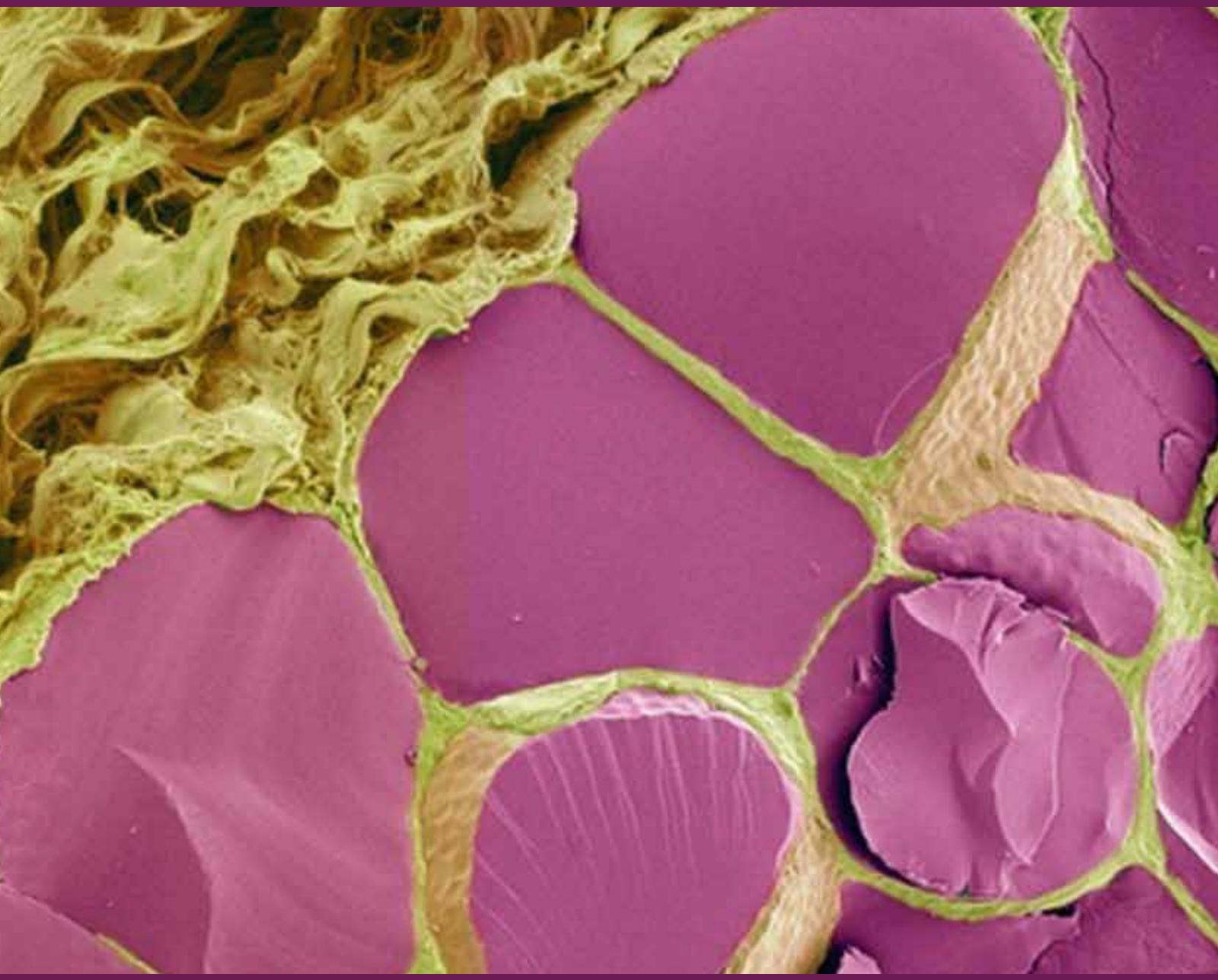
International Journal of Endocrinology

Disorders of Sex Development and Hypogonadism: Genetics, Mechanism, and Therapies

Guest Editors: Gil Guerra-Junior, Ana Claudia Latronico, Olaf Hiort, and Rodolfo Rey



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Editorial Board

Contents

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Editorial

Disorders of Sex Development and Hypogonadism: Genetics, Mechanism, and Therapies

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Disorders of sex development (DSD) and hypogonadism are genetically heterogeneous and include a broad spectrum of phenotypes. Recent advances in biology and medicine have introduced impressive improvements in both clinical management and structured research, mainly in new technologies to study their genetic features and the mechanisms underlying their pathologies. Knowledge and understanding of these conditions have led to the development of successful therapies and novel tools to characterize them and provide better care to patients. Therefore, both DSD and hypogonadism represent an important field in research and clinical setting.

This *special issue* presents a series of articles mainly reflecting the difficulties in characterization of DSD in four papers, advances in genetics of DSD in two papers, hypogonadotropic hypogonadism in one paper, clinical presentation of male primary hypogonadism, like Klinefelter's syndrome in one paper, and long-term followup of female hypergonadotropic hypogonadism in one paper, as well as clinical and molecular data of aromatase excess syndrome in one paper and an interesting review about the complex association of androgens and adipose tissue in males in one paper.

Juniarto and colleagues in their paper "Application of the new classification on patients with a disorder of sex development in Indonesia" from Indonesia showed how patients' and general society's opinion on DSD, economic background of the patients, and lack of access to health insurance can affect the complex management of DSD in a negative way. Probably, these situations are not restricted to Indonesia, but to all underdeveloped countries, and education of primary health care workers is necessary to prevent morbidity and mortality in some cases. Hersmus and colleagues in their paper "Delayed recognition of disorders of sex development (DSD): a missed opportunity for early diagnosis of malignant germ cell tumors" reported three independent male patients with germ cell tumors retrospectively recognized as DSD, showing again, but now in a developed country, the underdiagnosis of DSD in the newborn and the significant relevance of early and correct evaluation for identification of individuals at increased risk for development a gonadal tumor. Wünsch's paper "Checklist for the structural description of the deep phenotypes in disorders of sexual development" suggest an interesting checklist for the structural description of gonads and internal sex ducts using video endoscopy and laparoscopy, which can contribute to a comprehensive definition of the variations of deep phenotype in DSD. Veiga-Junior and colleagues in their paper "Clinical and laboratorial features that may differentiate 46,XY DSD due to partial androgen insensitivity and 5α-reductase type 2 deficiency", evaluating 58 patients with sex ambiguity, 46,XY karyotype, and normal testosterone secretion, reported clinical and laboratorial features, like consanguinity, familial recurrence, severity of ambiguous genitalia, penile length, and T/DHT ratio, which may differentiate 46,XY DSD due to partial androgen insensitivity and 5α-reductase deficiency.

Barbaro and colleagues in their paper "Multigeneration inheritance through fertile XX carriers of an *NR0B1* (*DAX1*) locus duplication in a kindred of females with isolated XY gonadal dysgenesis" identified another *NR0B1* duplication in two sisters with isolated XY gonadal dysgenesis and an X-linked inheritance pattern. They also studied three fertile female carriers of three different small *NR0B1* locus duplications and did not find obvious skewing of X-chromosome inactivation, suggesting that *NR0B1* overexpression does not impair ovarian function. Mooslehner and colleagues in their paper "A cell model for conditional profiling of androgen-receptor-interacting proteins" reported preliminary results that allow future studies to focus on replacing wild-type androgen receptor (AR) with mutant AR to uncover differences in protein interactions caused by AR mutations involved in partial androgen insensitivity syndrome.

Beate and colleagues in their paper "Genetics of isolated hypogonadotropic hypogonadism: role of GnRH receptor and other genes" showed an interesting revision about the genetics of isolated hypogonadotropic hypogonadism and the importance of inactivating mutations in the pituitary GnRH receptor inducing GnRH resistance in patients with normosmic isolated hypogonadotropic hypogonadism. Pacenza and colleagues in their paper "Clinical presentation of Klinefelter's syndrome: differences according to age", evaluating 94 patients with Klinefelter's syndrome, showed that 46.8% were diagnosed between 11 and 20 years, probably because nowadays pediatricians are more aware that boys and adolescents with neurodevelopmental disorders and cryptorchidism should be investigated for this chromosome anomaly. Pantelis and colleagues in their paper "Long-term followup of adolescent and young adult females with hypergonadotropic hypogonadism" showed that all prepubertal girl, teenager, or young woman diagnosed with primary ovarian insufficiency should undergo extensive research by a group of specialists in a referral center, and the final decision of therapy should be taken by the patient and her clinician.

Fukami and colleagues in their paper "Molecular bases and phenotypic determinants of aromatase excess syndrome" showed that aromatase excess syndrome represents a novel model for gain-of-function mutation leading to human genetic disorders characterized by gynecomastia. Mammi and colleagues in their paper "Androgens and adipose tissue in males: a complex and reciprocal interplay" reported an interesting revision about the association between androgens and adipose tissue in males. The authors concluded that adequate levels and balance of circulating sex hormones are necessary to maintain a correct distribution and size of adipose tissue, which in turn is fundamental to keep a normal reproductive and sexual function.

Our understanding in DSD and hypogonadism is advancing, and we hope that the readers will find this *special issue* enjoyable and helpful in the clinical practice. In addition, we would like to take the opportunity to express our gratitude and thanks to all the authors involved in this selected issue for the high-level papers they submitted. We also thank the reviewers for their time and expertise as well as the Editor-Chief of International Journal of Endocrinology for giving us the honor to publish this important *special issue*. Enjoy this *special issue* of DSD and hypogonadism!

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Olaf Hiort
Rodolfo Rey

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Review Article

Hypogonadism in the Aging Male Diagnosis, Potential Benefits, and Risks of Testosterone Replacement Therapy

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Hypogonadism in older men is a syndrome characterized by low serum testosterone levels and clinical symptoms often seen in hypogonadal men of younger age. These symptoms include decreased libido, erectile dysfunction, decreased vitality, decreased muscle mass, increased adiposity, depressed mood, osteopenia, and osteoporosis. Hypogonadism is a common disorder in aging men with a significant percentage of men over 60 years of age having serum testosterone levels below the lower limits of young male adults. There are a variety of testosterone formulations available for treatment of hypogonadism. Data from many small studies indicate that testosterone therapy offers several potential benefits to older hypogonadal men. A large multicenter NIH supported double blind, placebo controlled study is ongoing, and this study should greatly enhance the information available on efficacy and side effects of treatment. While safety data is available across many age groups, there are still unresolved concerns associated with testosterone therapy. We have reviewed the diagnostic methods as well as benefits and risks of testosterone replacement therapy for hypogonadism in aging men.

1. Introduction

Hypogonadism in older men is a syndrome characterized by the presence of low testosterone levels and clinical signs and symptoms of hypogonadism. The symptoms of hypogonadism can include decreased libido, impaired erectile function, muscle weakness, increased adiposity, depressed mood, and decreased vitality. Hypogonadism is more common in aging men and is is also referred to as late-onset hypogonadism (LOH) [1], androgen deficiency in the aging male (ADAM) [2], partial androgen deficiency in the aging male (PADAM) [3], testosterone deficiency syndrome (TDS) [4], and andropause [5]. We prefer LOH over the other descriptors.

A significant percentage of men over 60 years of age have serum testosterone levels below the lower limits of young male adults (20 to 30 years) [6–9]. One longitudinal study has suggested that approximately 20% of men in their 60s and approximately 50% of men in their 80s have serum total testosterone (TT) levels significantly below those of the levels of normal young men [8]. European Male Aging Study (EMAS) estimated much lower prevalence (2.1%) of symptomatic late-onset hypogonadism in the in the population [10]. Several other studies have also noted a decline in TT with age [11]. In some instances, the clinical symptoms/manifestations are more difficult to recognize because they may be masked by comorbid illnesses. There has been some controversy as to the significance of falling testosterone levels with age. Most experts believe that it is a medically significant condition resulting in significant detriment to the quality of life and adversely affecting the function of multiple organ systems [6, 11]; while others suggest that it is a chemical marker of generalized illness [11].

Over the past two decades, significant advances have been made in improving the understanding of the pathophysiology of the hypogonadism, the diagnostic methods used to diagnose low testosterone levels, and testosterone replacement therapy. In spite of these advances, a great deal of confusion and misunderstanding still exists among clinicians and patients about diagnosis of hypogonadism in aging men,

and benefits and risks associated with testosterone therapy. In this paper, we have reviewed the studies reported in the literature on this subject and attempted to address the important questions pertaining to hypogonadism in older men. (1) How to diagnose LOH in aging males? (2) What are the best treatment options available today for clinicians to treat LOH? (3) Will older hypogonadal men benefit from testosterone treatment? (4) What are the risks associated with such an intervention?

2. Prevalence of Hypogonadism in Aging Males

Several longitudinal and cross sectional studies have been carried out to determine the prevalence of hypogonadism in men. Some of the important cross-sectional and longitudinal studies reported include Baltimore Longitudinal Study of Aging (BLSA), Boston Area Community Health Survey (BACHS, European Male Aging Study (EMAS), and Massachusetts Male Aging Study (MMAS) [8–10, 12]. These studies have reported different prevalence rates of hypogonadism in men. The differences may be in part due to different definitions of hypogonadism adopted by these studies. Variables include low testosterone level definition, clinical symptoms used for the diagnosis of hypogonadism, the population studied, and the inclusion or exclusion of comorbid conditions in older men.

