in Sertoli-stromal cell tumors of the ovary. *Int J GynecolPathol*. 2004 Apr;23(2):180-1.
3. Denny MC, Smith D, O'Shea D, McKenna TJ. Investigation of patients with atypical or severe hyperandrogenaemia including androgen-secreting ovarian teratoma. *Eur J Endocrinol*. 2010 Feb;162(2):213-20.
4. Gershenson DM, Young RH. Ovarian sex cord-stromal tumors. In: Hoskins WJ, editor. *Principles and practice of gynecologic oncology*. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2005. p. 1024.
5. Nardo LG, Ray DW, Laing I, Williams C, McVey RJ, Seif MW. Ovarian Leydig cell tumor in a peri-menopausal woman with severe hyperandrogenism and virilization. *GynecolEndocrinol*. 2005 Oct;21(4):238-41.
6. Yetkin DO, Demirsoy ET, Kadioglu P. Pure leydig cell tumour of the ovary in a post-menopausal patient with severe hyperandrogenism and erythrocytosis. *GynecolEndocrinol*. 2011 Apr;27(4):237-40.
7. Haji AG, Sharma S, Babu M, Vijaykumar D, Chitrathara K. Androgen secretingsteroid cell tumor of the ovary in a young lactating women with acute onset ofsevere hyperandrogenism: a case report and review of literature. *J Med CaseReports*. 2007 Dec 18;1:182.
8. Jung SE, Rha SE, Lee JM, Park SY, Oh SN, Cho KS, Lee EJ, Byun JY, Hahn ST. CT and MRI findings of sex cord-stromal tumor of the ovary. *AJR Am J Roentgenol*.2005 Jul;185(1):207-15. PubMed PMID: 15972425.
9. Situmorang A. Staying single in a married world. *Asian Population Studies*. 2007;3(3):287-304.
10. Albert I, Trommsdorff G, Mayer B, Schwarz B. Value of children in urban and rural Indonesia: socio-demographic indicators, cultural aspects, and empirical findings. In:Trommsdorff G, Nauck B, editors. *The value of children in cross-cultural perspective case studies from eight societies*. Lengerich: Pabst science; 2005. p. 171-207.
11. Ekback M, Wijma K, Benzein E. "It is always on my mind": women's experiences of their bodies when living with hirsutism. *Health Care Women Int*. 2009 May;30(5):358-72.

Chapter 10

Gonadal malignancy in 13 Consecutive Collected Patients with Disorders of Sex Development (DSD) from Semarang (Indonesia)

Achmad Zulfa Juniarto, Bestari A. Setyawati, Ika P Miranti, Ardy Santosa, Remko Hersmus, Hans Stoop, Martine Cools, J. Wolter Oosterhuis, Stenvert L.S. Drop, Sultana M.H. Faradz, Leendert H.J. Looijenga

J ClinPathol. 2013 Mar;66(3):198-204. doi: 10.1136/jclinpath-2012-201062. Epub 2012 Dec 25. PMID: 23268320

Abstract

Aims: Caucasian patients with Disorders of Sex Development (DSD) have increased risk for Germ Cell Cancer (GCC). GCC are prominent in young adults in Western countries, while the incidence is significantly lower in Asia. So far it is unknown what the risk of GCC in Asian DSD patients is.

Methods and Results: A detailed gonadal histology study was undertaken on 16 Indonesian DSD patients using morphologically and immunohistochemistry (OCT3/4, TSPY, VASA, SCF/KITLG, SOX9, FOXL2). 13 cases could be analyzed, including ovarian tissue (n=3); streak gonad (n=1); undifferentiated gonad (n=1) and testicular tissue (n=8), diagnosed as 46,XX (n=1), 46,XY (n=7) and sex chromosome DSD (n=5). The precursor lesion gonadoblastoma or carcinoma *in situ*, or a GCC was diagnosed in four cases (30.8%; three 46,XY and one sex chromosomal DSD). A hormone producing ovarian Leydig cell tumor was identified in a 46,XX patient, supposed to be a late onset congenital adrenal hyperplasia. **Conclusions:** In spite of the significant lower risk of GCC in the general Asian population, DSD is a dominant risk factor. The study demonstrates the power of immunohistochemical markers for (early) diagnosis. This knowledge deepen understanding of the patho-biology of GCC and clinical handling of patients with DSD globally.

INTRODUCTION

Testicular Germ Cell Cancer (GCC) are the most frequent malignancy found in Caucasian males aged 20-45 years and has increased over the last decades (1, 2). The highest incidence exists in Nordic countries (11.5 per 100,000 men), although also heterogeneously, while it accounts for the lowest rates (1 to 2 per 100,000) among the black and Asian population. The rates are notably lower in East Asia (0.7-1.6 per 100,000) compared to West Asian (4.1 per 100,000) (3). Among the known risk factors for GCC are a previous GCC, cryptorchidism, infertility (suggested to be part of the Testicular Dysgenesis Syndrome, TDS) and Disorder of Sex Development (DSD) (4,5). DSD patients are currently classified based on a three root system, in which the first step divides the patients into 46,XX DSD, 46,XY DSD, and sex chromosomal DSD (6,7,8). Certain DSD subgroups are prone to develop a GCC, even up to 60% of the patients (9). This risk is related to the presence of a specific region of the Y chromosome, referred to as gonadoblastoma locus on the Y chromosome (GBY), for which the Testis Specific Protein-Y encoded (TSPY) is the main candidate (10,11). High risk is shared among gonadal dysgenesis (GD) patients who have the GBY region in their genome combined with intra-abdominal gonads (15-35%); non-scrotal gonads specifically in partial androgen insensitivity syndrome patients (PAIS) (50%). Intermediate risk includes variants of Turner syndrome variant (Y+) (12%) as well as 17 β -hydroxysteroid dehydrogenase deficiency patients (28%). These fore-mentioned DSD variants are all part of the 46,XY DSD entity (7,8). At low risk are patients with complete androgen insensitivity syndrome (CAIS), ovo-testicular DSD and Turner syndrome without Y-chromosomal material, with 2%, 3% and 1% respectively. Patients with GD and mosaic GD (45X/46, XY) show a prevalence of 30% and 15-40%, respectively, while the risk in cases of 5 α -reductase deficiency and Leydig cell hypoplasia are still unknown (12).

Development of an invasive GCC is preceded by CIS (carcinoma *in situ*)/IGCNU (intratubular germ cell neoplasia unclassified) of the testis or gonadoblastoma (GB) of the dysgenetic gonad (13). The formation of these precursors is dependent on the level of testicularization of the gonad, directly related to the presence of SOX9 as marker for Sertoli cells and FOXL2 as marker for Granulosa cells (14). Long term effects on quality of life, including

fatigue, metabolic syndrome, heart and vascular diseases, as well as secondary cancers, have been identified related to application of irradiation and chemotherapy at relative young age (15). Therefore, identification of the GCC as early as possible is of relevance for optimal treatment with long term effect (16).

Here we present results of the first series ever of histopathological characterization of consecutively collected gonadal tissues of Indonesian DSD patients. Various germ cell and stromal markers were applied, informative for the identification of GCC at the earliest possible pathogenetic stage.

MATERIAL AND METHODS

Patient selection and tissue samples

Indonesian DSD patients with the presence of ambiguous genitalia or any anatomical abnormality of external or internal genitalia, including penoscrotal hypospadias, with or without descended testes were examined in the Center for Biomedical Research, Diponegoro University (CEBIOR)/Dr. Kariadi Hospital from 2004-2011 and diagnosed in accordance with the recent consensus on DSD (7). Chromosome analysis was conducted using a G-banding technique in CEBIOR as described (17). Hormonal and mutation analysis was performed at the Departments of Endocrinology and Clinical Genetics Laboratories (Erasmus MC), respectively. Supposed gonadal tissue samples of 16 patients with androgen action disorders ($n=2$), androgen excess ($n=1$), and gonadal dysgenesis ($n=13$) aged from 0.9 to 30.9 yr (median age at time of operation was: 19.9 yr) were obtained after biopsy or gonadectomy. Bilateral specimens were available from five (31.2%), while the others were from unilateral biopsy or gonadectomy. Only 13 patients were included in the final examination. Characteristics of patients are summarized in Table 1. All gonadal samples were acquired for prophylactic and diagnostic purposes with written informed consent. Histological and immunohistochemical analysis (OCT3/4, TSPY, VASA, SCF, SOX9, FOXL2 and INH α subunit) were done at the Department of Pathology Diponegoro University Indonesia and Erasmus MC, Josephine Nefkens Institute, Rotterdam (the Netherlands).

Histological analysis

Specimens were first examined using routine H&E staining. Gonadal differentiation was determined per sample (testis/ovary/streak/undifferentiated gonad) as described previously (18). General morphology and maturity of seminiferous tubules and germ cells, presence of supporting cells, e.g. Sertoli cells, Leydig cells, and granulosa cells, adnexal structures e.g. fallopian tube or uterus were assayed. A Johnson score was attributed to each sample containing seminiferous tubules.

Diagnosis of the precursor lesion (CIS and GB) and their invasive GCC counterpart was made according to the World Health Organization Classification by an experienced pathologist (JWO). On the basis of morphology, germ cells were classified as immature or mature (1).

RESULTS

General histology

Histological examination of the available gonadal samples demonstrated the following pattern: ovarian structures (n=3); testicular structures (n=8); streak gonad (n=1) and undifferentiated gonads (n=1) (Table 2). Overall, the anatomical localization of the gonads was 50% abdominal, 46,2% inguinal, and 3.8% scrotal (one out of 26). However, within the group of 46,XY DSD the anatomical localization was mainly inguinal (78,6%) (Table 1). In case of the presence of testicular tissue (n=8), atrophic seminiferous tubules (based on Johnson score) were found in nearly all cases (n=7) (Table 2). Maturation of spermatogenesis was variable, however elongated spermatids were never found. The Johnson score ranged from 1 to 6 with the mean of 2.5. Two patients with sex chromosome DSD had a Johnson scored of 2 and 2.5, respectively.