The actual prevalence of low-serum testosterone in aging men is not known with certainty, but it is projected to be up to 25% [8, 10, 13, 14]. The EMAS noted an overall prevalence of hypogonadism of 2.1% [10]. The study noted an increase with age from 0.1% for 40- to 49-year-old men to 5.1% for 70- to 79-year old men. The MMAS reported that the overall prevalence of symptomatic androgen deficiency was 5.6% with an increased prevalence of 18.4% among 70-year-old men. In the Boston Area Community Health Survey, the overall prevalence of hypogonadism was 5.6% among older men of age 30–79, and the survey also suggested that the prevalence among 70-year-old men could be 18.4% [9]. In a study of men in Hong Kong, the prevalence of symptomatic hypogonadism was 9.5% with an increased prevalence of 16.7% in the older age group (60–64 yrs) [15]. The BLSA reported that 19% of men over 60 years had low testosterone levels with the average decline of TT was 3.2 ng/dL per year among men who had an average age of 53 years at entry into BSLA [8].

A summary of these studies is given in Table 1. Some of the important findings of these studies are (1) prevalence hypogonadism in men (based on symptoms and/or total testosterone levels) increases with age starting from the fourth decade, and (2) the hypogonadism was projected to be much higher in aging men with comorbidities such as metabolic syndrome, type 2 diabetes (T2DM), and cardiovascular disease (CVD). Some other studies have pointed out that much of the increase in prevalence of hypogonadism with age can be ascribed to comorbid conditions [16–18].

3. Causes

Hypogonadism can be classified as primary, secondary, and mixed hypogonadism. Primary Hypogonadism results from disorders of the testes that lead to low testosterone production and impaired fertility. The laboratory values for patients with primary hypogonadism show low testosterone and elevated LH and FSH levels. Secondary hypogonadism results from disorders of the hypothalamus and the pituitary. The laboratory values for men with secondary hypogonadism show low testosterone and low or inappropriately normal LH and FSH levels. Mixed hypogonadism can result from dual defects in the testes and in the pituitary-hypothalamic axis. The laboratory values for mixed hypogonadism can be varied including cases with low testosterone with mild increases in LH and FSH levels.

Often the type of hypogonadism in older men is either secondary or mixed hypogonadism. The decline in testosterone levels can be due to several factors including (1) decline in Leydig cell function, (2) decline in pituitaryhypothalamic axis function with loss of circadian variation (3) increase in the levels of SHBG, (4) changes in testosterone receptors sensitivity, and (5) effects of altered cardiometabolic and inflammatory markers [20–22].

Most aging males do not have congenital etiology as their cause hypogonadism. These congenital processes would show up much earlier than later in life. Aging males are more likely to have an acquired cause or idiopathic etiology of hypogonadism. The diagnosis of hypogonadism in aging men requires the clinician to evaluate for other causes of secondary and mixed hypogonadism such as hypothalamic-pituitary disease, hyperprolactinemia, depression, chronic alcoholism, diabetes mellitus, and infiltrative diseases such as hemochromatosis and medications (e.g., opioids, anabolic steroids, and glucocorticosteroid, opioid analgesics, antidepressants cimetidine, spironolactone, and antifungal drugs).

4. Diagnosis of Hypogonadism in Aging Males

Questionnaires have been developed to help identify aging males with hypogonadism. These questionnaires include (1) Androgen Deficiency in Aging Male (ADAM) questionnaire, (2) Aging Male survey (AMS), and (3) MMAS questionnaire [23, 24]. While these questionnaires can have high sensitivity, they have low specificity [25]. Because of their low specificity, most guidelines do not recommend their use. A new questionnaire has recently been validated for hypogonadal men and its usefulness is not yet established in older men [26]. Testosterone deficiency should be confirmed by laboratory measurements for older men who are identified as hypogonadal by means of these questionnaires [23, 24]. The Endocrine Society Consensus Committee recommends that the diagnosis of hypogonadism should be based on identification of symptoms and signs suggestive of testosterone deficiency and presence of low testosterone levels measured by a reliable assay on two or more occasions [27]. The algorithm suggested for the diagnosis of hypogonadism in aging males described in Figure 1.

Table 1: Cross-Sectional and Longitudinal Studies of Hypogonadism in Aging Men.

Study	Population	Results	Notes
European Male Aging Study (Cross-sectional) [10]	3219 men ages 40 to 79 years.	(1) Overall prevalence of hypogonadism was 2.1%. (2) Hypogonadism increases with age 0.1% (40 to 49 yrs) 0.6% (50 to 59 yrs), 3.2% (60 to 69 yrs) 5.1% (70 to 79 yrs). (3) Prevalence is higher with increasing number of coexisting illnesses and BMI	Total testosterone <320 ng/dL (11 nmol/L), and free testosterone <64 pg/mL (220 pmol/l). (LCMS method)
The Baltimore Longitudinal Study of Aging (longitudinal) [8]	890 men; average age 53.8 + 16 (samples during time period 1961 to 1995).	(1) Serum testosterone decreased at a fairly constant rate, independent of other clinical variables. (2) Average change of T is about 3.2 ng/dL (−0.124 nmol/L) per yr. (3) Incidence of hypogonadism:: ∼20% in 60s, ∼30% in 70s, and ∼50% in 80s.	Androgen deficiency was defined as total testosterone less than 325 ng/dL. (RIA method)
The Massachusetts Male Aging Study (longitudinal) [12]	1667 men aged 40 to 70 at baseline (1987–1989).	(1) Crude prevalence of androgen deficiency at baseline and followup is 6.0 and 12.3%. (2) Crude incidence rate of androgen deficiency was 12.3 per 1,000 P-Yr. (3) Prevalence and Incidence rate increased with age. (4) T declines associated with aging −10.1% decline in TT per decade −23.8% decline in FT per decade.	Total testosterone less than 200 ng/dL or total testosterone 200–400 ng/dL and free testosterone less than 8.91 ng/dL. (RIA method)
Boston Area Community Health Survey [9]	1475 men ages of 30–79 yr; 47.3 ± 12.5 yr.	(1) Crude prevalence of symptomatic androgen deficiency was 5.6%. (2) Prevalence increases with age a. 3.1–7.0% in men less than 70 yr b. 18.4% among 70 yr old. (3) 24% of subjects had total testosterone less than 300 ng/dL, (4) 11% of subjects had free testosterone less than 5 ng/dL	Total concentration <300 ng/dL and free testosterone <5 ng/dL.
New Mexico Aging Process Study (longitudinal) [19]	77 men in the age group 66–80. 15 years of the study period.	(1) Observed a longitudinal decline in T and an increase in LH and FSH. (2) The increasing levels of FSH suggest that hypogonadism in aging males is probably due to secondary hypogonadism. (3) Average rate of decrement in testosterone concentration is about 11 ng/dL (0.382 nmol/L) per year	Note, this study varies in rate of testosterone decline from the other studies

Abbreviations: T: Testosterone; TT: Total Testosterone; FT: free testosterone; YRS: years of age; P-Yr: person years; LCMS: Liquid Chromatography tandem Mass Spectrometry (LCMS); RIA: Radio Immunometric Assay.

The symptoms and signs of hypogonadism in aging men vary depending upon the age, severity and duration of andro gen deficiency, comorbid illnesses, androgen sensitivity, and previous testosterone therapy [28]. Symptoms and signs suggestive of hypogonadism (Table 2) include loss of vitality, visceral obesity, decreased muscle mass and strength, osteopenia and bone pain, and mood changes and depression [1, 2, 5]. Other nonspecific symptoms are decreased energy, motivation, and initiative, poor concentration and memory, sleep disturbance, increased sleepiness, increased body fat, and diminished physical or work capacity [1, 2, 5, 10].

The measurement of the serum testosterone concentration is usually the most important single diagnostic test for male hypogonadism. The important factors that need to be considered in testosterone measurement are (1) types/forms of testosterone to be measured, (2) time of measurement, and (3) frequency of measurement.

Three different forms of testosterone can be measured and they include (a) total testosterone level, (b) free or unbound testosterone, and (c) bioavailable testosterone. About 30% to 50% of testosterone is bound to albumin with low affinity. The free testosterone is about 1% to 2%. The bioavailable fractions of testosterone are composed of both the albumin-bound and the free testosterone. The rest of the testosterone is bound to sex hormone-binding globulin (SHBG), and this portion is not available for use by most target organs [21, 27–29].

Measurement of the serum testosterone concentration is dependent on the time of measurement. In healthy males, the circadian rhythm affects GnRH secretion and causes testosterone levels to change throughout the day. Testosterone levels are highest in the morning and start to decline by 10 am. The lowest value of testosterone is observed approximately by 10 PM [30]. The variability between morning and evening testosterone levels decreases in older men because of changes to the circadian rhythm. Testosterone levels should be measured in the morning to obtain peak testosterone results. If a single morning value is low or borderline low

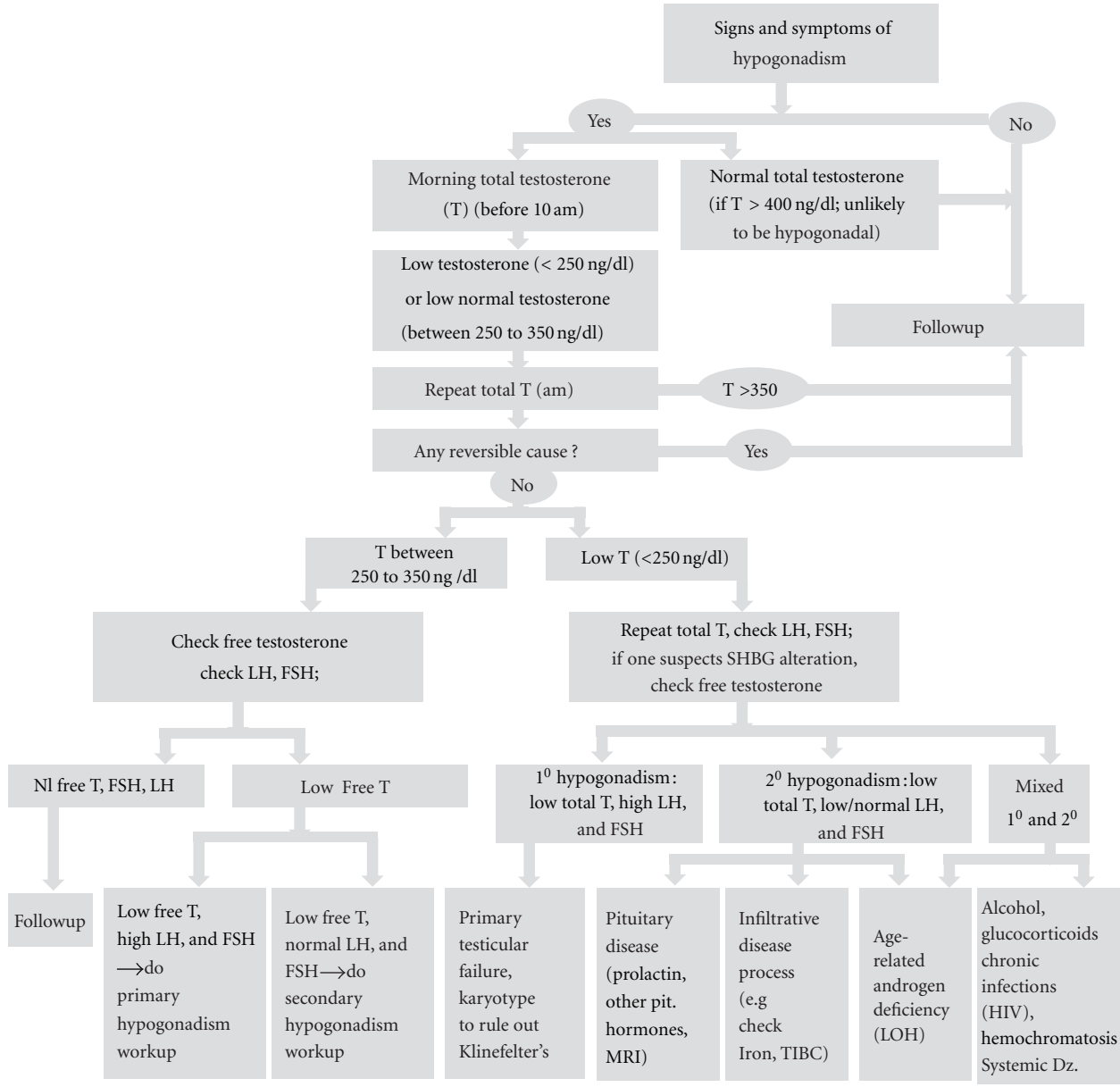

Figure 1: Algorithm for the diagnosis of hypogonadism in aging males.

or does not fit with the clinical findings, the measurement should be repeated once or twice before making the diagnosis of hypogonadism.