Table 1. Summary of clinical data and cytogenetic result for each patient

CASE	AGE * (19)	GENDER AT DIAGNOSIS TIME	KARYOTYPE	DIAGNOSIS	GENETIC ANALYSIS	PHALLUS (cm)**	RIGHT GONAD		LEFT GONAD		QUIGLEY	POSITION OF URETHRAE
							VOL (ml)	LOCATION	VOL (ml)	LOCATION		
46,XX DSD												
1	30.9	F	46, XX	Hypervirilizatione.c. Androgen producing tumor		2,5	NA	abdominal	NA	abdominal	4	Perineal
46, XY DSD												
2	13.8	F	46, XY	Gonadal dysgenesis	No SRY mutation	4,1	NA	inguinal	NA	inguinal	3	Perineal
3	23.3	F	46, XY	Gonadal dysgenesis	No SRY mutation	normal clitoris	<1	inguinal	<1	inguinal	6/7	Perineal
4	1.9	M	46, XY	Androgen action disorder	NoAR Mutation	3,5	1	inguinal	NA	abdominal	2	Scrotal
5	16	F	46, XY	Androgen action disorder	859delG	normal clitoris	NA	abdominal	NA	abdominal	6/7	Perineal
6	1.8	M	46, XY	Gonadal dysgenesis	No SRY mutation	2.5	<1	inguinal	NA	inguinal	3	Scrotal
7	18.2	F	46, XY	Gonadal dysgenesis	DAX- 1duplication	5,0	NA	inguinal	NA	inguinal	4	Perineal
8	27	F	46, XY	Gonadal dysgenesis	No SRY mutation	5,0	NA	inguinal	1	inguinal	3	Perineal
CHROMOSOMAL DSD												
9	3.8	M	46,XY/46,XX	Gonadal dysgenesis	No SRY mutation	3,5	NA	abdominal	1	inguinal	2	Glans
10	22	F	46,XY/46,XX	Gonadal dysgenesis		normal clitoris	NA	abdominal	NA	abdominal	6/7	Perineal
11	13	M	45,X/46,XY	Gonadal dysgenesis		4,0	NA	abdominal	8	scrotal	2	Scrotal
12	20.8	F	46X,idicY	Gonadal dysgenesis		6,0	NA	abdominal	NA	abdominal	3	Scrotal
13	19.8	F	45,X /46,XY	Gonadal dysgenesis		5.5	NA	abdominal	NA	abdominal	3	Perineal

*Age: Age when gonadectomized or biopsed; ** According to Judith G Hall., et.al. http://www.amazon.com/Hanbook-Physical_Measurements-Medical-Publications/dp/019261696X-#Handbook of Normal Physical Measurements. Oxford University Pers.2003 ; NA = not available;

*** No gonadal histology

Table 2. Summary of patient's characteristic, histological finding and immunohistochemistry staining

CASE	AGE * (19)	SIDE GONAD	LOCATIO N OF GONAD	MATE RIAL FROM	MORPHOLOGY (JOHNSON SCORE)	OCT3/ 4	T S P Y	VASA	SC F	SOX 9	FOXL2	DOUBLE STAINING	OTHERS	HISTOLOGY
46, XX DSD														
1	30.9	L	Abdominal	B	Ovary with gonadal stromal tumor compatible with a Leydig cell tumor lacking histological signs of malignancy. The histology does not reliably predict the clinical behavior of the tumor(NP)	-	-	+(infollicles)	NP	-	+	NP	Inh (+); LH-R (-)	Ovary with Leydig cell tumour
46, XY DSD														
2	13.8	L	Inguinal	G	Testicular tissue with many germ cells at different stages of maturation (6)	+	+	+	+	+	NP	OCT/VASA (+)	c-KIT (+); PLAP (+)	CIS
		R	Inguinal			-	-	-	-	+	NP	NP	NP	-
3	23.3	L	Inguinal	G	Testis with leydig cell hyperplasia. Most seminiferous tubules are atrophic (2-3)	-	+	+	-	+	NP	NP	NP	Leydig cell hyperplasia
		R	Inguinal			-	+	+	-	+	NP	NP	NP	Leydig cell hyperplasia
4	1.9	R	Inguinal	G	Dysgenetic gonad, nogerm cells	-	-	-	-	+	-	NP	NP	Streak gonad, remnant sex cords, adjacent fallopian tube
5	16	L	Abdominal	G	Atrophic testis (1)	-	-	-	-	+	NP	NP	NP	Leydig cell hyperplasia
		R	Abdominal			-	-	-	-	+	NP	NP	NP	Leydig cell hyperplasia
6	1.8	L	Inguinal	G	UGT (NP)	+	+	+	+	+	+	OCT/TSPY (+)	NP	Gonadoblastoma
7	18.2	L	Inguinal	G	Testis with leydig cell hyperplasia. Most seminiferous tubules are atrophic (1-2)	-	-	-	-	+	NP	NP	NP	Leydig cell hyperplasia
		R	Inguinal			-	-	-	-	+	NP	NP	NP	Leydig cell hyperplasia
8	27	L	Inguinal	G	Atrophic testes with seminoma and carcinoma in situ on both sides (1-2)	+	+	+	+	+	+	OCT/TSPY (+)	NP	CIS and seminoma
		R	Inguinal			+	+	+	+	+	+			CIS and seminoma
CHROMOSOMAL DSD														
9	3.8	R (L in situ)	Abdominal	G	Ovary with multiple cysts, follicles and granulosa cells, including primordial follicles (NP)	-	-	+(in cysts/ follicles)	NP	-	+	NP	c-KIT (-); PLAP (+)	Ovary with multiple cysts, follicles and granulose cells
10	22	R(L in situ)	Abdominal	G	normal ovary (NP)	-	-	+(follicles)	NP	-	+	NP	NP	Normal ovary
11	13	R(L in situ)	Abdominal	G	Atrophic testis, Johnson 1, Leydig cell hyperplasia, no CIS (2)	-	-	-	-	+	NP	NP	NP	Sertoli cells only
12	20.8	L(R in situ)	Abdominal	B	No germ cells; no malignancy; diffuse Leydig cell hyperplasia (2-3)	-	-	-	-	+	NP	NP	NP	Leydig cell hyperplasia
13	19.8	L(R in situ)	Abdominal	B	Testicular (NP)	+	+	NP	NP	NP	NP	NP	PLAP (+)	Seminoma

* Age: Age when gonadectomized or biopsied; L: left; R: right; B: Biopsy; G: Gonadectomy; NA: not available; NP: not performed; UGT: undifferentiated gonadal tissue

GCC, *in situ* and invasive

The presence of germ cells was recognized by cytoplasmic staining for VASA and TSPY. In total, a GCC was identified in four cases (30,7%), predominantly in the 46,XY DSD group (42,9%), and one in the sex chromosomal DSD group (20%). All contained the Y chromosome in their karyotype, in agreement with the results of TSPY staining (see below). The presence of malignant cells was visualized using the OCT3/4 staining, showing a nuclear localization in all cases. In the 46,XY DSD patients, all GCC containing gonads were located at an inguinal position, while the sex chromosomal DSD patient with the GCC affected an abdominal gonad.

CIS was identified in two patients with high level of LH and FSH, low testosterone and no response for hCG test. One presented unilaterally (case 2, Figure 1), while the other was bilaterally, adjacent to an invasive seminoma (case 8, Figure 2), all at an inguinal position. Another pattern observed in a single patient (46, XY DSD) was the previously described undifferentiated gonadal tissue (UGT), consisting gonadal tissue with germ cells without seminiferous tubule structures or follicles, mixed with Sertoli cells or granulosa cells in cord like structures, supposed to be the precursor of GB (case 6, Figure 3) (18). Indeed, a combined expression of SOX9 and FOXL2 in GB derived from UGT has been reported previously (18).

No OCT3/4 positive germ cells were found in fully matured ovarian structures. SCF as marker for early malignant germ cells was consistently expressed in both invasive and precursor lesions (19). OCT3/4 –TSPY or VASA co-expression was encountered in CIS as well as GB as seen in our double staining experiments (Figure 4).

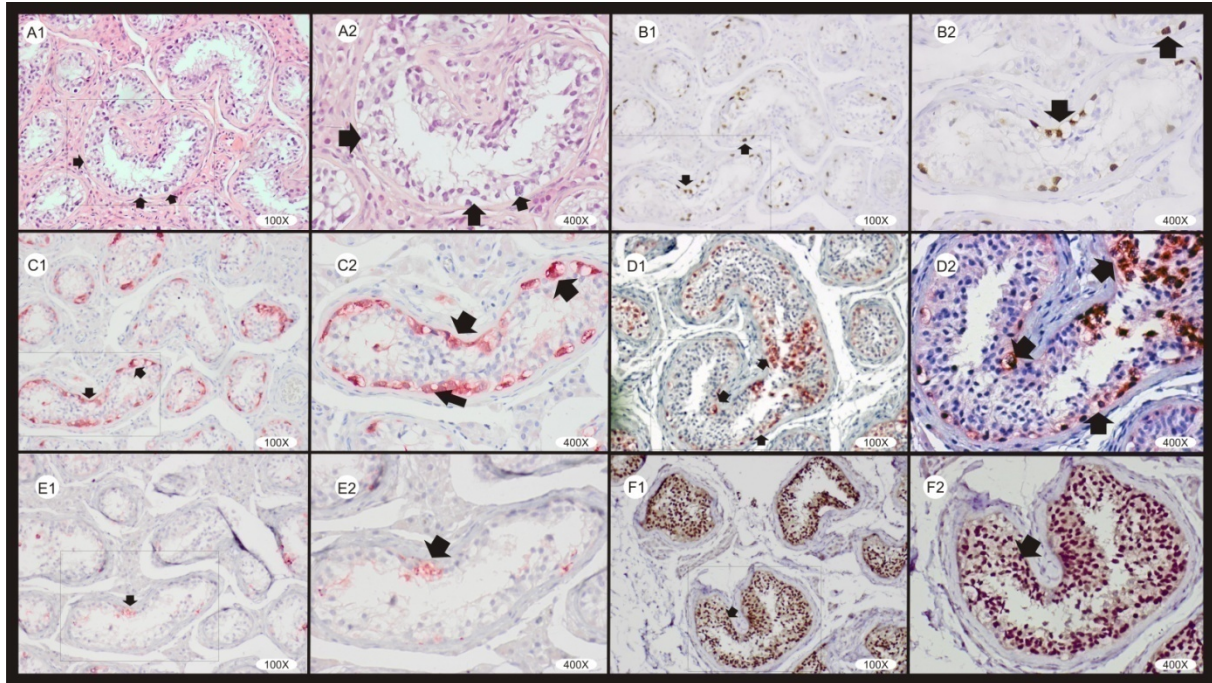


Figure 1. Case 2: 46, XY female with androgen action disorder. Breast development was present. After gonadectomy the patient received estrogen hormonal therapy. H&E staining (A1-2), morphology of the gonad showing testicular tissue with germ cells at different stages of maturation. Presence of carcinoma *in situ* (black arrow) confirmed by morphology and immunohistochemical staining for OCT3/4 (B1-2), TSPY (C1-2), VASA (D1-2), SCF (E1-2) and SOX9 (F1-2). Magnifications are as indicated in each pictures.

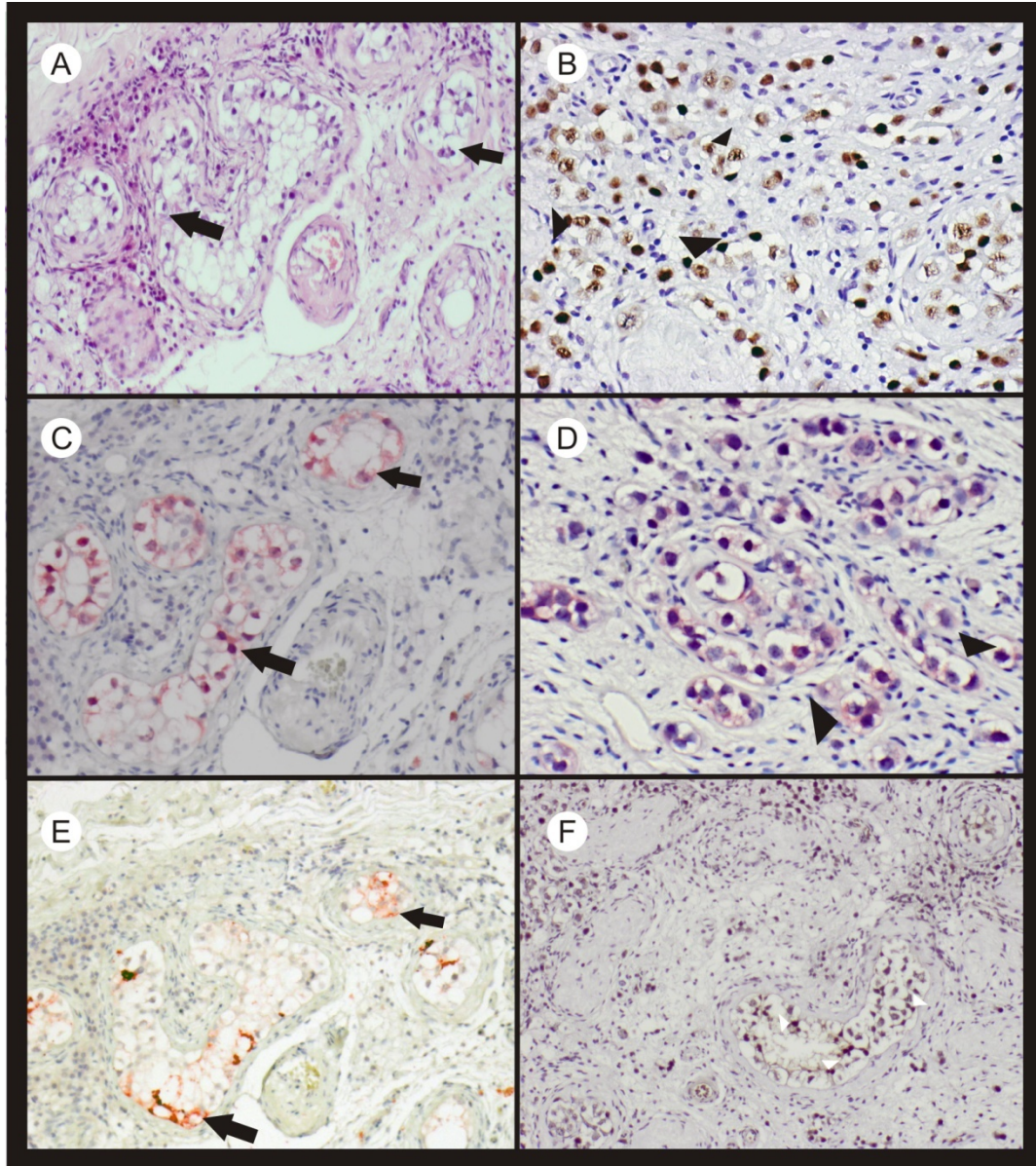


Figure 2. Case 8: 46, XY female with androgen action disorder. No breast development. After gonadectomy the patient received estrogen hormonal therapy. Carcinoma *in situ* (CIS) (A,C,E) was found adjacent to seminoma (B,D). H&E staining (A) showing almost complete atrophy of the seminiferous tubules containing extensive carcinoma *in situ* (CIS) (black arrow), which also expressed TSPY (C) and SCF (E). Seminoma (black arrow head) found in the same sample expressed OCT3/4 (B) and VASA (D). Sertoli cells (white arrow head) identified by SOX9 (F). Magnifications are 200x.

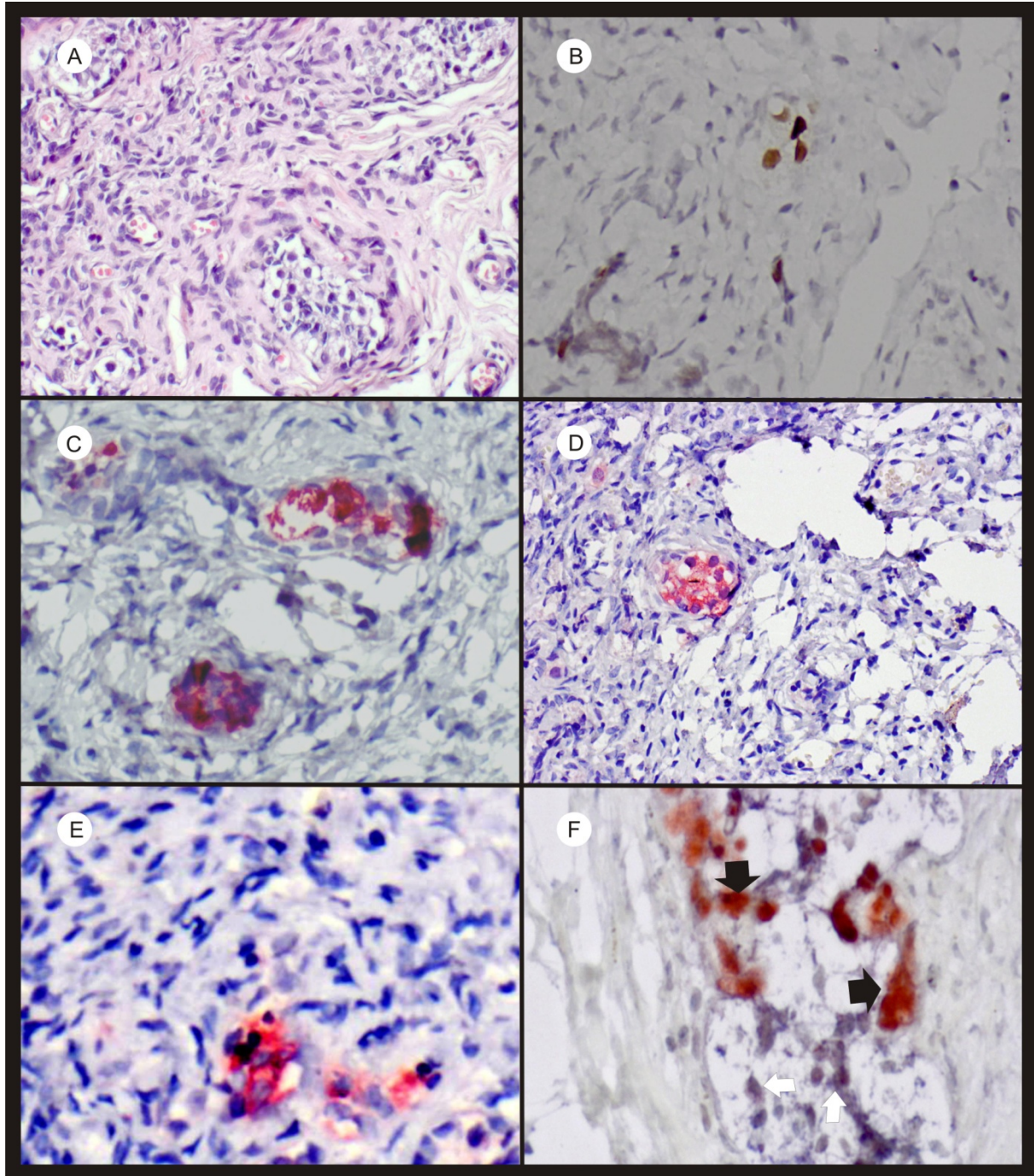


Figure 3. Case 6: 46, XY male with severe hypospadias and cryptorchidism staining depicting undifferentiated gonad with gonadoblastoma nest (black arrow) in H&E staining (A). Presence of immature germ cells confirmed by immunohistochemistry staining: OCT3/4 (B), TSPY (C), VASA (D), SCF (E). Supportive cells (F) are stained both for SOX9 (white arrow) and FOXL2 (black arrow) indicative for Sertoli cells and granulosa cells, respectively. Magnifications are 200x.