The normal reference range for TT in adult men is approximately 300–1000 ng/dL. If the early morning serum TT level is less than 250 ng/dL, the patient is likely to be hypogonadal. A repeat TT measurement is required to confirm the diagnosis. Further evaluation is required if the TT is in the grey zone of 250 to 350 ng/dL. The follow-up tests required include a repeat of TT levels. One should evaluate the FT levels when TT values are in the grey zone. If the results indicate a low TT and/or low FT levels, then the patient is hypogonadal. These labs then should be followed with testing of the serum gonadotrophins (LH, FSH) levels to help ascertain the anatomical level of hypogonadism (e.g., primary testicular or hypothalamic/pituitary). If a TT level is <150 ng/dL or there are signs or symptoms of possible mass lesion in the pituitary, then one should order pituitary imaging to exclude pituitary and/or hypothalamic tumor or infiltrative disease. The diagnosis of hypogonadism in aging men should never be undertaken during an acute illness as it can result in temporarily low testosterone levels [27, 31].

TT levels can be measured directly by automated immunoassays and immunometric assay methods [32–35]. However, there is a growing concern about the accuracy of automated immunometric assays especially for measurements in the low testosterone concentration range [36–38]. Currently, the most accurate method for determining the TT to

Table 2: Symptoms and associated morbidities with low testosterone levels.

Symptoms and associated morbidities	
Sexual function	Cognition and vitality
Loss of libido	Decline in verbal and visual memory
Erections: reduced quality and frequency, including nocturnal erections	Decline in visuospatial performance
Oligospermia or azoospermia	Depressed mood
Gynecomastia/breast discomfort	Decreased energy
Changes in secondary hair characteristics (e.g., shaving)	Decline in feelings of initiative
Changes in size of testes	Decreased sense of vitality
Decreased fertility	
Muscle, bone, and body composition	Other
Progressive decrease in muscle mass	Sleep disturbance
Decreased physical function	Lipid abnormalities
Decrease in bone mineral density; osteopenia, osteoporosis, increased risk of bone fractures	Mild anemia (normochromic, normocytic)
Increase in visceral fat	Decreased response to PDE5 inhibitors

differentiate eugonadal from hypogonadal males is liquid chromatography-tandem mass spectrometry [38]. Bioavailable testosterone is measured by the ammonium sulfate precipitation method. The equilibrium dialysis is the reference method for the measurement of free testosterone concentrations [28]. This latter method is very complex and tedious and thus a routine measurement is only available in reference laboratories. Free and bioavailable testosterone concentrations can also be estimated (calculated) from TT and SHBG concentrations. The calculated FT concentrations derived from using the law of mass action equations provide a convenient and reasonable alternative to equilibrium dialysis methods [39–41]. The calculations of FT are limited by assumptions made for the equilibrium dissociation constants (Kd) for the binding of SHBG and T and albumin and T. In addition, there is no agreed standard for determining the SHBG. The tracer analog displacement assays that are available in many hospital laboratories are inaccurate and their use is not recommended [31].

5. Treatment of Hypogonadism with Testosterone

There must be a definitive diagnosis of hypogonadism before the treatment is initiated. Borderline testosterone levels alone are not necessarily an indication to begin testosterone replacement therapy. There must be a combination of signs, symptoms and issues with patient's quality of life [27, 42, 43]. The goal of testosterone therapy is to raise serum testosterone level into the midnormal range (400–700 ng/dL) and resolution or reduction in symptoms of hypogonadism. However, the ultimate goals of therapy are to reduce disease and disability, maintain or improve quality of life, and hopefully add vitality to the years [44].

The Endocrinology Society Guidelines recommend that the health care provider considers avoiding testosterone replacement therapy in men with certain conditions. The guidelines do not recommend testosterone replacement therapy for those who still desire fertility [27]. Aging males with a history of severe lower urinary tract obstruction, untreated sleep apnea, prostate cancer, or breast cancer should not be considered for testosterone replacement therapy [27]. Individuals with an abnormal digital rectal examination suggestive of cancer, and/or elevated prostate-specific antigen should have a careful evaluation by an urologist before considering treatment. An elevated hematocrit (e.g., >50%) is also considered to be a relative contraindication for testosterone replacement therapy [27]. Other contraindications include poorly controlled heart failure and an American Urological Association International Prostate Symptom Score (IPSS) >19. Testosterone replacement therapy can be considered, for individuals that are treated with medications to decrease the urinary obstructive symptoms of BPH or congestive heart failure.

There are several types of testosterone preparations that are currently available in the United States including testosterone injections, scrotal and nonscrotal transdermal patches, oral testosterone, buccal testosterone, and testosterone gel preparations. Advantages and disadvantages associated with different formulations are given in Table 3. Currently, testosterone injections and testosterone gel preparations are more commonly used in the United States. Medications that stimulate the production of endogenous testosterone (i.e., hCG, clomiphene) may be used in the treatment of older men when fertility is an issue.

The buccal testosterone preparation had several drawbacks including a fixed dose of 30 mg and problems with adhesion of the tablets to the gums [45]. There are men who have not been able to tolerate the feeling of local presence of the buccal testosterone tablet. In the United States, an oral testosterone is currently not in use because preparations of oral 17-alkylated androgens (e.g., methyltestosterone and fluoxymesterone) have an increased risk for hepatic toxicity and abnormal lipid profile (elevated LDL, low HDL) [46]. Oral testosterone undecanoate (TU) is available in many parts of the world but is not approved for use in the United States [46, 47]. The presently available oral TU has a short pharmacokinetic profile and must be taken with food. It leads to variable serum testosterone levels, elevated DHT/T ratio, and variable clinical response [46, 47].

There are several intramuscular injectable preparations available including testosterone enanthate, testosterone cypionate, and TU. Testosterone enanthate or testosterone cypionate injections can be administered with 200 mg every 2 weeks [48–50]. The peak levels of testosterone can be achieved within 2-3 days after administration of testosterone enanthate and cypionate. Serum testosterone levels have to be measured midway between injections, in individuals that are

Table 3: Advantages and disadvantages of testosterone preparations.

Administration method	Formulation	Advantages	Disadvantages
Transdermal agents	Testosterone patches	Mimics circadian rhythm; simple administration	Skin irritation, occasional allergic contact dermatitis, daily administration
	Testosterone gel 1-2%	Easy to apply, readily absorbed into skin. Flexible-dose modifications, skin irritation less common, T levels maintained in normal range.	Transfer during intimate contact; direct contact with children and women should be avoided; skin irritation at the application site in a small number of men, daily administration
	Underarm testosterone gel	Skin irritation less common, T levels maintained in normal range	Transfer during intimate contact; direct contact with children and women should be avoided, daily administration
Subcutaneous agents	Implants	Implants are inserted every 16 to 24 weeks	Invasive procedure with risk of extrusion and infection
Intramuscular injections	Testosterone cypionate	Relatively low cost	Pain and redness at injection site; fluctuations in circulating T levels high risk of polycythemia;
	Testosterone enanthate	Relatively low cost	Pain and redness at injection site; fluctuations in circulating T levels, high risk of polycythemia
	Testosterone undecanoate	Less frequent administration, T levels maintained in normal range	Pain at intramuscular injection site
Buccal formulation agents	Buccal testosterone	Provides sustained release of T; through the buccal mucosa	Unpleasant taste, can stick to gums, gum pain, or tenderness, headache
Oral formulation agents	Methyltestosterone	Oral; modifiable dosage, relatively low cost	Potential hepatotoxicity, drug not in use, may adversely affect lipid profile, decreasing HDL, and increasing LDL
	Testosterone undecanoate	Oral; (approved in the Europe)	Variable clinical effects and testosterone levels must be taken with meals, nonaromatizable to estrogen, Underevaluation in the United States

Abbreviations: T: Testosterone; High Density Lipoprotein: HDL; Low Density Lipoprotein: LDL.

on biweekly testosterone enanthate or cypionate injections. Adjustments to the administration of testosterone dosage have to be made when T is > 700 ng/dL or T < 350 ng/dL [27, 48–51]. The long acting TU injection has been approved in Europe but it is not yet approved in the United States. It can be administered in Europe as 1000 mg injection with a loading dose, followed by another injection of TU 1000 mg at week 6, and subsequently administered at every 12 to 14 weeks [51]. The serum testosterone levels rise to supraphysiologic levels for several days and gradually decline over a period of 10 to 14 weeks after administration of TU injection [27, 51–53]. The high peak levels can be avoided by administering 750 mg instead of 1000 mg of TU injections [54]. The levels can be checked prior to each subsequent injection. The TU injection requires a large volume and can cause cough in a small number of cases [27, 51–53]. The testosterone levels from the longer acting TU cannot be reduced quickly if PSA levels start to rise. This can pose a risk for an aging male.

The transdermal method of testosterone administration has been used as scrotal and nonscrotal patches. Scrotal patches are not currently used because of the need to shave or cut scrotal hair to maintain adequate patch adhesion to the skin [51, 55–57]. In addition, some individuals complained of scrotal itching or discomfort. Transdermal patches can deliver 5 to 10 mg of testosterone. The levels of TT should be checked 3–12 h after application of the patch, and dose adjustments should be made to achieve testosterone level in the midnormal range [51, 55–57]. While nonscrotal patch can help achieve normal serum testosterone levels with a diurnal variation, skin irritation can occur in up to 30% of patients [55–57]. Other drawbacks include the need for up to two patches per day in some men. A testosterone-in-adhesive matrix patch is also available. It can deliver approximately 4.8 mg of T/d and last for 2 days. Like other transdermal patches, it can cause some skin irritation [58].

Most testosterone gels are hydroalcoholic-based gels and contain 1-2% testosterone. The testosterone is absorbed into

the skin and is slowly released into the body. It allows for a fairly steady level of serum testosterone that is as effective as the patch. Testosterone gel is packaged as sachet, metered pump, or underarm testosterone-gel preparation. The most widely used testosterone gel can come as 2.5 g, 5 g, 7.5 g, or 10 g gel with the usual dose being between 5–10 g per day. One application of the gel contains 50–100 mg of testosterone. It is intended to deliver approximately 5 to 10 mg testosterone to the body which can match the normal production of testosterone in healthy men. The dose adjustment of testosterone gel can be done after a patient has been treated for at least one week to achieve serum testosterone level in the midnormal range. Testosterone gel has minimal skin irritability. However, they is a potential of transfer of testosterone to others upon close skin contact. Transfer of testosterone can cause clinical virilization in females and children, and this can be minimized by showering or wearing clothing.

Of the transdermal testosterone preparations, the gel formulation is currently recommended for most hypogonadal men. The gel formulation is able to produce a steady serum testosterone concentration within the physiological range of adult men. Some men who desire freedom from daily application or lower cost can use intramuscular injections of testosterone. Oral testosterone formulations that do not have side effects of prior oral drugs are under development.

Aging males who are started on testosterone replacement therapy should be followed periodically. After the initiation of testosterone replacement therapy, subjects should have a clinic visit, within 3 months, to make needed dosage and formulation adjustments. Subjects should have regular visits (3 to 6 months after treatment initiation and then annually) for assessment of symptom improvement [27]. The visits should also focus on evaluating for erythrocytosis, prostate disease, difficulties with sleep apnea, and other adverse events. The urinary frequency or voiding difficulties can be affected by prostate size. The International Prostate Symptom Scale (IPSS) can be used to monitor for changes by using it at baseline and subsequent visits. An IPSS prostate symptom score of >19 should warrant a urological consultation [27]. In addition to TT levels, lab tests should include measures of liver function, hemoglobin, hematocrit, and PSA. A hematocrit is to be evaluated at baseline, 3 to 6 months, and then annually. When hematocrit rises above 54%, one should cease testosterone replacement therapy until hematocrit decreases to a safe level before reinitiating therapy at a lower dose as noted in the recent Endocrine Society Guidelines [27]. The patient with significantly elevated hematocrit should be monitored and evaluated for symptoms of sleep apnea, cardiovascular events, and hypoxic complaints.

6. Possible Benefits of Testosterone Therapy

In the past two decades, several studies have been carried out to determine the benefits of testosterone replacement therapy for hypogonadal men. The results of these studies indicate that testosterone therapy provides several benefits including improvements in muscle mass and strength, bone mineral density (BMD), adiposity, lipid abnormalities, glucose control, cardiovascular disorders, sexual function, mood, and cognitive function. A summary of the benefits of testosterone therapy for aging hypogonadal males is given below.

6.1. Muscle Mass and Strength. In aging males, falling testosterone levels have been associated with declining strength and muscle mass [59, 60]. The New Mexico Aging Process Study also noted correlations between total and free testosterone levels and muscle strength [19, 61]. A cross sectional study of 118 men on androgen deprivation therapy for prostate cancer showed impaired physical and functional musculoskeletal performance when they were compared with age-matched controls who were not hypogonadal [62].

Several studies found testosterone replacement therapy to be beneficial in improving muscle strength in hypogonadal older men. Svartberg et al. noted an improvement in hand grip in older men with TU treatment [63]. Page et al. found improvements in both hand grip and physical function for hypogonadal men that were on testosterone enanthate treatment [64]. A modest increase in muscle mass and an improved leg extensor strength were also observed in some studies [65, 66]. A double-blinded placebo-controlled study found that elderly men on testosterone replacement therapy for 6 months improved their lower limb muscle strength (isometric knee extension peak torque) when compared with subjects on placebo [67]. The improvement in muscle strength was accompanied by other measurable benefits in gait and balance, aggregate locomotor function test, physical performance test, and 6-min walk test at 6-month assessment in the testosterone group [67]. However, the improvements in muscle strength did not result in significant changes in functional ability. Meta-analyses of randomized trials in middle-aged and older men have confirmed that testosterone administration is associated with an increase in lean body mass (LBM), reduction in fat mass, and increase in grip strength when compared with placebo.