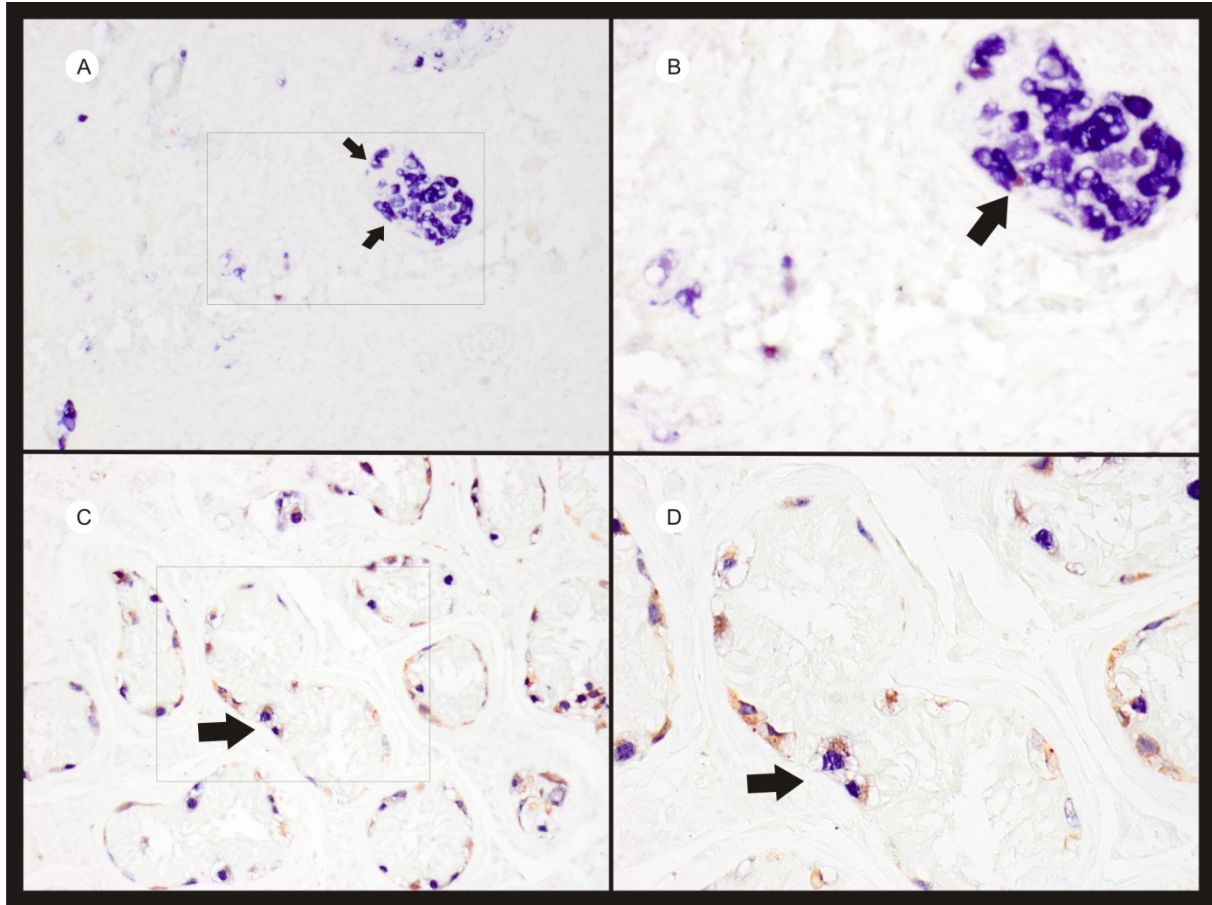


Figure 4. Representative examples of double staining experiments. Shown are TSPY (blue cytoplasmic signal) and OCT3/4 (red nuclear signal) in case gonadoblastoma in undifferentiated gonad (case 6) (A-B); VASA (red cytoplasmic signal) and OCT3/4 (blue nuclear signal) in case of carcinoma *in situ* (case 2) (C-D). Note the double positive cells are indicated by a black arrow. Magnifications are 100x (left panel) and 400x (right panel).

An unexpected Leydig cell tumor is a supposed late onset CAH patient

Within the series of patients investigated, a 46,XX virilized female presented with virilization and secondary amenorrhea. The testosterone value was tremendously increased (59.5 nmol/L, normal level 3.3nmol/L) despite a normal level of LH and FSH (12,3 IU/L, and 8.1 IU/L, respectively). Abdominal laparoscopy revealed a right normal ovary and a tumor lesion of the left, subsequently demonstrated to be a Leydig cell tumor confirmed by immunohistochemical detection of inhibin and LH receptor (LHr) (data not shown). No mutation within exon 11 of the LHr was identified (20).

DISCUSSION

We performed a detailed investigation based on histology and immunohistochemistry of a unique series of gonadal tissues from 13 DSD patients from the Indonesian population. To our knowledge, this represents the first of such a series and shows the high incidence of GCC and its precursor. Three malignancies were found in patients belonging to the category of 46,XY DSD and one case from sex chromosome DSD (46,XY/45,X). Indeed, these have been reported to show the highest risk for malignant transformation of germ cells leading to a GCC (9,21,22). It is important to consider the presence of gonadal malignancy (ovarian Leydig cell tumor) in case of adult women with virilization, which is a rare finding (21).

Despite varying histopathology features, the general morphology of the DSD gonads and germ cells did not differ from previous findings in Caucasian populations (22,23). The majority of the gonads had a non-scrotal localization. Most GCC or precursors were identified in gonads localized in the inguinal region and one in the abdominal. This highlights the potent malignancy of partially descended testes, as compared to abdominal testes. In the latter position the germ cells are probably undergoing apoptotic cell death before they can give rise to malignancy (23). Regarding age, 75% of GCC and their precursor lesions were at pre-pubertal and pubertal age ranges. The only two invasive GCC (seminomas) were found in two postpubertal patients (aged 19.8 and 27 years). This demonstrated the timing of progression from an *in situ* lesion to invasive growth happening after puberty.

Infertility is a well-established feature of cryptorchid testis, also confirmed by this study based on the low Johnson score in inguinal and abdominal gonads, although increased proliferation of fetal gonocytes has been reported in case of testicular feminization (24, 25). In general germ cells express markers like TSPY and VASA (26, 27), while OCT3/4 and SCF depends on the maturity of the germ cells, and their transformed phenotype. All GCC and the precursors, including dysgenetic and fetal gonads show presence of OCT3/4, which is absent on fully matured (non-embryonic) germ cells (11). OCT3/4 is reported to prevent apoptosis in embryonic germ cells, while in embryonic stem cells it mainly regulates pluripotency (28). Again demonstrated in this study, CIS, seminoma and GB can be diagnosed based on OCT3/4

staining, also in the undervirilised gonad (11). Similar to previous findings abundant and abnormal expression of TSPY, which proposed to accelerate proliferation and promote tumorigenicity, was identified in all our cases (11,25,29). However, TSPY can be lost upon progression to invasiveness (11). This situation also applies to germ cells in an unfavorable environment in DSD situations as nicely illustrated in case 8 displayed a CIS adjacent to seminoma. TSPY was abundantly expressed in CIS component but strongly reduced in seminoma as it gained its invasive capability. All gonads with high risk for malignant transformation also expressed SCF/KITLG, confirming the use of this marker for detection of early malignant characteristics of a GCC (19).

Taken together, it is obvious that the use of specific immunohistochemical markers is highly informative for detection of GCC and its precursors; which is rarely applied in Indonesia. However, OCT3/4 alone, which could be done in Indonesia, is sufficient to detect early malignancy.

Incidence of GCC is low in most Asian countries like Indonesia (2,3,30). However, and highly relevant, within the group of DSD patients, the incidence is as high as found in the Caucasian DSD population (1). This indicates that the combination of genetic and environmental factors, we refer to as “GENVIRONMENT”, which supposedly contribute to the high incidence of GCC in the Caucasian population, and which are less prominent in the Indonesian population, are compensated for in specific variants of DSD independent of ethnic background. Two recent studies indicate an association between specific genetic variants of the KITLG (as well as others) and the risk for GCC in the general population (29,31). It is an intriguing hypothesis that the effects of environmental factors are likely modulated by genomic variation (polymorphisms), thus explaining the individual susceptibility and population-level differences in the incidence of GCC. It might be of interest to study the distribution of high risk alleles both in the general - as well as in Asian DSD population. Based on the first description of gonadal histology of Indonesian DSD patients, it can be concluded that DSD is a dominant determinant for development of GCC. This insight opens relevant new perspectives in the diagnosis and investigation of the pathogenesis of this type of cancer.

ACKNOWLEDGEMENT

AZJ is the recipient of DGHE postgraduate scholarship from Indonesian Government. **BAS** is the recipient of a Nuffic scholarship program from The Dutch government. **MC** is supported by the Flanders Research Foundation (FWO).¹

References

1. Oosterhuis JW, Looijenga LH. Testicular germ-cell tumours in a broader perspective. *Nat Rev Cancer*. 2005 Mar;5(3):210-22.
2. Huyghe E, Matsuda T, Thonneau P. Increasing incidence of testicular cancer worldwide: a review. *J Urol*. 2003 Jul;170(1):5-11.
3. Chia VM, Quraishi SM, Devesa SS, Purdue MP, Cook MB, McGlynn KA. International trends in the incidence of testicular cancer, 1973-2002. *Cancer Epidemiol Biomarkers Prev*. 2010 May;19(5):1151-9.
4. Looijenga LH. Human testicular (non)seminomatous germ cell tumours: the clinical implications of recent pathobiological insights. *J Pathol*. 2009 Jun;218(2):146-62.
5. Skakkebaek NE, Rajpert-De Meyts E, Main KM. Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. *Hum Reprod*. 2001 May;16(5):972-8.
6. Lee PA, Houk CP. Disorders of sexual differentiation in the adolescent. *Ann N Y Acad Sci*. 2008;1135:67-75.
7. Hughes IA, Houk C, Ahmed SF, Lee PA. Consensus statement on management of intersex disorders. *J Pediatr Urol*. 2006 Jun;2(3):148-62.
8. Hughes IA. Disorders of sex development: a new definition and classification. *Best Pract Res ClinEndocrinolMetab*. 2008 Feb;22(1):119-34.
9. Cools M, Looijenga LH, Wolffenbuttel KP, Drop SL. Disorders of sex development: update on the genetic background, terminology and risk for the development of germ cell tumors. *World J Pediatr*. 2009 May;5(2):93-102.
10. Lau Y, Chou P, Iezzoni J, Alonzo J, Komuves L. Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma. *Cytogenet Cell Genet*. 2000;91(1-4):160-4.
11. Kersemaekers AM, Honecker F, Stoop H, Cools M, Molier M, Wolffenbuttel K, et al. Identification of germ cells at risk for neoplastic transformation in gonadoblastoma: an immunohistochemical study for OCT3/4 and TSPY. *Hum Pathol*. 2005 May;36(5):512-21.
12. Looijenga LH, Hersmus R, Oosterhuis JW, Cools M, Drop SL, Wolffenbuttel KP. Tumor risk in disorders of sex development (DSD). *Best Pract Res ClinEndocrinolMetab*. 2007 Sep;21(3):480-95.
13. Woodward PJ, A. H, Looijenga LH, Oosterhuis JW, McLeod DG, Moller H, et al. Classification of Tumours. Lyon: *World Health Organization IARC Press* 2004.
14. Hersmus R, Kalfa N, de Leeuw B, Stoop H, Oosterhuis JW, de Krijger R, et al. FOXL2 and SOX9 as parameters of female and male gonadal differentiation in patients with various forms of disorders of sex development (DSD). *J Pathol*. 2008 May;215(1):31-8.
15. Markman M, Bookman MA. Second-line treatment of ovarian cancer. *Oncologist*. 2000;5(1):26-35.