Some other studies did not observe any significant improvements in muscle strength with testosterone therapy. A small study by Clague et al. found no significant improvements in handgrip strength, isometric strength of knee flexors, and extensors or leg extensor power [68]. Meta analysis studies by Isidori et al. also did not find any significant improvement in muscle strength when middle-aged men were treated with testosterone replacement therapy [69]. Similar results were reported by Nair et al. with testosterone patch replacement study in elderly men [70].

Physical function is affected by many factors including muscle strength. Improvements in physical function have also been studied. Changes in performance-based measures of physical function have been inconsistent across testosterone trials that recruited healthy older men. Testosterone therapy did not significantly affect overall quality of life scores [27, 71]. However, a Spanish study confirmed that physical function and the ability to participate in physical activity safely are related to feelings of well being in the elderly [72]. The long-term benefit of testosterone on functional improvements requires further investigation.

6.2. Bone. Testosterone plays an important role in BMD by increasing osteoblastic activity and reducing osteoclastic activity [73–75]. Some of the androgen effects on bone are partially indirect. The reduction in osteoclastic activity appears to be mediated via testosterone's aromatization product to estradiol (e.g., effects on cortical bone) [73–78]. There appears to be associations between testosterone levels and 25 (OH) Vitamin D levels and testosterone and phosphate levels [79, 80]. There is a strong association between low bone density, bone loss, osteoporosis, and low testosterone levels in aging males [81, 82]. There has also been an association of increased risk of fractures in men with hypogonadal states [83–85].

Testosterone replacement therapy was found to increase bone density in hypogonadal men [63, 86]. The bone density increases, however, it may not reach normal adult bone mass [87]. Meta-analysis studies have shown testosterone replacement therapy positively affects bone density and reduces the rate of bone loss [69, 88]. Testosterone therapy appears to have a positive effect on bone markers with a reduction in bone resorption markers [24, 69]. While a few studies have not shown a clear benefit in bone density, many studies with exogenous testosterone have noted increases in BMD in hypogonadal aging males [89, 90]. The improvement in BMD was noted in most types of exogenous testosterone administration including the more commonly used gel preparations [70, 91].

Some studies have reported improvements in lumbar bone density [63, 91–93]. This has also been noted in the meta-analysis by Tracz et al. [88] and Isidori et al. [69]. The studies by Wang et al. [91], Amory et al. [90] and Nair et al. [70] suggested improvements in hip BMD while the meta-analysis studies by Tracz et al [88] and Isidori et al. [69] found femoral neck improvements to be inconclusive. While BMD improves, the effect of testosterone replacement therapy on fracture risk is still unclear. None of the studies have been large enough to show a fracture risk reduction with testosterone replacement therapy. Further, investigations are required to confirm the long-term benefits of testosterone therapy for improving bone strength and its properties.

6.3. Adiposity, Lipid Abnormalities, and Glucose Metabolism. Men with obesity, metabolic syndrome, and type-2 diabetes have low total and free testosterone and low sex hormone-binding globulin (SHBG). Low testosterone is associated with dyslipidemia, hypertension, obesity, and diabetes, all of which increase the risk of cardiovascular disease [93, 94]. It appears that testosterone levels are involved with obesity in a complex relationship. Testosterones levels could be as a causal factor of obesity and could also be a consequence of excess adipose tissue itself. A meta-analysis of observational studies noted that males with metabolic syndrome had lower TT and free testosterone levels [94].

Obesity has effects on the hypothalamus, pituitary gland, and testes with resultant hormonal abnormalities [95]. An increase in regional adiposity appears to be related to levels of low testosterone [96]. This can be associated with lower TT and sex hormone-binding globulin in obese individuals when compared with nonobese individuals [10, 95]. It appears that there is a negative correlation between serum TT and FT levels and visceral fat mass [97, 98].

Several studies have noted that testosterone replacement therapy results in a reduction of body fat mass and waist circumference in hypogonadal men with and without obesity [99, 100]. The studies have also noted some positive changes in total body fat and regional fat distribution with testosterone replacement therapy [95, 100–102]. Adiposity was also noted to decrease along with an increase in lean body mass in a muscle function trial of hypogonadal men in the testosterone-treated group [67]. BMI improved in only one trial [103] and body fat decreased in other studies [104, 105]. It has also been noted that the leptin levels correlate with body fat content and leptin levels decrease with testosterone replacement in T2DM and metabolic syndrome [104, 106]. Several studies indicate a decrease in central adiposity in men with metabolic syndrome and/or T2DM with testosterone treatment [103, 104, 107].

Testosterone plays a role in lipid metabolism. It does affect the actions of lipoprotein lipase and lipolysis [108]. Low testosterone levels can lead to changes in triglycerides and high-density lipoprotein cholesterol. It can also affect total cholesterol levels [69]. The effect of testosterone on lipid profile was investigated in several studies including those on coronary heart disease, metabolic syndrome, and diabetes [100]. Testosterone therapy results in a small but significant fall in total cholesterol and in some LDL cholesterol [100, 105, 107]. In one study, it was noted that the use of testosterone showed a dose-dependent trend toward lower HDL, LDL, and total cholesterol [109]. Most reports found no change in triglycerides. However, one meta-analysis noted that the testosterone and placebo/nonintervention groups did not differ significantly in the changes from baseline in total cholesterol, low-density lipoprotein (LDL), and triglycerides [110]. The observed decrease in LDL in a number of studies may be of some benefit to individuals with hypogonadism and other cardiovascular risk factors. In general, the effects on lipids are observed more after oral-and higher-dose testosterone treatment. The effects are less with transdermal preparation and lower doses of replacement.

Hypogonadism with low total or free testosterone correlates with low HDL cholesterol [111]. The MMAS study noted a strong, positive relationship between HDL and testosterone in men with CVD [111]. However, treatment with testosterone does not seem to increase HDL. Some studies have observed that supraphysiologic doses of testosterone will lower HDL [110, 112]. High-density lipoprotein (HDL) cholesterol levels were also found to decrease in patients that were on oral testosterone therapy [57, 113]. In a meta-analysis study, the high-density lipoprotein (HDL) cholesterol levels were significantly lower in the testosterone-treated group than the control group [110]. There is some evidence that after an initial decrease, HDL cholesterol levels then return to baseline [105]. It is concerning to note that any cardiovascular benefit in lowering LDL is tempered by undesired changes to HDL levels.

There have been studies that have noted associations among hypogonadism, insulin resistance, T2DM, and

metabolic syndrome. Low testosterone concentrations have been noted in individuals with T2DM [17, 114]. This was also noted in a review of the NHANES database [115]. While individuals with T2DM did not appear to have linear correlations between testosterone concentration and degree of glucose control, studies with testosterone treatment in hypogonadal men have shown some reductions in glucose levels and insulin resistance [103, 107, 114, 116]. Wang et al. has stated that the mechanisms that connect hypogonadism, insulin resistance, and T2DM are complicated and include inflammatory markers, oxidative stress, and many other possible underlying causes [117]. A mechanistic paradigm that may be involved between testosterone, obesity, and T2DM has been reviewed by Wang et al. [117].

The effects of testosterone on insulin sensitivity and glucose control have been noted in studies. Low TT or SHBG levels are associated with T2DM [118, 119]. Marin et al. reported that testosterone improved insulin sensitivity assessed by euglycemic insulin clamp studies in obese men while reduc-ing central adiposity [99]. A randomized double blind crossover trial demonstrated a significant reduction in insulin resistance in hypogonadal men with T2DM on testosterone replacement therapy [107]. Several other studies have also shown that testosterone therapy improves insulin sensitivity in hypogonadal men with and without T2DM [117]. Another study has noted improvements in insulin sensitivity once testosterone levels were normalized [120]. In one study, healthy men who had induced hypogonadism had a reduction in insulin sensitivity when there was an acute withdrawal of testosterone [121]. A small longitudinal study of TU versus placebo noted improvement in fasting glucose and decreased insulin resistance in the TU group [122]. The improvements in insulin sensitivity and glucose control are corroborated in other studies which also noted improvements in HOMA when hypogonadal men with metabolic syndrome and/or T2DM were treated with testosterone replacement therapy [70–72]. A prospective trial, by Jones etal., reported that hypogonadal men with metabolic syndrome and/or T2DM on transdermal TRT had reductions in HOMA-IR in addition to beneficial effects on total and LDL-cholesterol and lipoprotein-a [105]. Improvements in hemoglobin A1C (HbA1C is a glycated hemoglobin that reflects average plasma glucose concentration over several weeks) were also observed in two trials [103, 107]. A meta-analysis study has also noted improvements in fasting plasma glucose, and HOMA in the testosterone-replacement therapy group [118]. These studies suggest the normalizing testosterone levels may be helpful in individuals who have T2DM and hypogonadism.

6.4. Cardiovascular Disease. High levels of testosterone do not contribute to the etiology of cardiovascular disease and increased incidence of coronary atherosclerosis in men undergoing coronary angiography [123, 124]. However, cardiovascular risk factors may be affected by the presence of low testosterone levels. Low testosterone is associated with dyslipidemia, hypertension, obesity, and diabetes, all of which increase the risk of CVD [125, 126]. Lower testosterone levels were associated with adverse changes to carotid intima medial thickness and ankle/brachial index as a measure of peripheral arterial disease and calcific aortic atheroma [127–129].

Some observational studies show a correlation between low testosterone and CVD, and others show no correlation. Several epidemiological studies have noted that low testosterone levels were associated with increased mortality in older men [130–135]. In contrast, some studies such as the MMAS study did not show that TT levels were clearly related to all-cause mortality [136, 137].

Aging males are at particular risk for CVD. There are studies that testosterone therapy may be beneficial in several ways. English et al. were the first to report that testosterone-replacement therapy may be beneficial for men with cardiac disease [138]. They found that 22 men with chronic stable angina who were treated with transdermal testosterone-replacement therapy had greater angina-free exercise tolerance than 24 placebo-treated controls. TRT administration in hypogonadal men was reported to improve exercise tolerance (decreased exercise-associated ischemia) in aging males with coronary artery disease [139]. A small longitudinal study of TU versus placebo noted improvements in the carotid medial thickness in the TU group [122]. Testosterone treatment was also found to decrease lipoprotein—A levels in subjects with metabolic syndrome and or T2DM [105, 140]. Another randomized control study showed testosterone treatment in elderly patients with chronic heart failure improved various cardio, respiratory, and muscular outcomes [94, 141].

A meta-analysis study showed no significant differences in the rates of death, myocardial infarction, revascularization procedures, or cardiac arrhythmias between the testosterone and the placebo/nonintervention groups [110]. Another meta-analysis also found no association between testosterone replacement therapy and cardiac events [142]. However, these meta-analyses trials of testosterone therapy generally have not been designed or adequately powered to detect effects on clinically significant cardiovascular events [143]. Other studies of testosterone-replacement therapy have not demonstrated an increased incidence of cardiovascular disease or events such as myocardial infarction, stroke, or angina [144]. However, the true benefits of normalizing testosterone levels in aging hypogonadal men who have underlying cardiac disease are not fully understood and require further investigation.

6.5. Sexual Function. When plasma testosterone levels are below a minimum level, many aging men experience symptoms of low libido, changes in erectile function, and possibly changes in morning erection frequency [145]. Low testosterone levels can lead to reduced sperm production (oligospermia), decreased libido and sexual satisfaction, and strength of erections in elderly men. In hypogonadal younger males, the restoration of serum testosterone levels to the normal levels show benefits on sexual function outcomes [146]. There are reports of improvements in sleep-related erections, cavernous venous leakage, and enhancing production of nitric oxide synthase following testosterone replacement

therapy [147–149]. Many studies in younger men have reported improvements in libido in the testosterone-treated groups when compared with placebo groups [144]. While improvements in libido appear to be more consistent, the improvements to erectile function appear to be varied among trials [150–152]. The meta-analysis study by Isidori et al. reported that moderate improvement in sexual function was noted in men with testosterone levels below 346 ng/dL [152]. The overall impact on sexual satisfaction, however, appears to be unclear as to degree of improvement after testosterone replacement therapy [151]. Long term studies are required to further evaluate the effects of testosterone-replacement therapy on erectile function in older men.

6.6. Mood. Aging hypogonadal men are at increased risk for developing depression [153]. In hypogonadal men, testosterone replacement was associated with improved mood and feelings of wellbeing [44, 154–156]. Despite improvements in mood with testosterone-replacement-gel therapy, the beneficial effect from concomitant testosterone-replacement therapy and SSRIs cannot be clearly differentiated [157, 158]. A meta analysis showed some beneficial effects of testosterone replacement therapy on depression scores [159]. Testosterone replacement therapy's beneficial effects on quality of life and depressive mood have not been consistent across trials [138, 154]. Meta-analysis studies and reviews of androgen replacement therapy do not appear to support testosterone replacement as an antidepressant for the general population [160, 161]. Additional studies are needed to assess the effects of testosterone on clinical depression.