16. van Casteren NJ, Looijenga LH, Dohle GR. Testicular microlithiasis and carcinoma in situ overview and proposed clinical guideline. *Int J Androl*. 2009 Aug;32(4):279-87.
17. Scarpato R, Migliore L. Comparison of spontaneous structural chromosome aberration frequency in 48 h-cultured human lymphocytes mitotically arrested by different colcemid treatments. *Mutat Res*. 1996 Sep 26;361(1):35-9.
18. Cools M, Stoop H, Kersemaekers AM, Drop SL, Wolffenbuttel KP, Bourguignon JP, et al. Gonadoblastoma arising in undifferentiated gonadal tissue within dysgenetic gonads. *J ClinEndocrinolMetab*. 2006 Jun;91(6):2404-13.
19. Stoop H, Honecker F, van de Geijn GJ, Gillis AJ, Cools MC, de Boer M, et al. Stem cell factor as a novel diagnostic marker for early malignant germ cells. *J Pathol*. 2008 Sep;216(1):43-54.
20. Cui J, Miner BM, Eldredge JB, Warrenfeltz SW, Dam P, Xu Y, et al. Regulation of gene expression in ovarian cancer cells by luteinizing hormone receptor expression and activation. *BMC Cancer*. 2011;11:280.
21. Hoffman JG, Strickland JL, Yin J. Virilizing ovarian dermoid cyst with leydig cells. *J PediatrAdolesc Gynecol*. 2009 Jun;22(3):e39-40.
22. Schreiber L, Lifschitz-Mercer B, Paz G, Yavetz H, Zadik Z, Kula K, et al. Lack of RBM expression as a marker for carcinoma in situ of prepubertaldysgenetic testis. *J Androl*. 2003 Jan-Feb;24(1):78-84.
23. Cools M, van Aerde K, Kersemaekers AM, Boter M, Drop SL, Wolffenbuttel KP, et al. Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilization syndromes. *J ClinEndocrinolMetab*. 2005 Sep;90(9):5295-303.
24. Kogan SJ. Fertility in cryptorchidism. An overview in 1987. *Eur J Pediatr*. 1987;146 Suppl 2:S21-4.
25. Merlet J, Racine C, Moreau E, Moreno SG, Habert R. Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *Proceedings of the NationalAcademy of Sciences*. 2007 February 27, 2007;104(9):3615-20.
26. Castrillon DH, Quade BJ, Wang TY, Quigley C, Crum CP. The human VASA gene is specifically expressed in the germ cell lineage. *ProcNatlAcadSci U S A*. 2000 Aug 15;97(17):9585-90.
27. Schnieders F, Dork T, Arnemann J, Vogel T, Werner M, Schmidtke J. Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues. *Hum Mol Genet*. 1996 Nov;5(11):1801-7.
28. Skakkebaek NE. Possible carcinoma-in-situ of the testis. *Lancet*. 1972 Sep 9;2(7776):516-7.
29. Kanetsky PA, Mitra N, Vardhanabhuti S, Li M, Vaughn DJ, Letrero R, et al. Common variation in KITLG and at 5q31.3 predisposes to testicular germ cell cancer. *Nat Genet*. 2009 Jul;41(7):811-5.
30. Andayani YD, Safei S. Pattern of germ cell testicular carcinoma in DharmaisCancerHospital between January 2000-December 2004. *Acta Med Indones*. 2008 Jan;40(1):11-3.
31. Rapley EA, Turnbull C, Al Olama AA, Dermitzakis ET, Linger R, Huddart RA, et al. A genome-wide association study of testicular germ cell tumor. *Nat Genet*. 2009 Jul;41(7):807-10.

Chapter 11

**General Discussion and Future Organization of the
Management of Disorders of Sex Development in Indonesia**

Introduction

The term Disorder of Sex Development is presently used as an umbrella term including previous terms such as hermaphrodite, intersex, ambiguous genitalia. The history of the nomenclature is reflecting the confusion not only of experts but also for sure, for lay people.

Although the older terms are still included in this thesis for reference, they have been replaced by most experts (and patients and families) because they are misleading, confusing, and insensitive. Increasingly this group of conditions is being called disorders of sex development (DSD) in order to avoid conflicting anatomy with identity. Members of The Lawson Wilkins Pediatric Endocrine Society and the European Society for Pediatric Endocrinology accepted the term "disorders of sex development" in their "Consensus statement on management of intersex disorders" published in the Archives of Disease in Children and in Pediatrics in 2006. The term is defined by congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical. However, this has been met with criticism from activists who question a disease/disability model and advocate no legal definition of sexes, no formal gender assignments, no legal sex on birth certificates, and no official sexual orientation categories. Alternatives to labelling these as "disorders" have also been suggested, including "variations of sex development"

Genital anomalies are estimated to occur in 1 in 4500 births (1). Where in 46,XY individual the ratio is 1: 16.000 in every life birth. In 46 XX individuals the most common form of DSD is congenital adrenal hyperplasia (CAH) with an occurrence of 1 : 9000-16000 in every life birth.

The subjects of this research are 286 patients referred to the Kariadi Hospital/Diponegoro University Semarang, Indonesia during the period 2004-2010.

A number of patients with mild genital anomalies such as isolated mild hypospadias and other mild sexual disorders, amounting to 347 subjects, were excluded from the main research.

Among these 286 patients of RSDK, the occurrence of 46,XY DSD was 68.2%, 46, XX DSD was 23,4% and the rest is accounted for by Sex Chromosome DSD.

The DSD treatment in RSDK did not cover all of the patients. Only 50.7 % of patients were treated with hypospadias correction in boys and CAH therapy in girls being the most common. It became clear that most of the reasons for patients not to undergo the therapy were problems such as lacking financial resources, a too large distance to the center, hesitance for the surgery process, while some of the patients obtained too little information.

This research is novel in Indonesia, and as far as we know, the multidisciplinary treatment of DSD as in this research has never been performed before.

1. The development of Management of DSD in Indonesia

The management of DSD has been set up to guide the medical community in treating DSD patients; however, the implementation and the outcome is variable and depends on many factors such as type of the disorder, the patient's general health and the financial condition of the patient or his family, and patient's and family's reaction and understanding towards DSD.

In Indonesia, the Ministry of Health (or government) did not pay much attention to the management of DSD since infectious diseases are still a priority, being the major cause of morbidity and death. Actually, six major hospitals in Indonesia were recommended to have a gender team since the year of 1989; however due to lack of expertise and laboratory equipment as well as financial constraints development has been slow. Moreover, awareness of medical personnel and of the community for the particular problems related to DSD remained limited. In order to improve the management of DSD in Indonesia, understanding of this last issue is important. At the same time, extensive research of the etiology of DSD of Indonesian patients and identification of the structure of the ethnic and cultural background is necessary.

The general guidelines of DSD management should be set and standardized nationally and be accountable. Hence it will provide adjustable implementation for the various conditions of the patients and their families. Therefore, DSD management should be employed differently depending on the diversity of cultural and demographic background.

1.1 Conditions of the DSD Patients in Indonesia

As mentioned above, the background of the patient is an important factor contributing to the intensity (length and depth) of the care provided, including the decision making process. Several factors such are affecting the management of DSD.

First, the general community, of which the patient and family are part in daily social life is still considering DSD as a taboo. Sexual related problems such as DSD are not well understood and just ignored. Most of the parents and adult patients are reluctant to seek medical attention. The majority of the parents are embarrassed of having children with a sexual abnormality. Thus they consider it as a disgrace which should be concealed from the others. This is one of the reasons why there are still undetected DSD patients. The clinical condition of DSD in Indonesia is mostly identified because patients or parents seek medical attention due to urgent matters, such as severe dehydration (salt loosing crisis in CAH), hernia inguinalis incarcerata, delay or absence of puberty, gender related problems such as desire to get married, or getting an identity card. For some people in Indonesian society the term DSD is misleading. A lot of people confuse a DSD patient with a transgender or transsexual individual or with a homosexual (gay, lesbian) or bisexual person. In the Indonesian population these forms of sexuality are considered to be against nature and individuals are often labelled as condemned persons and discriminated in the community.

The Bugis tribe in South Sulawesi, Indonesia is one community that acknowledge three sexes, which is male, female and hermaphrodite. There are four genders; the makunrai (female women), oroane (male-men), calalai (biological females who serve the roles and functions expected from men), calabai (men who behave close to what is expected of women) and in addition the bissu (the meta gender). The bissu are assumed to be hermaphrodites by embodying both female and male elements, and usually fit as a specific group acting as priests. The Bugis believe that a bissu who appears externally as a male internally is a female, and vice versa. This combination of sexes leads to communal acceptance of 'meta-gender' as the third sex among them (2-4).

Second, most of the patients who are seen by our sex adjustment team in Semarang are from middle and lower economic classes. Therefore their financial situation limits their access to health care. In addition their often limited educational level influences their capability to receive new information and to communicate on further procedures. These limits affect the implementation of diagnostic procedures, particularly as procedures are often rather expensive.

Third, most patients do not have health insurance; and even if they have health insurance it often does not cover genetic testing. This situation is adding to the complexities of the problem.

1.2 Current Management of DSD in Indonesia

A guideline for DSD diagnostic implementation has been created and published by ESPE in 2006, which is continuously improved (1). In principle, there are several stages in the diagnostic process of a DSD patient (1,5,6), which are listed below :

1. General physical examination
2. Chromosomal analysis
3. Hormonal analysis
4. Determination of electrolyte levels in serum and urine essential for the urgent management of CAH
5. Hormonal stimulation tests (HCG, ACTH) as a second line of investigation specifically to determine disorders of steroidogenesis.
6. Additional investigations such as ultrasound, MRI, and endoscopy to check the anatomy of the internal genitalia
7. Specific molecular genetic analysis such as gene mutation analysis to confirm a genetic diagnosis.

However, despite recent developments, there are several factors that are slowing the pace of the further development of structured management of DSD patients in Indonesia:

1. The term DSD is unknown and even considered unpopular among health care professionals in Indonesia
2. DSD management in Indonesia is not yet integrated in centers other than Semarang

3. There is a lack of specialised diagnostic facilities in medical centres
4. It is still unclear whether the final decision regarding gender choice and genital surgery is decided by the patient / parent or by the doctor (medical team).
5. There are no certain rules or ethical guidelines regarding surgical management of DSD patients; it is unclear whether definitive surgery should be performed at an earlier age with informed consent (mostly by the parent) or should be postponed until the patient has the age to decide for her/himself.

These potentially inhibiting factors will be explained here in more detail:

First, for health care professionals, the terminology of DSD as well as the clinical diagnosis is still not widely understood and relevant information is available only in the major cities. Most medical personnel is familiar with the term ambiguous genitalia or “double sex” (*kelamin ganda*) or hermaphroditism rather than DSD. The referral centre / hospital for proper diagnosis and multidisciplinary management is unknown to them. Thus this indicates a need for education regarding the recognition of clinical signs and early treatment as well as of the referral system relating to DSD.

Second, the mechanism of DSD management is not yet integrated in the Indonesian health care system. A multidisciplinary team approach to treat DSD cases is exceptional in Indonesia. However in Semarang a multidisciplinary approach by the gender team called Tim Penyesuaian Kelamin is in use since 1989. DSD patients are usually extensively treated in only one department with limited collaboration assistance from the other departments. Moreover extensive hormonal and genetic analysis is rarely performed because of limited laboratory facilities and financial constraints. This situation needs to be improved so that management of DSD patients can be implemented comprehensively.

Third, not every medical centre has adequate facilities. Taking into consideration the diagnostic complexities, this gap in medical centres’ facilities is likely to affect the accuracy of diagnosis and treatment.

Fourth, in the case of gender assignment or gender alteration especially in very young age patients, it is still unclear whether the final decision is taken by the team of medical professionals, or by the parents. The treatment of patients needs to be initiated immediately after

diagnostic tests are conducted. Problems usually occur when diagnostic tests are conflicting or in mismatch with patient's current gender or with parent's wish. Extensive explanation and instruction with subsequent adaptation is then required. In typical situations like this, the medical team is not supposed to force the decision making respecting the feeling of the patient and family. The opinion and the needs of the patients and family are to be listened to. Most importantly, the decision for surgical therapy, often a key part of therapy, should be taken only following thorough consideration and without rushing.

In conditions where consensus is not reached, treatment relating to gender assignment can be delayed until consensus is reached between patient and family (parents) and the medical team. When elective surgery and hormonal treatment is considered there is consensus that the preferred option in typical DSD is to delay treatment until the patient can actively participate in the decision-making about how his or her own body will look, feel, and function.

Fifth, taking into consideration that the clinical spectrum of DSD is very broad and varied, the individual management of each patient is important since it cannot be generalized. Even when the clinical appearance is quite similar, there may be a situation where a different decision regarding outcome is taken. There are no general recommendations for each case except comprehensive management procedures, and the rule of thumb to prioritize on longevity and quality of life of the patient. This requires not only a profound and specialized medical knowledge of the medical team, but also experience.

2. Recommendations for Management of DSD Patients in Indonesia:

2.1 Treatment

Ideally, a professional team of DSD experts should collaborate to treat DSD patients in a comprehensive and understanding manner, including genetic and psychosocial counselling of both patients and his or her family.

Parents must be able to grasp the controversies in management of DSD patients. Gender assignment that is decided too early based just on the anatomy of the external genitalia could harness further problems on the child. The phenotype does not always depict the actual conditions due to intrauterine mal-development. Gender assignment in a hasty way will directly

impact on the child's rearing. If a mismatch occurs between sex of rearing and the biological condition of the child in the future life then the solution to these problems will become more complicated.

Chromosomal (genetic), hormonal, neurological, psychological and behavioural factors all affect one's gender. Presently, the majority of experts prefers to delay the surgical interventions for gender assignment as long as the patient is in healthy condition, in order to involve the individual him/herself in the decision making process. However, our experiences with Indonesian parents indicate that they are very dominant to decide gender assignment and surgical treatment as soon as possible. They will push the medical gender team to initiate treatment directly for their own wish and to hide their shame.

2.2 Follow up

After the diagnostic procedures and when treatment is initiated, a regular follow up is beneficial to record the development of patient's health; any required action could be taken whenever related problems occur during or after hormonal treatment or surgical therapy. Maintaining quality of life of a patient is a doctor's main priority. Many of the patients are living in small villages and far from the medical centre. Sometimes their compliance is limited and thus the team should actively contact them for medical follow up; it is not rare that their cost of transportation has to be provided.

2.3 Prognosis

Prognosis of DSD is heavily dependent on the specific cause. A correct understanding of the etiology, effective medication and comprehensive support from every involved party will result in the best possible prognosis. In Indonesia the prognosis is difficult to predict because comprehensive management cannot be provided to the whole archipelago and not even to the major cities.

2.4 Support Group

The existence of a support group will provide an essential prop up for patients. They will feel that they are not alone in facing their problems. There are others who are dealing with the same issues. It will ease the psychological burden of the patients. It is not very common in Indonesia as most of the patients do not want to be disclosing their anomaly, however in Semarang we established a patient support group named “Forkis” with initial funding from the Central Java governor. This support group is comprised of patients and volunteers who will accompany a new patient to alleviate the psychological burden by sharing their experiences. Creating a website that contains information related to DSD and to common concerns of patients , should be seriously considered for implementation..

2.5 Support from the Government (Policy Maker)

DSD is not the main priority of the health sector in a developing country such as Indonesia. However, supportive action from the government is expected. Efforts in providing health insurance for poor people are on-going for several years and have been proven to be advantageous in treating the DSD patients. Budget allocation for research and treatment of general medical problems also including DSD should be taken into consideration for overall health improvement of all Indonesian people. Pharmaceutical preparations such as hydrocortisone and fludrocortisone, presently on the WHO list of essential drugs are not available in Indonesia. Efforts are underway to request the Ministry of Health and National agency for food and drug control (NAFDC) to make these drugs available. Awareness of the medical personnel and pharmacists to the need of these medication should be encouraged.

2.6 The need for an MDT (multidiscipline team) organization

In order to manage DSD patients the organization of a multidisciplinary team should have adequate manpower (7):

- **Team Leader** as the formal institutional head of the team. He or she assembles the team and oversees and steers the work of the team.
- **Team Coordinator** will keep team members connected (including organizing consultations, case discussions, and team meetings) and ensure timelines and continuity in case management.
- **Team Liaison** will serve as the contact person for the patients and family, ensuring that parents and or patients are kept informed of all care and treatment options and linking them with resources and support services. In addition, a religious figure needs to be included in the MDT in Indonesia. As a religious society, spiritual perspectives weigh heavily in every decision of daily life. The role of a religious expert is vital, especially in the final decision of assigning gender prior to surgical therapy.

2.7 The need to establish a network of medical centres

In view of the current situation of a substantial gap of facilities between medical centres in major cities, it is recommended to establish a network of collaborations regarding certain diagnostic examinations and procedures. This may mean that a patient who requires a certain test needs to be referred to another medical centre for a specific examination. This is important to improve the treatment quality and the accuracy of diagnosis based on the current protocol (1).

2.8 Cooperating with medical centre in other countries

Another important action is to cooperate with medical centres from other countries, preferably those who are more advanced in the competency of health care professionals as well as research and diagnostic facilities. It is expected that health care professionals in Indonesia could be assisted in performing the management of DSD patients, as well as receiving assistance in the form of expert opinion on DSD cases. The development of websites or networking through internet either jointly with Asia Pacific countries or with European countries will greatly facilitate further improvement of care and will be of great benefit of DSD patients in Indonesia (8).

References

1. Hughes IA, Houk C, Ahmed SF, Lee PA, Lawson Wilkins Pediatric Endocrine Society/European Society for Paediatric Endocrinology Consensus G. Consensus statement on management of intersex disorders. *J Pediatr Urol.* 2006 Jun;2(3):148-62.
2. Andaya LY. Kingship - adat rivalry and the role of islam in south sulawesi. *Journal of Southeast Asian Studies.* 1984 Mar.;15(1):22-42.
3. Graham S. Sex, Gender, and Priests in South Sulawesi, Indonesia 2002; 29.
4. Graham S. Sulawesi's fifth gender. *Inside Indonesia.* 2001 APR-JUN 2001.
5. Faisal Ahmed S, Achermann JC, Arlt W, Balen A, Conway G, Edwards Z, et al. UK Guidance On The Initial Evaluation Of An Infant Or An Adolescent With A Suspected Disorder Of Sex Development. *Clin Endocrinol (Oxf).* 2011 Apr 16.
6. Warne GL, Raza J. Disorders of sex development (DSDs), their presentation and management in different cultures. *Rev Endocr Metab Disord.* 2008 Sep;9(3):227-36.
7. Consortium on the Management of Disorders of Sex Differentiation, editor. *Clinical Guidelines for the Management of Disorders of Sex Development in Childhood: Accord Alliance; 2006.*
8. Drop SL, Mure PY, Wood D, El-Ghoneimi A, Faisal Ahmed S. E-consultation for DSD: a global platform for access to expert advice. *J Pediatr Urol.* 2012 Dec;8(6):629-32.

Chapter 12

Summary

This thesis elaborates the stepwise diagnostic procedure in DSD patients using a multidiscipline approach to obtain the diagnosis of these patients which includes clinical assessment, hormonal, genetic, and pathological investigations, so that it is beneficial as the background for decisions on the required therapy.

Most of the patients in this research had a 46,XY karyotype where Androgen Action Disorder was found to affect the majority of the cases. In the majority of the 46,XX DSD cases, CAH required immediate comprehensive treatment because its nature often leads to a life threatening situation.

In a developing country such as Indonesia, it is rare to have a complete diagnostic procedure where a molecular evaluation is needed for DSD confirmation. A health care center providing molecular analyses as its service is considered very rare, if not nonexistent. Such luxury is limited to a research center in a capital town. Early diagnosis and integrated treatment is vital for DSD patients, in order to obtain the proper outcome in terms of medical, psychological and social aspects.

DSD management in Indonesia is still striving to reach the ideal state, which is explicable by the country's priority on epidemic health diseases such as infectious or degenerative diseases which affects the nation to a large extent. However, by taking into account the long term effect on the patient, increasing concern over this matter is inevitable.

In order to put the results in proper international perspective, in chapter 2 normal sexual differentiation and development as well as concepts, definitions and classifications of DSD were discussed in detail.

Results of the research is reported in 4 sections

A. Stepwise diagnostic evaluation of DSD

In chapter 3 and 4 the results of a stepwise diagnostic evaluation are given. Patients with various forms and degrees of DSD were evaluated. They were referred to the department of Human Genetics Center for Biomedical Research, Faculty of Medicine Diponegoro University, Semarang, Indonesia for chromosomal analysis by clinicians of the departments of urology, pediatrics, internal medicine and obstetrics of the Dr Kariadi Hospital, Semarang between 2004 and 2010. Reason for referral was the presence of ambiguous genitalia or any anatomical

abnormality of external or internal genitalia including penoscrotal hypospadias with or without descended testes.

In chapter 4 data pertaining to 88 patients are reported. In chapter 5 the number of patients is expanded to 286 and additional data on gene mutations are included. Mutations in the androgen receptor (AR) gene, rendering the AR protein partially or completely inactive, cause androgen insensitivity syndrome, which is a form of a 46,XY disorder of sex development (DSD). In chapter 5, three novel AR variants found in Indonesian DSD patients are presented. The aim of this study was to determine the possible pathogenic nature of these newly found unclassified variants. Indeed reduced functionality of the mutated androgen receptors was demonstrated.

B. Congenital Adrenal Hyperplasia, diagnosis and management

In chapter 6, the long-term effects of 21-hydroxylase deficiency on phenotype in 15 patients with ages between 1 and 33 years who had not been treated for prolonged periods were studied to assess the correlation between genotype and phenotype. Results suggest that a correlation does exist between the concentration of androgens and the extent of virilization. However, no clear correlation between genotype and phenotype could be established.

In chapter 7, saliva of 24 CAH patients who received glucocorticoid replacement therapy was studied. The question was whether determination of salivary androstenedione and 17-hydroxyprogesterone in CAH patients would be a useful alternative to the measurement of these hormones in serum. The results suggest that this is indeed the case.