6.7. Cognitive Function. The impact of testosterone on aspects cognitive function has been studied in trials [162–164]. Lower levels of testosterone appear to have an effect on abilities such as spatial abilities, verbal abilities, and cognitive function [162–166]. This is of particular importance to the aging male who may experience changes in cognitive ability from other comorbidities such as vascular disease and neurological pathology.

The effects of testosterone replacement therapy on measures of cognitive function and memory have shown mixed results [167, 168]. In studies by Janowsky et al., Cherrier et al., and Tan and Pu, testosterone replacement improved verbal and spatial memory and constructional abilities in nonhypogonadal men with mild cognitive impairment, hypogonadal men and Alzheimer's disease [165, 166, 169–171]. Transdermal testosterone treatment (5 to 10 mg of testosterone) of men aged 34 to 70 years appears to improve their verbal memory and spatial memory [170, 171]. Another study of healthy men aged 50 to 90 years investigated the efficacy of intramuscular testosterone (alone or in combination with the aromatase inhibitor anastrozole) in improving the cognitive function. This study noted improvements in spatial memory in both groups but verbal memory only improved in testosterone-treated men without anastrozole [171]. The beneficial effects on cognition, memory, and visuospatial abilities were not seen in other randomized studies [172–175]. The evidence in support of and against the improvements in cognition, memory, and visuospatial abilities is not uniform. This could be in part due to the short duration and smaller sample size. Randomized control trials of a longer duration are needed particularly in older hypogonadal men who are on testosterone replacement therapy to fully ascertain the benefits on cognitive performance.

7. Possible Risks of Testosterone Therapy for Elderly Men

Testosterone replacement therapy should be utilized with full awareness of both its potential benefits and possible risks. Several clinical studies have been carried out to determine the potential risks associated with the testosterone therapy to various organs including cardiovascular, respiratory, blood, prostate, and testes. Many of these studies were based on limited number of subjects and of short to medium duration. In spite of these limitations, these studies have identified several potential risks associated with the testosterone therapy. Some of the potential risks include possible effects on cardiovascular complications, sleep apnea, polycythemia, and prostate cancer.Elderly men seem to be at increased risk for fluid retention, increased risk of polycythemia, changes in sleep apnea, and acceleration of benign or malignant prostatic disease [5]. Testosterone-replacement therapy is not recommended for those who still desire fertility. A brief review of some of the main concerns associated with testosterone replacement therapy is given below.

7.1. Cardiovascular Disease. In the near past, there was a prevailing idea that androgens can have an atherogenic effect. This concern arose from the observation that aging men had higher incidence of CVD compared to aging women. Androgen administration drew a concern because it could subsequently add to the risk of developing CVD in men because there were already higher levels of testosterone in aging men compared to aging women. Over the last decade several papers have examined the relationship of androgens with CVD. Many of these studies suggest that there may be even neutral to beneficial effect of testosterone-replacement therapy on the cardiovascular risk factors and adverse cardiovascular complications (e.g., angina) [142, 176–179]. Testosterone replacement is not without risks for the aging males. TRT can lead to water retention and edema [176]. However, in one interventional trial, there were an increased number of cardiovascular-related adverse events in a population of older men who had significant chronic disease and with limitations with mobility [180]. However, such an increase was not noted in another study on testosterone treatment in frail older men [67]. We need longitudinal studies that will assess coronary artery changes to help us attain a better understanding of the effects of testosterone-replacement therapy on the cardiovascular system in aging hypogonadal men.

7.2. Sleep Apnea. Obstructive sleep apnea syndrome (OSA) can result in hypoxemia, sleep fragmentation, and excessive daytime sleepiness [181, 182]. Individuals with OSA can have an increased incidence of visceral obesity, insulin resistance, hypertension, and cardiovascular complications such

as atrial fibrillation, stroke, and cardiac ischemia [181, 183]. OSA and metabolic syndrome are also associated with increased incidence of reduced-circulating testosterone values and erectile dysfunction [184–186]. Androgens may play role in contributing to the pathogenesis of obstructive sleep apnea as there are associations between men with abnormal sleep patterns and increased visceral adiposity with low plasma testosterone values [187, 188]. The presence of low testosterone levels with preexisting OSA can further complicate cardiometabolic risk factors.

Some early studies have suggested supraphysiologic doses of testosterone seem to be more often associated with exacerbation of OSA [189–191]. Elderly individuals who are not treated for OSA should not be started on TRT till OSA is treated. This is because of the increased risk of polycythemia and its possible effects on other comorbid conditions.

Individuals with treated OSA and hypogonadism have been considered for testosterone-replacement therapy. In a meta-analysis of placebo-controlled trials of testosterone-replacement therapy to aging men, they found that there was no significant difference in frequency of sleep apnea between the placebo and testosterone-replacement therapy groups [101]. A small study has noted that testosterone-replacement therapy in individuals with idiopathic hypogonadotropic hypogonadism improves slow wave sleep and does not increase the frequency of OSA [192]. Another study noted that testosterone replacement therapy appears to improve erectile dysfunction in men with OSA and hypogonadism [187]. Even though small studies have shown benefits of testosterone-replacement therapy for individuals with OSA, one should exercise caution in giving testosterone-replacement therapy to individuals with severe untreated or poorly treated OSA [27, 101, 191]. Further longitudinal studies are needed to ascertain the effects of testosterone-replacement therapy in men with hypogonadism and OSA.

7.3. Polycythemia. Men with hypogonadism have lower hemoglobin levels than age-matched controls. The anemia, observed in the aging hypogonadal men, has been suggested to be partly due to declining testosterone levels and also partly due to effects on erythropoietin and erythroid progenitor cells [193–196]. Testosterone-replacement therapy can restore the hemoglobin levels of older men to the normal range. There appears to be a direct relation between testosterone dosage and the incidence of erythrocytosis with testosterone gel [157]. This dose-dependency was noted in another study [57], which compared the effects of transdermal versus intramuscular testosterone. It was noted that the intramuscular testosterone raised the hematocrit more than transdermal testosterone. The dose dependency was also seen in testosterone pellets [197]. The stimulation of hematopoiesis has been noted to be influenced by age and appears to be more pronounced in older men [198, 199].

Although an increase in the hematocrit is generally beneficial for hypogonadal men with anemia, an elevation of hematocrit above the normal range may lead to an increase in blood viscosity. The elevations in hemoglobin can result in adverse outcomes, particularly in elderly due to increases in viscosity that can exacerbate vascular disease (coronary, cerebrovascular, or peripheral vascular circulation) [27, 193, 200–202]. Individuals with increased blood viscosity have been known to be at an increased risk for thrombotic complications such as stroke, myocardial infarction, deep vein thrombosis, stroke, and pulmonary embolism [203–208]. This finding has raised awareness of the importance of monitoring hematocrit when on testosterone-replacement therapy. While there are legitimate concerns regarding polycythemia in individuals with testosterone supplementation, there is no clear evidence of significant complications in a recent meta-analysis of placebo-controlled trials [101].

The risk of polycythemia can be managed through careful monitoring of individuals on testosterone treatment [27, 180, 194, 209]. Individuals who are on testosterone-replacement therapy need a check of their hematocrit levels every 3 to 6 months and then annually [27]. The testosterone-replacement therapy should be held if the hematocrit is $\geq 54\%$ until the hematocrit can return to a safe level; subsequently testosterone-replacement therapy is reinitiated at a reduced dose [27]. It is recommended that individuals with baseline hematocrit values (pre initiation of treatment with testosterone replacement therapy) above 50% should undergo a workup prior to testosterone-replacement therapy because these men have an increased chance of developing hematocrit levels above 54% [27].

7.4. Prostate. There are reports of metastatic prostate cancer after testosterone administration in (elderly) men [210–212]. This has raised concern that testosterone-replacement therapy should be given to aging men who do not have significantly high risk of developing prostate cancer. The current Endocrine Society Guidelines have been developed to render testosterone administration to elderly men acceptably safe therapy in men without a prior history of prostate carcinoma or without evidence of harboring a prostate carcinoma [27].

The concern for prostate cancer has led to the relative contraindication of testosterone-replacement therapy in some individuals [27]. If an individual's medical history reveals an increased risk of prostate cancer (e.g., African-Americans or men with first-degree relatives with prostate cancer) with PSA levels that are greater than 3 ng/mL, then the subject should have a workup with an urologist or other physician experienced in prostate gland evaluation. A workup with an urologist is also warranted in individuals with palpable prostate nodule or induration. Caution should be exercised in men over 40 years of age with increase in PSA concentration greater than 1.4 ng/mL within a 12-month period, elevated PSA levels (>3 or >4 ng/mL), or with high-risk prostate history. These individuals need to be evaluated with a digital rectal examination of the prostate to look for nodules and enlargement [27].

While there are relative contraindications to testosterone-replacement therapy, most studies (using different testosterone formulations over periods ranging from several months to 15 years in men with a wide range of ages) have not revealed an increased risk of prostate cancer [148, 213–215]. A meta-analysis found that testosterone treatment in older men

compared to placebo was not associated with a significantly higher risk of prostate cancer [101]. Two small studies have reported no significant prostate-specific antigen (PSA) rise or prostate cancer recurrence in a total of 17 men, following radical prostatectomy in men with undetectable PSA [216, 217]. Another small study reported no cancer recurrence in 31 hypogonadal men treated with brachytherapy with a followup of approximately 5 years [218].

7.5. Other Potential Adverse Effects of Testosterone-Replacement Therapy. Due to an imbalance in the testosterone to estrogen ratio, hypogonadal men have reported experiencing softer testes, gynecomastia, and increased visceral obesity. Testosterone-replacement therapy does appear to be helpful in men to reduce some of these effects such reduced visceral obesity [107]. However, some men have reported adverse physical changes in their breast, testes, and skin after receiving testosterone-replacement therapy [5].

The increase in serum testosterone levels can suppress gonadotrophin release from the pituitary. The reduction in the production of intratesticular testosterone can also lead to reduced sperm production. Men should be advised that fertility can be adversely affected during testosterone-replacement therapy [219]. When men become hypogonadal, there is an alteration in the free estrogen to free androgen ratio and this can affect the breast [220]. Some men have noted experiencing breast tenderness and swelling or worsening breast tenderness in men with preexisting gynecomastia. The evidence for this association, however, is considered to be weak.

A change in testosterone levels through the use of testosterone replacement therapy appears to affect the skin and hair in men. Some men have noted increased oiliness of skin and acne. The increases in testosterone levels following testosterone-replacement therapy can increase secretion of sebum [221, 222]. This can increase the incidence of minor inconveniences such as the reappearance of acne and oiliness of skin. While there are anecdotal reports of changes in hair pattern, it has not been extensively reported in randomized double blind control trials. The transdermal formulations of testosterone replacement therapy can have administration specific skin reactions. Individuals who have used gels and patches have complained of skin irritation. However, there are more complaints of skin irritations (e.g., erythema, pruritus) that have been reported with patches than with gel preparations [223]. Intramuscular injections of testosterone can cause local reactions such as soreness, erythema, and bruising [224, 225]. The adverse affects of different formulations are listed in Table 3.

8. Summary and Recommendations

Longitudinal studies have shown that prevalence of hypogonadism in the aging men is increasing with each additional decade. Hypogonadism in older men is a syndrome characterized by low serum testosterone levels and clinical symptoms often seen in hypogonadal men of younger age. The patient is likely to be hypogonadal if the early morning serum total testosterone level is less than 250 ng/dL. Since the serum testosterone threshold for a given symptom may vary among symptoms and individuals, it is possible that this threshold is too low in some cases. More large scale data are required to clarify this uncertainty. Aging men with hypogonadism may experience many symptoms including decreased sexual function, decreased cognitive function, elevated LDL in the lipid profile, increased visceral adiposity, changes to the bone density and strength, and muscle weakness and atrophy. Late-onset hypogonadism may also have effects on diabetes and the cardiovascular system. The Endocrine Society Guidelines recommend that one should have symptoms of androgen deficiency and low testosterone levels for the diagnosis of hypogonadism. These guidelines also recommend that testosterone levels should be measured on more than one occasion and the samples for analysis need to be obtained in the morning before 10 am. The diagnosis of late-onset hypogonadism can be less certain in aging men who have comorbid conditions with borderline low testosterone levels.

Several testosterone formulations have been developed for treatment of hypogonadism, and these formulations include testosterone injections, transdermal patches, oral testosterone, buccal testosterone, and transdermal testosterone gel preparations. Currently, testosterone injections and testosterone gel preparations are more commonly used in the United States. Limited clinical trials carried out with these medications indicate that testosterone-replacement therapy provides significant improvements in symptoms for men with late-onset hypogonadism. The long-term benefits and risks of testosterone-replacement therapy will become clearer when the effects of testosterone are studied on all health-related outcomes over an extended period of time. A NIH-sponsored large multicenter randomized control trial of testosterone in aging men with low testosterone levels is currently underway. This trial may provide answers to potential benefits and risks of testosterone replacement in aging males. One limitation of this trial is that it is not powered to fully assess potential risks of prostate cancer and cardiovascular events.