Chapter 8, describes the result of hydrocortisone therapy in CAH patients especially by taking their saliva for hormonal analysis during various periods of treatment. Among 43 CAH patients, clinical aspects, mutations found, therapy as well as the follow up which was carried out in 25 patients were described.

C. Gonadal tumor risk

Chapter 9 consist of a case report describing the diagnostic evaluation of a 32 yr old women presenting with severe virilization including clinical, hormonal, imaging and histological parameters and psychological assessment. Blood analysis showed a testosterone level in the high-normal male range. The presence of an ovarian tumor was confirmed by laparoscopy followed by biopsy of the left ovary since patient refused ovariectomy. Pathology showed a Leydig cell tumor without histological signs of malignancy. In spite of extensive explanation and psychological counselling, psychosocial and cultural barriers prevented appropriate treatment.

Caucasian patients with Disorders of Sex Development (DSD) have increased risk for Germ Cell Cancer (GCC). GCC are prominent in young adults in Western countries, while the incidence is significantly lower in Asia. So far it is unknown what the risk of GCC in Asian DSD patients is.

In chapter 10, a detailed gonadal histology study was undertaken on 16 Indonesian DSD patients using morphological studies and immunohistochemistry (OCT3/4, TSPY, VASA, SCF/KITLG, SOX9, FOXL2). 13 cases could be analyzed, including ovarian tissue (n=3); streak gonad (n=1); undifferentiated gonad (n=1) and testicular tissue (n=8), from patients diagnosed as 46,XX (n= 1), 46,XY (n=7) and sex chromosome DSD (n=5). The precursor lesions gonadoblastoma, carcinoma *in situ*, or GCC were diagnosed in four cases (30.8%; three 46,XY and one sex chromosomal DSD). A hormone producing ovarian Leydig cell tumor was identified in a 46,XX patient.

In spite of the significantly lower risk of GCC in the general Asian population, DSD is a dominant risk factor. This study demonstrates the power of immunohistochemical markers for (early) diagnosis. This knowledge deepens understanding of the pathobiology of GCC and clinical handling of patients with DSD globally.

General Discussion and Future Organization of Management on Disorder of Sex Development in Indonesia

In chapter 11, the implementation of comprehensive management of DSD in Indonesia is discussed in more general. The outcome varies and depends on many factors such as the level and type of the disorders, general health of patients, financial conditions, patient's and family's reaction and understanding towards DSD.

Medical, psychosexual, social and economic factors are discussed that are influencing management of DSD in Indonesia. Based on the studies described in the present thesis, subsequently recommendations for future improvements are formulated.

Summary (in Bahasa Indonesia)

Thesis ini membahas tahapan secara multi disiplin untuk mencari dan mendapatkan diagnosis pada pasien DSD yang meliputi pemeriksaan klinik, hormon, genetik, pathologi sehingga bisa digunakan sebagai pijakan dalam menentukan macam terapi yang diperlukan.

Pada riset ini, sebagian besar pasien mempunyai karyotype 46 XY dimana androgen action disorder menempati diagnosa terbanyak dari kelompok ini. CAH yang merupakan bagian terbesar dari 46 XX DSD, juga merupakan suatu keadaan yang harus diperhatikan mengingat kedaruratan yang bisa ditimbulkannya yang tidak jarang menimbulkan kematian.

Di negara yang sedang berkembang seperti Indonesia, masih sangat susah untuk bisa mendiagnosis sampai tahapan akhir prosedur dimana diperlukan pemeriksaan molekuler untuk konfirmasi penyakit. Sangat jarang, kalau tidak bisa dikatakan tidak ada, pusat pelayanan kesehatan di Indonesia yang menyediakan pemeriksaan molekuler sebagai bagian dari produk pelayanannya. Pemeriksaan molekuler ini masih terbatas pada pusat pusat riset yang hanya ada di kota kota besar di tanah air. Diagnosis dini dan penanganan secara terpadu sangat diperlukan pada pasien DSD ini, sehingga permasalahan medis, psikologis dan sosial dapat segera diantisipasi.

Penanganan kasus DSD di Indonesia masih membutuhkan perjuangan untuk bisa dikatakan ideal, hal ini dapat dimaklumi oleh karena DSD bukanlah merupakan prioritas kesehatan seperti halnya penyakit infeksi atau degenerasi yang memang mengenai banyak orang. Walaupun demikian, perhatian pada masalah ini tetap harus ditingkatkan mengingat efek jangka panjangnya yang sangat mengganggu penderita.

Hasil dari penelitian ini dilaporkan dalam 4 bagian.

A. Evaluasi diagnostik DSD secara bertahap

Dalam bab 3 dan 4 hasil evaluasi diagnostik bertahap diuraikan secara lebih terinci. Pasien dengan berbagai bentuk dan derajat DSD dievaluasi. Mereka dirujuk ke departemen Genetika Pusat Penelitian Biomedis, Fakultas Kedokteran Universitas Diponegoro, Semarang, Indonesia untuk analisis kromosom . Rujukan berasal dari dokter dari departemen urologi, pediatri, penyakit dalam dan kebidanan dan kandungan dari Dr Kariadi, Semarang antara tahun 2004 dan 2010. Alasan rujukan adalah adanya alat kelamin yang ambigu atau kelainan anatomi alat kelamin eksternal maupun internal termasuk hipospadia penoscrotal dengan atau tanpa penurunan testis.

Dalam bab 4 dilaporkan hasil penelitian terhadap 88 pasien DSD. Dalam bab 5 jumlah pasien diperluas menjadi 286 dan dilengkapi tambahan data pada mutasi gen. Mutasi pada reseptor androgen (AR) gen, memberikan perubahan protein pada AR sehingga menyebabkan sebagian atau seluruhnya tidak aktif sehingga mengakibatkan sindrom insensitivitas androgen, yang merupakan bentuk dari 46, XY DSD. Dalam bab 5, ditemukan tiga varian baru mutasi pada gen AR yang ternyata terbukti memberikan efek terjadinya gangguan perkembangan kelamin.

B. Diagnosis dan manajemen Congenital Adrenal Hyperplasia,

Dalam bab 6 , dilakukan penelitian untuk menilai korelasi antara genotipe dan fenotipe terhadap efek jangka panjang defisiensi 21 - hidroksilase pada 15 pasien dengan usia antara 1 dan 33 tahun yang tidak pernah diobati. Hasil menunjukkan bahwa terdapat korelasi antara konsentrasi androgen dan tingkat virilisasi . Namun, tidak ditemukan adanya korelasi yang signifikan antara genotipe dan fenotipe.

Dalam bab 7 , diteliti air liur dari 24 pasien CAH yang menerima terapi glukokortikoid. Pertanyaannya adalah apakah penentuan androstenedion saliva dan 17 – hydroxyprogesterone pada pasien CAH akan menjadi alternatif yang berguna untuk pengukuran hormon-hormon dalam serum . Hasil penelitian menunjukkan bahwa hal ini memang dapat dibuktikan kebenarannya .

Bab 8 , menjelaskan hasil terapi hidrokortison pada pasien CAH terutama dengan mengambil air liur mereka untuk analisis hormon selama berbagai periode pengobatan. Dari 43 pasien CAH yang ada, hanya 25 pasien yang bisa dilakukan penelitian lebih lanjut mengenai aspek klinis, terapi serta tindak lanjut yang dilakukan .

C. Risiko Tumor Gonad

Bab 9, berisi laporan kasus evaluasi diagnostik dari wanita 32 thn yang mengalami virilisasi berat yang meliputi parameter klinis, hormonal, pencitraan, histologis dan psikologis. Analisis hormonal menunjukkan kadar testosteron dalam kisaran batas atas dari normal laki-laki tinggi. Adanya tumor ovarium dikonfirmasi dengan laparoscopi diikuti oleh biopsi dari ovarium kiri karena pasien menolak ovariectomi. Pemeriksaan patologi menunjukkan tumor sel Leydig tanpa tanda-tanda histologis keganasan. Dalam kasus ini hambatan psikososial dan budaya menjadi penghalang pasien untuk mendapatkan pengobatan yang tepat..

Pasien DSD pada bangsa Kaukasia mempunyai risiko peningkatan terjadinya kanker dari germ cel. Jenis kanker ini sangat menonjol terjadi pada dewasa muda di negara barat dimana hal tersebut tidak terjadi di Asia. Sejauh ini tidak diketahui apa risiko apa yang membuat fenomena ini diantara pasien DSD di Asia.

Didalam bab 10, penelitian secara detil gambaran histology gonad pada 16 pasien DSD di Indonesia dengan menggunakan study morfologi dan imunohistokimia (OCT3/4, TSPY, VASA, SCF/KITLG, SOX9, FOXL2). Terdapat 13 jaringan yang dapat dianalisis, didapatkan jaringan ovarium (n=3), streak gonad (n=1), gonad tak berdiferensiasi (n=1) dan jaringan testis (n=8) dari pasien dengan 46,XX (n= 1), 46,XY (n=7) dan sex kromosom DSD (n=5). Lesi precursor gonadoblastoma, karsinoma in situ, atau karsinoma germ sel didapatkan pada 4 kasus (30.8%; tiga 46,XY and satu sex kromosom DSD). Terdapat satu pasien dengan tumor sel Leydig yang memproduksi androgen ditemukan pada pasien dengan 46,XX.

Terlepas dari risiko yang rendah pada populasi Asia secara umum, DSD merupakan faktor risiko yang dominan terjadinya karsinoma germ sel. Studi ini menunjukkan kegunaan penanda imunohistokimia untuk diagnosis(awal). Pengetahuan ini memperdalam pemahaman tentang pathobiologi dari karsinoma germ sel dan penanganan klinis pasien dengan DSD secara global.

Diskusi Umum dan Organisasi Manajemen masa depan dari Gangguan perkembangan Kelamin di Indonesia

Di dalam bab 11, pelaksanaan pengelolaan DSD secara komperhensif di Indonesia dibicarakan secara lebih umum. Hasilnya bervariasi dan tergantung pada banyak faktor seperti tingkat dan jenis gangguan, kesehatan umum pasien, kondisi keuangan, pasien dan keluarga reaksi dan pemahaman terhadap DSD

Sisi medis, psikoseksual, sosial dan ekonomi dibahas sebagai factor yang mempengaruhi pengelolaan DSD di Indonesia. Berdasarkan penellitian yang dihasilkan dalam tesis ini, kemudian dirumuskan rekomendasi untuk perbaikan masa depan pengelolaan DSD di Indonesia.

Chapter 13

ACKNOWLEDGEMENT

My works would be incomplete without this section of my thesis. Many people have contributed to this study. I would like to thank all people, teachers, colleagues, friends, family and even acquaintances- without them it would not have been possible for me to present this

First and foremost, I would like to express my sincere gratitude and high appreciation for Prof.dr.Stenvert L.S. Drop, PhD, my promoter, whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

Dear Prof. Drop, without your help, guidance and encouragement, I am sure this work of mine was not possible. You were not only generous with your time, at the department as well as home. Your dedication to work has been an inspiration

I owe my gratitude and it can not thank enough to my Indonesian promoter Prof Sultana M.H Fardz MD, PhD for tireless support and guidance to me. You are the initiator all of this. Countless thanks for your encouragement to always enthusiastic in doing this research.

I am heartily thankful to Prof. Dr. F.H. de Jong, PhD. Your comments, suggestions proved valuable in the course of this study. Frank, depth of knowledge you have is absolutely stunning so as to open the way for me to solve the problems

My sincere thanks to Prof. Dr. L.H.J. Looijenga, PhD for supporting my work and let me to involve in LEPO meeting.

I would like to take this opportunity to show appreciation to Prof Axel Themmen PhD, Dr. H. T. Bruggenwirth, PhD, Coby Steenbergen, Mariana Timmerman, Remco Hersmus, Ronald van der wal, Peter Elfferich for supporting me in the various laboratories against all odds, Katja P.Wolffenbuttel, MD for clinical support

With gratitude, I acknowledge Nani Maharani, Muflihatul Muniroh, Bestari Setyawati, widagdo Sidiq, Mahayu, Hermawan, and all Lab. Cebior staff (dr Iin, Nanik, Wiwik, Lusi, Intus, Rita, Dina, Evi) for your endless support and handling the patient during the study. I would be happy if we could continue to collaborate with in years to come. We will be a great team.

I would like to express my thanks to the member of sexual adjustment team Faculty of Medicine Diponegoro University-Kariadi Hospital Semarang Indonesia

I would like to express gratitude to my former, present colleagues with whom I spend a wonderful time namely: Kristel Gossens, Evelien hamelink, Yvonne van der Schwann

To Annastasia Ediati, thanks for your lunch time discussions and wish you all the best.

Furthermore, my stay in The Netherlands was not so relaxed and comfortable if I do not mention Mr.Syamsuddin and the big family of you (Tika, Reta, Solichin, Amanda, Tony, Malik, Morgan), who over the time became so close to me that we almost feel that we are living in Indonesia. You are more than brother to me. No words could express my feeling to you. Dear Abang, I love you.

Special thanks to Donnie Ahmad, mbakYuyul, Niken Iwani, dr. Henry and dr Maya.

To my parents Moecharom Aziz, Nanik Sri Mulyani and mother in law Roehati, thank you for caring my family during my stay in Rotterdam and your relentless pray for us.

To the parents and children who have suffered this disease, I hope this work can give a little contribution in addressing the DSD better and relief your hardship.

I owe special thanks to my dearest family since without their encouragement, love and understanding it would have been impossible for me to finish my work. My beloved wife Krisma Irmajanti, for unrelenting support and love helped me a lot to pass many tides during this assignment. I count myself truly blessed to have you by my side on our journey through life.

To my wonderful children: Zadit, Hasna and Aing. I am blessed to have you. I am indebted a lot of things for not accompanying you during which time the desperately need my presence.

This research project would not have been possible without the support of many people. Lastly I offer my regards and blessings to all of those who supported me in any respect during the completion of the project. I salute and thank all of you who have provided me with inspiration, information and useful suggestions.

To be a good doctor one has to be a good listener first.

Achmad Zulfa Juniarto

Curriculum Vitae

Achmad Zulfa Juniarto was born in Semarang Indonesia, 8 June 1970. Living in Semarang Indonesia, he graduated at SMA 3 (high school) in 1989 and entered medical school in Diponegoro University in the same city.

After graduating from the Faculty of medicine in 1995, he became a lecturer at the medical biology and continued his master programme in the field of reproductive medicine at the same university until 2004. In 2006, he became an andrologist and have additional position at the Kariadi hospital. Between the period of 2007-2010, he became The head department of Medical Biology Diponegoro University/ Dr.Kariadi Hospital, Semarang, Indonesia.

He joined Center for Biomedical Research Faculty of Medicine Diponegoro University and Sexual Adjustment Team Faculty of Medicine Diponegoro University - Kariadi Hospital Semarang Indonesia in 1999 till present.

Since then, together with Prof Sultana MH Faradz, he was active in the clinical care of patients with ambiguous genitalia and developed researches in disorder of sexual development at Kariadi hospital Semarang in which in 2004, research collaboration with Erasmus Medical Center Rotterdam The Netherlands (headed by Prof Stenvert L.S Drop) were initiated.

He is a member of Indonesian Medical Doctor Association, Indonesian Andrology Association, Indonesian Sexology Assosiation.

List of Publications

1. Elfferich P, Juniarto AZ, Dubbink HJ, van Royen ME, Molier M, Hoogerbrugge J, Houtsmuller AB, Trapman J, Santosa A, de Jong FH, Drop SL, Faradz SM, Bruggenwirth H, Brinkmann AO. Functional analysis of novel androgen receptor mutations in a unique cohort of Indonesian patients with a disorder of sex development. *Sex Dev.* 2009;3(5):237-44. doi: 10.1159/000252814. Epub 2009 Oct 23. PubMed PMID: 19851057.
2. Goossens K, Juniarto AZ, Timmerman MA, Faradz SM, Wolffenbuttel KP, Drop SL, de Jong FH. Lack of correlation between phenotype and genotype in untreated 21-hydroxylase-deficient Indonesian patients. *ClinEndocrinol (Oxf).* 2009Nov;71(5):628-35. doi: 10.1111/j.1365-2265.2009.03550.x. Epub 2009 Feb 18. PubMed PMID: 19226270.
3. Juniarto AZ, van der Zwan YG, Santosa A, Hersmus R, de Jong FH, Olmer R, Bruggenwirth HT, Themmen AP, Wolffenbuttel KP, Looijenga LH, Faradz SM, Drop SL. Application of the new classification on patients with a disorder of sex development in Indonesia. *Int J Endocrinol.* 2012;2012:237084. doi:10.1155/2012/237084. Epub 2011 Dec 29. PubMed PMID: 22253624; PubMed CentralPMCID: PMC3255103.
4. Juniarto AZ, Goossens K, Setyawati BA, Drop SL, de Jong FH, Faradz SM. Correlation between androstenedione and 17-hydroxyprogesterone levels in the saliva and plasma of patients with congenital adrenal hyperplasia. *Singapore Med J.* 2011 Nov;52(11):810-3. PubMed PMID: 22173250.

5. Juniarto AZ, Setyawati BA, Miranti IP, Santosa A, Hersmus R, Stoop H, Cools M, Oosterhuis JW, Drop SL, Faradz SM, Looijenga LH. Gonadal malignancy in 13 consecutive collected patients with disorders of sex development (DSD) from Semarang (Indonesia). *J ClinPathol*. 2013 Mar;66(3):198-204. doi:10.1136/jclinpath-2012-201062. Epub 2012 Dec 25. PubMed PMID: 23268320.
6. Achmad Zulfa Juniarto, Gerard Noppe, Nani Maharani, Erica van den Akker, Rudy Susanto, Sultana MH Faradz, Frank H. de Jong, Stenvert L.S. Drop. Salivary 17-hydroxyprogesterone (17-OHP) and Androstenedione in Monitoring Efficacy of Treatment Among Indonesian Congenital Adrenal Hyperplasia Patients. (ISSN 0857-1074 Printed in the Philippines Copyright © 2012 by the JAFES Received July 26, 2012. Accepted October 29, 2012. Vol. 27 No. 2 November 2012) : http://www.asean-endocrinejournal.org/index.php?option=com_content&task=view&id=26
7. Achmad Zulfa Juniarto, Bestari A Setyawati, Annastasia Ediati, Yvonne G. van der Zwan, Leendert H.J. Looijenga. Virilization due to Androgen Hypersecretion in a Patient with an Ovarian Leydig Cell Tumor: Diagnostic and Psychosocial Implications. *Acta Med Indones*. 2013 Apr;45(2):130-5. PubMed PMID: 23770793

PhD Portfolio

Name PhD student: : **Achmad Zulfa Juniarto**
PhD period: : September 2010 – January 2014
Promotors: : Prof.dr. S.L.S Drop
Prof.dr. S.M.H. Faradz
Prof. Frank H de Jong

PhD Training	Year
General Academic Skills	
- Basic SPSS	2011
- The Ensemble Workshop	2011
- Systematic Literature Retrieval using PubMed (by Medical Library)	2012
- Systematic Literature Retrieval using Medline/PsycInfo (by Medical Library)	2012
In-depth courses (e.g. Research School, Medical Training).	
- The Course on Basic and Translational Endocrinology	2011
- The Course on Molecular Medicine	2011
Seminars and workshop	
- PhD day of the Erasmus-MC	2011
Presentations	
- Indonesian Student Association (PPI) Rotterdam (Oral)	2012
- DSD symposium, Erasmus-MC (Oral)	2012
- 2 nd International seminar and workshop on DSD, Semarang, Indonesia (Oral)	2012
Grant/reward	
- DIKTI scholarship from the Directorate of Higher Education, Ministry of Education and Culture, Indonesia	2010

Abbreviations

AR	: Androgen Receptor
AAD	: Androgen Action Disorder
CIS	: Carcinoma in Situ
CNVs	: Copy Number Variations
CAH	: Congenital Adrenal Hyperplasia
DSD	: Disorder of Sex Development
DBD	: DNA binding Domain
FOXL2	: Forkhead box L2
GB	: Gonadoblastoma
GCC	: Germ Cell Cancer
GR	: Glucocorticoid Receptor
IGCNU	: Intratubular Germ Cell Neoplasia Unclassified
LCH	: Leydig Cell Hypoplasia
LHCGR	: Luteinizing Hormone/Chorio Gonadotropin Receptor
LBD	: Ligand Binding Domain
MRKH	: Mayer-Rokitansky-Küster-Hauser syndrome
NTD	: NH ₂ -Terminal Domain
OCT3/4	: Octamer-binding transcription factor 4
SCF	: Stem cell factor, also known as KITLG
SOX9	: SRY-related HMG-box, gene 9
TSPY	: Testis-specific Y-encoded protein 1
UMU	: Unknown Male Undermasculinization
UGT	: Undifferentiated Gonadal Tissue
WNT4	: Wingless-type MMTV integration site family, member 4



*Ngudia Dalam Sing Bener
Aja Mingar Minger
Yen Minger
Mundhak Keblinger*

(Find the right path in order not to get lost)