If an aging male is diagnosed with late-onset hypogonadism, the health care provider should engage in a discussion regarding the benefits and potential risks of testosterone therapy in older men. Older men who have significant erythrocytosis, untreated sleep apnea, prostate cancer, and high risk of cardiovascular events should be excluded from testosterone-replacement therapy. Currently, there is not enough evidence to clearly state that the benefits of testosterone-replacement therapy outweighs the risks of testosterone-replacement therapy in aging males. One cannot make a recommendation that testosterone-replacement therapy can be given to all aging males with low testosterone levels independent of significant signs or symptoms.

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Research Article

Multigeneration Inheritance through Fertile XX Carriers of an *NR0B1* (*DAX1*) Locus Duplication in a Kindred of Females with Isolated XY Gonadal Dysgenesis

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A 160 kb minimal common region in Xp21 has been determined as the cause of XY gonadal dysgenesis, if duplicated. The region contains the *MAGEB* genes and the *NR0B1* gene; this is the candidate for gonadal dysgenesis if overexpressed. Most patients present gonadal dysgenesis within a more complex phenotype. However, few independent cases have recently been described presenting with isolated XY gonadal dysgenesis caused by relatively small *NR0B1* locus duplications. We have identified another *NR0B1* duplication in two sisters with isolated XY gonadal dysgenesis with an X-linked inheritance pattern. We performed X-inactivation studies in three fertile female carriers of three different small *NR0B1* locus duplications identified by our group. The carrier mothers did not show obvious skewing of X-chromosome inactivation, suggesting that *NR0B1* overexpression does not impair ovarian function. We furthermore emphasize the importance to investigate the *NR0B1* locus also in patients with isolated XY gonadal dysgenesis.

1. Introduction

Xp21 duplications containing the *NR0B1* (*DAX1*) locus have long been known to be associated with XY gonadal dysgenesis (GD). A 160-kb minimal common region that, if duplicated, causes XY GD has also been determined [1] by comparing several patients with Xp rearrangements. This minimal region contains the *MAGEB* genes and the *NR0B1* (*DAX1*) gene, with *NR0B1* as the strongest candidate to cause gonadal dysgenesis if overexpressed [1, 2]. In fact *NR0B1* has an embryonic expression compatible with a role in sex determination and in adrenal and hypothalamic function in mice [3], and several functional roles in the hypothalamic-pituitary-adrenal-gonadal axis have been reported [4]. However, XY mice transgenic for *Dax1* show delayed testis development and sex reversal only if the transgene is tested against a weak *Sry* allele [5]. In humans, the effect of *NR0B1* overexpression is also shown by XY patients with 1p duplications including the *WNT4* gene; WNT4 is a signalling molecule that has been shown to upregulate *NR0B1* and XY patients with *WNT4* duplications present with abnormal gonadal development [6]. Another indirect proof in human patients is provided by patients with XY GD caused by *NR5A1* (*SF1*) haploinsufficiency. *NR0B1* has been reported to inhibit NR5A1, with consequent reduction of steroidogenesis and AMH production, thus it can be hypothesized that overexpression of *NR0B1* leads to gonadal dysgenesis via inhibition of NR5A1.

Although the aforementioned data support *NR0B1* as the gene responsible for GD, a direct proof in a patient is still missing as a duplication containing only the *NR0B1* gene has not been identified yet in XY patients with GD. All duplications reported so far also contain at least some of the *MAGEB* genes that have specific testis expression but yet unknown function; thus, they cannot be ignored.

Most patients reported before the development of array-CGH had XY GD as part of a more complex phenotype

which also included dysmorphic features and/or mental retardation (for review [7, 8]). This is explained by the fact that most patients have been identified by conventional karyotyping; thus, they carry large genomic rearrangements involving several genes.

We have previously described two *NR0B1* locus duplications in three patients with isolated XY GD; two sisters with complete XY GD [9] and a boy with partial XY GD [10]. Both duplications were of a relatively small size (≤800 kb). In fact they were not identified by conventional karyotyping but by array-CGH and MLPA, respectively. This indicates that *NR0B1* locus duplications should be investigated also in cases with isolated XY GD and not only when GD, in partial or complete form, is part of a more complex phenotype. In fact two other groups have applied array-CGH to patients with 46,XY GD and have identified additional *NR0B1* locus duplications [11, 12].

We developed a DSD probe set for MLPA analysis to screen for dosage imbalances of several genes involved in DSD, with particular attention to genes involved in GD [10]. This probe set includes probes for *NR0B1* and also the *MAGEB1* gene, thus enabling us to detect small *NR0B1* locus duplications. Here, we present a *NR0B1* locus duplication responsible for isolated XY GD identified in a large English family. We also analysed the X-inactivation patterns in fertile female carriers of each of the three small *NR0B1* locus duplications identified by our group. Female carriers of macroscopic Xp21 duplications are thought to be healthy and fertile due to skewed X-inactivation, preferentially inactivating the duplicated chromosome and thereby protecting the individual from increased gene expression. Similarly, a skewed inactivation in carriers of the small duplications would indicate a deleterious effect on increased *NR0B1* (*DAX1*) gene expression on ovarian function.

2. Materials and Methods

2.1. Patients. We studied an English family (Figure 1) with two sisters with isolated 46,XY GD. An X-linked inheritance of the defect was suspected on the basis that, through the maternal line, there is a relative who is an XY female. Informed consent and ethical approval were obtained.

Patient VI.1 (*proband*) presented with primary amenorrhea at 16 years of age. She had female external genitalia. The endocrine profile showed markedly elevated gonadotropins and low oestradiol. The testosterone level was 1 nmol/L and androstenedione was 2.4 nmol/L. Chromosomal examination and revealed a 46,XY karyotype. At ultrasound examination and the vagina was normal with a small prepubertal uterus of 1.8 mL of volume. A small amount of tissue of 0.5 mL volume was visible in the region of the right gonad, no follicles were identified within it. No ovarian tissue was identified on the leftside; however, this may be due to technical problem rather than the gonad being absent. At 17 years and 5 months of age, she underwent gonadectomy. Histological examination revealed fallopian tubes, and streak gonads. Ovarian-type stroma could be detected, and in one area some follicle cells could be demonstrated.

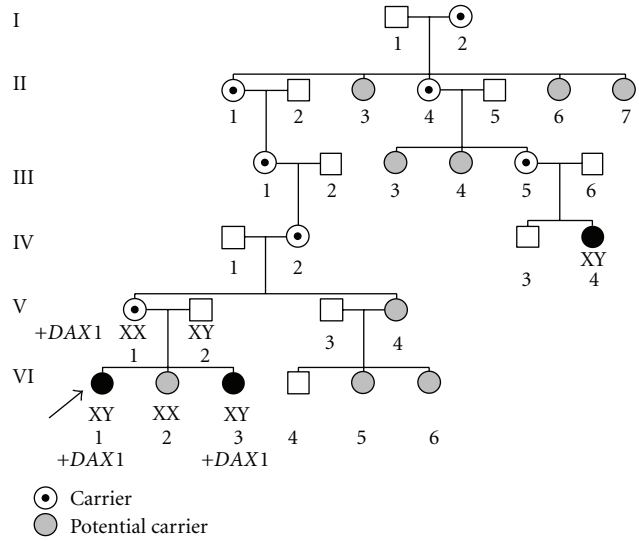

Figure 1: Family pedigree. Pedigree of a family with two sisters with 46,XY gonadal dysgenesis. +DAX1 indicates the subjects that carry the X chromosome containing the *NR0B1* locus duplication. XX and XY have been added underneath some subjects indicating a 46,XX or 46,XY karyotype, respectively. Mandatory female carriers and potential carriers are marked.

Patient VI.3 is the younger sister of patient I1. She also had a 46,XY karyotype. Ultrasound examination, at 12 years and 4 months of age, revealed a prepubertal uterus, the right gonad had a 0.2 mL volume, while no left gonad was seen. Histological examination after gonadectomy at 12 years and 11 months of age showed the presence of unremarkable fallopian tubes, ovarian stroma cells, and gonadoblastoma, bilaterally.

Subject VI.2 has a 46,XX karyotype, she is an adult, and at the moment she does not wish to know if she is a carrier or not. After genetic counselling, no genetic investigation has been performed for this subject.

Patient VI.4 is a female with a 46,XY karyotype; however, no more information is available. No genetic investigation was performed as DNA was not available. It is very likely that she carries the same genetic defect as patient I1 and I3.

2.2. DNA. DNA was extracted from blood and/or Epstein-Barr virus-immortalized cell lines by phenol/chloroform extraction.

2.3. MLPA Analysis. For the initial MLPA analysis, the in-house designed synthetic probe set DSD together with the EK1 reagent kit from MRC Holland (Amsterdam, The Nederland) was used as previously described [10]. Several probe mixes (Table 1) with different combinations of probes targeting sequences within the Xp21 region were used to further narrow down the breakpoint regions.

2.4. Fluorescence In Situ Hybridization. Fluorescence in situ hybridization (FISH) analysis on metaphase nuclei prepared from EBV-immortalized lymphocytes was performed as previously described [9], using the BAC clone RP11-662D2

Table 1: MLPA probes and results.

Probe name[a]	Size (bp)	Results[b]			Sequence[c]	
		This study	[9]	[10]	5′ half probe	3′ half probe
IRLAPL1	126	−	−	−	CAGCTCTAGCCACTGCCCATCCAGATCTCCGTTCTACCTTTCACA	ACACGTACCATTCACAAATGCGTCAGAAACACTACTACG
IGS10	93	−	−	−	CTATGCTGCCAGAAGTCTTAGCAA	AATGCCACATGTGCCCAAGAGAGATTC
IGS11	90	−	−	−	GGAAAATCTCTCTGGGCAGCTGCA	GCCACAGTGCAGGTTGCTGACTTC
IGS15	117	−	−	−	CACAATTTGGAGCAACTTAAAGAAGATATCAGAAATT	TTCTCCAAATTCTGTAGAAAGCACAAATTAAAACAGAG
IGS16	102	−	−	−	GTTTTCTAGAGCCATGTATGAGATTGCAGA	TGTGAATATTGTTCCAGTGAGAGGATACTG
IGS28	99	−	−	−	CTAAATGCTAAAGTAATGGTGCCAGTAA	AAGTGACATTGGTAACTTTGGAAACTCTG
IGS29	93	−	−	−	GCACTGCGGCCTTCTGACATCAGGTATG	ACTCCCTGCCCTGATCTTTCTAC
IGS17	114	−	−	+	GAGTAGATCTCCTTACAGAGAAGCTAGTTGTGGATG	TTGTCCCCTTCTTCTTCCTGAATGCATTCCTTCAGG
IGS12	111	−	−	+	GAAGGAATTCTAATATCAGCAGTCTGTGAACA	ATGTGAAAATCACTGCCTAAGTCTATTGCAGGCACAG
IGS13	117	−	−	+	GCTTCCCCCTTGGATTCTTTATAGCCAATGCACTTA	AAATAGGTTGTTTTGGCACAGCCTGCACAGTTTGATACC
IGS1	99	−	−	+	GGAGGAAAGTAGGACAATTTGGAGGA	ATTTGGGTTCTCAGTGGGGTGGCATTGTAGG
IGS2	117	−	−	+	GTGTATGATAACTGCGTGTCCTCACTGTGAATAATAGCTTG	TGGAACACAAACTGATAGAAATAGACGCATATTG
IGS3	111	−	−	+	CAGCAGCTGGTGAGTGTTCAGCATTAGATATGAGAAGTTA	AGCTGAATAAACTGGAGGAAAACCTTCTG
IGS21	108	+	−	+	GTATCAATGATTTGGGGTTCTGTGTTC	TATAAGCTACAAGACCTGCCCACCACATATTTACACTTC
IGS22	90	+	−	+	CCCAATTCCTCAGTTACTCACTGG	TAGTAAGCTGGACCCTTAGGGAGG
IGS23	126	+	−	+	GAGCATATTTACATGCACCTGGAGGAAGTATCTGATACTATTT	CTAAAGATTAAGTAACAGTGCCTCTTCACTCTTGAATCTAG
IGS24	120	+	−	+	CTTATCCAACTAGGCCTTACATAACAAGTAATAGTCTCCAG	ATGGAATGGCAAAACAACTCTACCAGTGCACTACATC
IGS26	93	+	−	+	GATTAGACAATGTGGGCAGCTATCT	TGGCAACATGACATAATTGGAGCTGC
IGS6	117	+	−	+	GTGGCATAAGGAGCTCACAGCTAGAGTTACT	ATCAAAAGCTAGGCAAGCAGAGAAATATTTATGCCACTATAGCC
IGS7	90	+	−	+	CTGCTAGATGAGCGCACCTCA	ACAGCAAGAGGCTATAGGATGTTCTGC
IGS8	102	+	−	+	CATCCTACATTAGATGATCTCAGTCAACCA	CTGACAAAGACTGAAGTTCAAATTCTGATG
IGS9	108	+	−	+	CCCTCAGATGGTAAGCTTCAGAGTTGAGAGACT	AAGCCTATAATGGAAAAGTGCTCTTAACTTTGG
MAGEB2-5′	96	+	−	+	GGACTGGCGGATTTGGGTCAGCACGCAT	ATTCGTCCCAGGCTGCTAGATACTGC
MAGEB2ex2	93	+	+	+	GGCCACACTTACACCTTCATCGACA	AGGTAGACCTCACTGATGAGGAATCC
MAGEB1	120	+	+	+	GAAGGAAGACAACCCTAGTGGCCACACCTAC	ACCCTCGTCAGTAAGCTAAACCTCACCAATGATGGAAACCTGAGCAG
MAGEB1last	113	+	+	+	GCTAAACCTCACCAATGATGGAAACCTGAGCAATGA	TTGGGACTTTCCCAGGAATGGGCTTCTGATGCCTCTCCTGG
DAX1	102	+	+	+	GCAGCCTCAGCGGGCCTGTTGAAGACGCTG	CGCTTCGTCAAGTACTTGCCCTGCTTCCAG
IGS4	132	+	+	+	CCAAGGAAGTCATAACAGCACAATAGCTATAC	AATCTGATAACCTTGTTAGCTCAAATCAAAGCTCCTAACAAGTAGGAGAGGTTAGTGC
CXorf21	90	+	+	+	CCAGCCACCTGCTCATTATAAGAG	GCACAACTCCAGTGGATGTCATTC
GK	96	+	+	+	CACTATATGTAGGTTATCTCTTCGGTGACA	TACACTGCAATTTGAGAGGGCTGG

Table 1: Continued.

Probe name[a]	Size (bp)	Results[b]			Sequence[c]	
		This study	[9]	[10]	5′ half probe	3′ half probe
IGS5	114	+	+	+	GCAAGCAAGTAAGTGCTGTATCTCCTAGCGAC	TCCCTCCATCACCATTCCTGGTACTCTGTTGTCAAA-TGGC
IGS14	96	+	+	+	GGCAGAGTCCAATCTATAGCAGAGGAA	*t* CAGCAGCAGTAGAGAGAGTATAAAGC
IGS18	99	+	+	+	GAGCTGGTGGAGAAACAGTTACAGAAGAT	AATGGAAAGCATGGGATCCTGGGAGTG*c*
IGS19	135	+	+	+	CCGATACTTGCATGTTGAGATGCCATCCACATTTAG-CCATTTAATAC	ATAATATTGGTTTAACATTTTATCGAGGAAAGCCTG-CCCTAGCACC
IGS33	96	+	+	+	GGTACTTGGGCCTGGGAAGACTGCAG	AATGGGGTTCCTGCATGCAGGCTTCGCC
IGS32	120	+	+	+	GTTTCTGTGGCAGAGATAGGCATCTTCCATTCCATT-GCTGG	AGATATGTGGGGCTCCCTCAGAGAAGACTTAGAGACC
IGS31	117	+	+	+	CATCTGCCTGACATTTAACAAAGCGCATAATGGTTA-GC	TGATCAATCTGGAGTCCCCACAACTTCATATATCCTC
IGS30	111	+	+	+	CAGATGTGGATATGCTGGTTTCCAATAGTAAA	CATGAATTGCTTTCAGGATTCACATTTAGGTACAAAG
IGS20	123	+	+	−	GATACATGTGACTATGGGTGATTACCTGGCATGTTTG	AGCTAAGCCTTTATCACATAATTCACATTTGTAAGG-CTCCTCAC
MAP3K7IP3ex12	108	+	+	−	GCTTCTGGTAGTGCTCAAAGTTTCACTTTCAA	CCTGGCGCAGACTTTTCTGAATTCAGGTGTACCG
MAP3K7IP3ex11	123	−	+	−	GTGTCATGGATGTCTGCCTGTACTTTGGAGGTCACG-CTA	ATTCTTCGGGCTTTTCTCTCAATTGTGCAGGGGTCT-GAGGAG
TAB3ex10	102	−	+	−	ATGGCTTTTGGATCAAAGTTTCCTATAAA	GAAAGTAAATTCATCAGGCATTTAAGAACTC
IGS27	99	−	+	−	GTAATTAGAACAGGAAGAATGGGGGGGAAT	AACTTGAGGGAGGGGGTAATTGGCAGC
MAP3K7IP3ex8	99	−	+	−	GGGATCGCAGTGGTGCAGCTGACTCTTC	TGAGCCGTCTCTGCATCAGGTCATGCTCC
MAP3K7IP3ex7	111	−	−	−	CGGCAACAGGCTTCAAGATTGTTGTTATTC	TAGGGGAGAAAAATGGTAAAAGTAACATTGGCAACA-TTC
MAP3K7IP3ex6	114	−	−	−	GTGGCCAAGTTTCTCTTAGGAAATGGATGTT	AACCGGCTTTCCAAAAGTAATGATCTTCTAGCACCA-CAGTC
MAP3K7IP3ex1	123	−	−	−	GCTTGGACTCTGAGGTTCTGACCGTAGCATCAGATC-ACACAGAGAAC	TACCTTGCTCGCCCGAATGTGTGGCTTTCTCTGC
DMD	135	−	−	−	CATCGCTCTGCCCAAATCATCTGCCATGT	GGAAAAGACTTCCTACATTGTGTCCTGGAAAACAAA-GAGAAAGAAAGACAGACTTTACAAAAGG

[a] The probes are listed according to their genetic location from the most telomeric to the most centromeric.
[b] Plus and minus signs indicate duplicated and nonduplicated regions, respectively.
[c] The 5′ half probes are preceded by the universal tag sequence GGGTTCCCTAAGGGTTGGA; the 3′ half-probes are followed by the universal tag sequence TCTAGATTGGATCTTGCTGGCAC and are phosphorylated at the 5′ end.

(CHORI BACPAC Resource Center, Oakland, CA, USA) which contains the *NR0B1* gene.

2.5. X-Inactivation Experiments. The X-inactivation pattern was examined by the methylation analysis of the polymorphic CAG repeat within the androgen receptor gene [13]. Briefly, 250 ng of DNA were digested using the methylation sensitive enzyme HpaI; digested and undigested DNA samples were amplified by PCR using a primer pair flanking the CAG repeat region as well the cleavage site, with the forward primer FAM labelled, Taq Gold (Applied Biosystems), 1X buffer II (Applied Biosystems), 200 μM of each dNTPs, and 1.5 mM $MgCl_2$. PCR products, together with the ROX HD400 size marker (Applied Biosystems), were size separated by capillary electrophoresis using a ABI3100 genetic analyser (Applied Biosystems, Warrington,UK). Trace data were analysed using the GeneMapper software (Applied Biosystems). Peak heights for the two digested alleles were corrected by the peak heights of the corresponding undigested alleles. The ratios of the skewed X-inactivation in digested samples were calculated by normalizing the sum of the two corrected alleles to 100%. DNA was obtained from EBV-immortalized cell line and from blood. In addition, DNA from a male sample was included as control for complete digestion. Data are presented as the average of three independent experiments.

3. Results

3.1. MLPA Detection and Fine Mapping of the Duplication. The MLPA analysis with the DSD probe set detected a duplication of the *DAX1* probe as well of the MAGEB1last probe, targeting the last exon of the *MAGEB1* gene, in both affected sisters. MLPA was also used to further characterize the breakpoint region, using several probe mixes with different combination of probes targeting the Xp21 locus. We determined that the telomeric breakpoint region (5.6 kb) is located approximately 63 kb upstream of the *MAGEB* genes, while the centromeric breakpoint region (2.6 kb) lies within intron 11 of the *MAP3K7IP3* gene. Thus, the duplication has a minimal and maximal size of 679 kb and 687 kb, respectively, and in addition to *NR0B1* it contains the *MAGEB* genes, *CXorf21*, *GK* and part of the 3′ region of the *MAP3K7IP3* gene (Figure 2).

Parental DNA analysis revealed that the mother is a healthy carrier of the duplication. Table 1 summarizes all the probe pairs used and the results obtained. Together with these English patients, we present, for comparison, the results obtained for two Iranian sisters [9] and for an Italian patient, in which we previously narrowed down the breakpoint region [10].

3.2. Analysis of the Duplication Using FISH. Metaphase FISH analysis was performed to establish the location of the extra copy. On metaphases from the patient, only one signal for the clone RP11-662D2 containing the *NR0B1* gene was detected; on metaphases from the mother only one signal per X chromosome was detected, thus indicating an interstitial duplication and excluding translocation of X chromosome material onto another chromosome (data not shown).

3.3. X-Inactivation Studies. We analysed the X-inactivation pattern in subject V-1, and the methylation ratio between the X-chromosome carrying the duplication and the normal chromosome, in EBV-immortalized lymphocytes, was 65% : 35%. We performed X-inactivation studies also on two other mothers carrying different *NR0B1* locus duplication that have been previously described by our group [9, 10]. Ratios between the X-chromosome carrying the duplication and the normal chromosome were 58% : 42% and 33% : 67%, respectively.

These results indicate that in lymphocytes there is not a strong preferential methylation for the X-chromosome carrying the duplication.

4. Discussion

We present here a family where a relatively small *NR0B1* locus duplication is the genetic cause of isolated complete 46,XY GD. The duplication extends from the *MAGEB* gene to part of the *MAP3K7IP3* gene, including *NR0B1*, *CXorf21*, and *GK* genes.

By bioinformatics evaluation of the breakpoint regions, we noted that within the telomeric breakpoint region there is a *AluSc* repeat of approximately 300 bp that shares 85% and 84% identity (BLASTN2.2.21 [14]) with two *AluY* repeats within intron 11 of the *MAP3K7IP3* gene. These evolutionary young *Alu* repeats may be involved in the duplication mechanism as they are found to be enriched near or within duplication junction [15]. We unfortunately have not been able to amplify and sequence the duplication junction so we could not determine the rearrangement mechanism and differentiate between a nonallelic homologous recombination (NAHR) or a nonhomologous end joining (NHEJ) mechanism. The latter was the mechanism of the duplication in a previously described family [9]. Actually, the failure, after several attempts, to amplify the duplication junction in two of the three duplications we have identified, makes us suspect that the duplications could be more complex and be generated by a FoSTes (Fork Stalling and Template Switching).

Xp21 duplications, as well deletions [16], including the *NR0B1* gene, are all different, thus they are caused by nonrecurrent rearrangements. This indicates that in this region there are several genomic elements that can lead to genomic rearrangements. These rearrangements will be different regarding size and genes involved. This is important to consider when a genetic test is chosen or developed to screen for these genomic disorders.

Two other groups have applied array-CGH to analyse patients with 46,XY gonadal dysgenesis and found additional Xp21 duplications [11, 12]. Patients with large duplications presented XY gonadal dysgenesis associated to syndromic feature, while two patients with relatively smaller duplications (<1 Mb) including *NR0B1* presented isolated 46,XY gonadal dysgenesis (Figure 2). Thus, confirming our findings

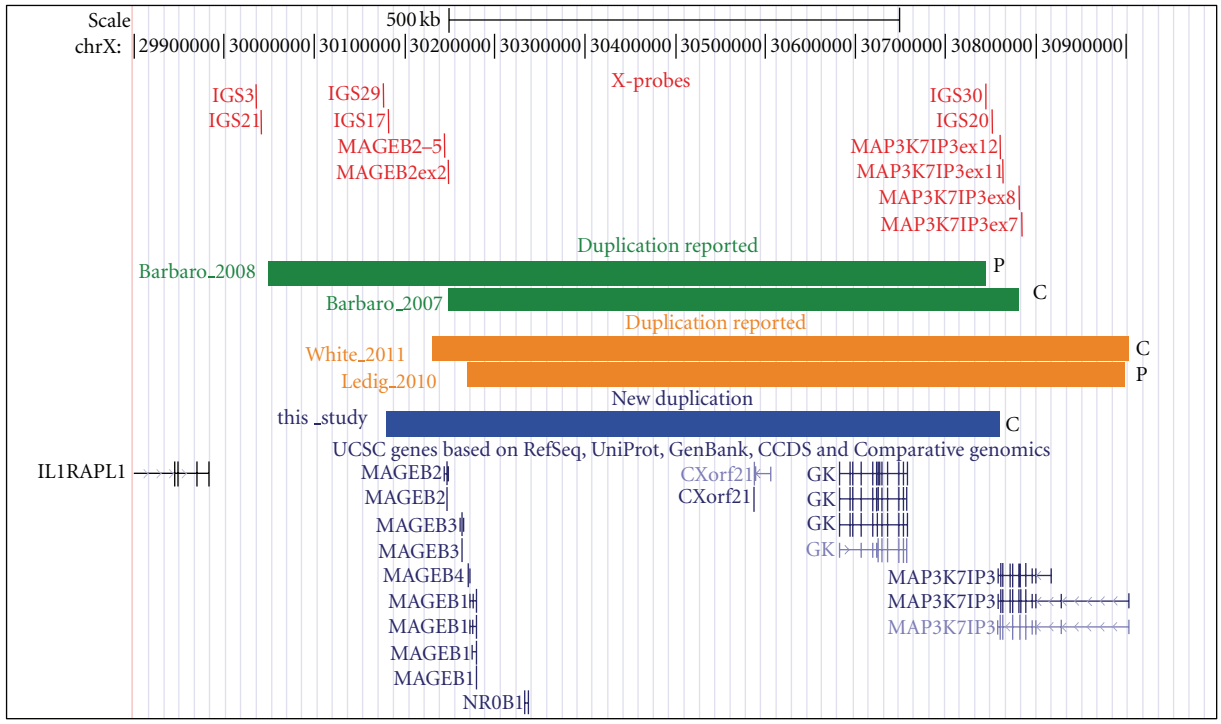

Figure 2: Comparison of *NR0B1* locus duplications. Representation from the UCSC genome browser (NCBI36/hg18) of the *NR0B1* locus on Xp21.2. The probes that delineate the breakpoints in the three independent families identified by our group are represented by red vertical lines. The horizontal blue line represents the extension of the duplication in the family here described, the green lines represent the duplicated regions previously described by our group, and the orange lines represent the two duplications identified by array CGH in patients with isolate 46,XY GD by other groups. The C and P at the right ends of the lines indicate if the GD was complete or partial, respectively.

that *NR0B1* locus duplication should be investigated in all cases of isolated 46,XY gonadal dysgenesis.

By comparing the three small Xp21.2 duplications identified by our group, together with the other two recently reported by Ledig et al. and White et al. [11, 12] (Figure 2), it is not possible to directly delineate a genotype-phenotype correlation for the partial or complete GD forms. It is known that dosage-sensitive genes with complex expression patterns are particularly sensitive to positional effects, and regulatory regions can lie far outside the transcription unit [17]. A regulatory region upstream *NR0B1* has been proposed after the identification of a 257 kb deletion in the region between *NR0B1* and *GK* gene in a patient with XY GD [18]. Furthermore, a small inversion immediately upstream of *NR0B1* has been identified in a patient with congenital adrenal hypoplasia [19]. However, patients with both partial and complete form share entirely these regions in their duplications. The interaction of *NR0B1* with other transcription factors could also modulate the final phenotype.

Interestingly, in all cases with isolated 46,XY GD, the *IL1RAPL1* gene, located immediately telomeric to the duplication containing *NR0B1*, is not disrupted (Figure 2). Deletions or mutations of this gene have been identified in patients with mental retardation [20]. Disruption of this gene could explain the mental retardation in some previously described patients with larger Xp21 duplications.

As all three duplications we identified were maternally inherited, we could use the material available to investigate the X-inactivation pattern of the duplicated X. In several studies where a larger Xp21 duplication was maternally inherited, it was shown that the duplicated X was preferentially inactivated [21, 22]. Thus carrier women are thought to be protected from a double *NR0B1* dose on the gene expression level. Therefore, we investigated if there was a preferential methylation of the duplicated X chromosome also in females carrying the small duplications which contain fewer deleterious genes. Analysis in lymphocytes of the three mothers showed that there is no obvious preferential inactivation of the duplicated X. Even though we could not investigate the situation directly in the ovary, we can hypothesize that healthy carrier women are fertile because the ovary, in contrast to the testis, can tolerate an extra dose of *NR0B1*.

In conclusion, the identification of *NR0B1* locus duplications in patients/families, originating from different countries (Iran [9], Italy [10], England (this study), Germany [11], and Australia [12] stresses the importance of using methods that can detect submicroscopic duplications of the region surrounding *NR0B1* in the evaluation of patients with isolated 46,XY GD (complete or partial). The lack of phenotype in the carrier mothers and the pedigree of the family here described also illustrate that such duplications can be

spread through the female line in the family. In the present family there are seven mandatory female carriers and nine potential carriers. In fact, some of these could even be affected XY females who have not been investigated. Small duplications are most probably more frequent, but have escaped detection due to the methods that have been used so far and the selection of the patients investigated. We believe that in a patient with isolated XY GD, the *NR0B1* locus should be carefully investigated. MLPA and array-CGH are two different techniques that can be applied. Array-CGH offers the advantage of a whole genome screening approach; however, the capacity to detect very small *NR0B1* duplications depends on the platform used (number and distance of probes within the *NR0B1* locus). *NR0B1* has a size of 5 kb, and the two nearest *AluSx* sequences on opposite side of *NR0B1* are only 14 kb apart. MLPA containing specific probes guarantees the identification, of isolated *NR0B1* duplications and the maternal sample can be simultaneously analysed for carrier status at a limited extra cost compared to array CGH.

Acknowledgments

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Research Article

A Cell Model for Conditional Profiling of Androgen-Receptor-Interacting Proteins

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Partial androgen insensitivity syndrome (PAIS) is associated with impaired male genital development and can be transmitted through mutations in the androgen receptor (AR). The aim of this study is to develop a cell model suitable for studying the impact AR mutations might have on AR interacting proteins. For this purpose, male genital development relevant mouse cell lines were genetically modified to express a tagged version of wild-type AR, allowing copurification of multiprotein complexes under native conditions followed by mass spectrometry. We report 57 known wild-type AR-interacting proteins identified in cells grown under proliferating and 65 under nonproliferating conditions. Of those, 47 were common to both samples suggesting different AR protein complex components in proliferating and proliferation-inhibited cells from the mouse proximal caput epididymus. These preliminary results now allow future studies to focus on replacing wild-type AR with mutant AR to uncover differences in protein interactions caused by AR mutations involved in PAIS.

1. Background

Androgen insensitivity gives rise to a wide spectrum of disorders in man, the most severe being complete sex reversal, to milder forms of PAIS associated with ambiguous or underdeveloped genitalia, or even milder forms causing "only" male infertility in otherwise healthy males. Often mutations in the androgen receptor (AR) are involved which interfere with ligand binding, DNA binding, or increase or decrease intramolecular interactions between AR domains [1]. Where no mutations are identified in the AR [2] mutations in AR coregulators may be implicated in failure to activate or repress androgen-regulated target genes. Although there are a number of mouse models available to study impaired AR function *in vivo* [3], the signalling networks are too complex to dissect without using simpler cell models. The aim of this study was to develop a cell model for the study of AR signalling in the urogenital tract. In turn this may identify disrupted signalling resulting from AR mutations associated with PAIS. Male genital development relevant murine cell lines PC1 (proximal caput epithelial cells from mouse epididymus) [4] and MFVD (mesenchymal fetal vas deferens cells) [5] were genetically modified to express a tagged wild-type AR to test the system. The modifications allow purification of multiprotein complexes associated with AR under native conditions and analysis of the copurified protein complexes by mass spectrometry. The data was analysed using readily available bioinformatics software: the pathway mining tool of "DAVID" bioinformatics resources [6, 7] and the gene group functional profiling tool of g : profiler [8]. By focussing solely on known AR coregulators, we were able (as a proof of principle) to uncover differences in the proteome of proliferating and nonproliferating epithelial PC1 cells.

2. Methods

2.1. N-Terminal Tandem Affinity Purification Tag (N-TAP). The N-TAP was designed by modifying the C-terminal tandem affinity tag (C-TAP) from Fernández et al., 2009 [9]. The HAT tag was amplified, using the C-terminal tag [9] as a template and PCR primers (Figure 1(a)) designed to add

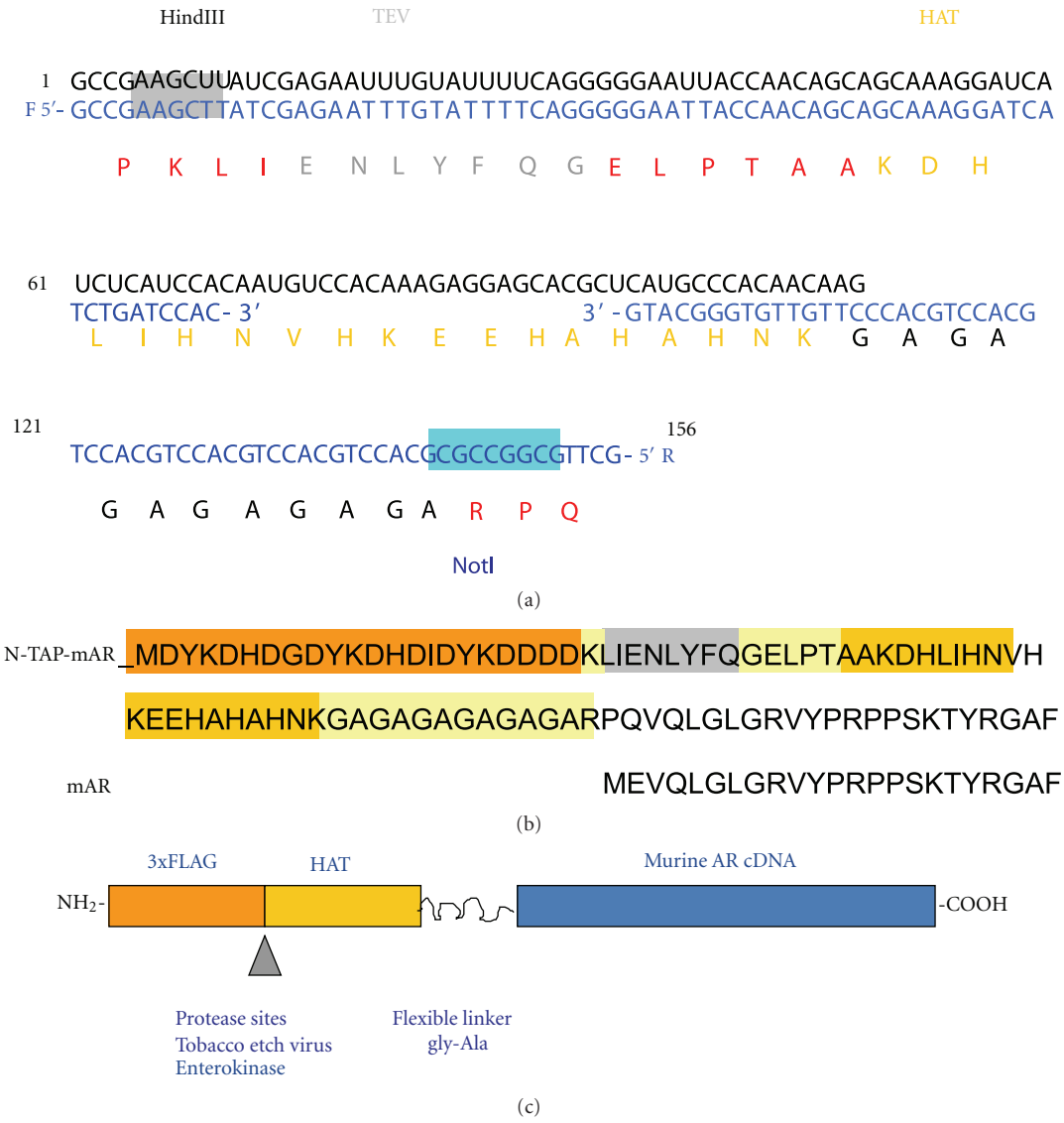

Figure 1: Creation of the mouse N-TAP-mAR. (a) Amplification of the TEV-HAT-glycine alanine repeat sequence with primers F and R using the C-TAP from Fernández et al., 2009 [9] as template. (b) Aminoacid sequence of the complete NH2-terminus of the androgen receptor. The N-TAP increases the size of the mAR by 70 amino acids and the molecular weight by 7.8 kDa (http://web.expasy.org/compute_pi/). (c) Schematic showing the tagged androgen receptor (N-TAP-mAR) cDNA clone. The N-terminal TAP tag was selected on the basis of small size. It contains three FLAG epitopes (3xFLAG) and 6xHistidine residues (HAT) located in an alpha helix.

the TEV protease cleavage site with the forward primer and the glycine-alanine repeat and an additional NotI-cloning site with the reverse primer. The HindIII-NotI fragment was then inserted into the polylinker region of p3XFLAG-CMV-10 (Sigma) in frame with 3XFLAG (Figure 1(b)). The mouse androgen receptor cDNA clone (gift from Professor Jan Trapman, Department of Pathology, Erasmus MC/JNI Rotterdam) was modified by replacing the start methionine ATG with an NotI site-by-site directed mutagenesis (Stratagene) and introducing the 2.787 kb NotI-BHI full length mouse cDNA into the N-TAP-CMV-10 vector (Figure 1(c)). The N-TAP-mAR fusion construct was confirmed by sequencing.

2.2. Transient Transfection and Luciferase Assay. In total 10^5 COS-1 or Hela cells/well were seeded into 12-well tissue culture plates in DMEM, containing 10% charcoal-stripped serum. Cells were transiently transfected using Fugene (Roche) or Lipofectamine 2000 (Invitrogen) with 25 ng AR or N-TAP-mAR, 500 ng of pGRE-luciferase and 25 ng pTK-RL according to manufacturer's instructions. 12–16 hours after transfection, the medium was replaced with DMEM, containing 10% charcoal-stripped serum + or −10 nM dihydrotestosterone (DHT; Sigma). 24 h later, cells were harvested and lysed in 25 mM glycine (pH 7.8), 15 mM $MgSO_4$, 4 mM EGTA, 1% triton × 100 and 1 mM dithiothreitol. Luciferase assays

were performed with reagents from nanolight technology and the ratio of luciferin : renillaluciferase activity was measured using a Turner TD-20/20 luminometer. Standard error bars relate to three independent transfection experiments.

2.3. Control Cell Lines. Androgen responsive cell lines PC1 and MFVD from the mouse urogenital tract served as control cell lines in their nonmodified state. The PC 1 cell line was a gift from Araki et al. [4] and the MFVD cell line a gift from Umar et al. [5]. The PC1 cell line is an epididymal cell line immortalized with SV 40 large T-antigen and has been characterized in great detail regarding morphology [4], epithelial and epididymus specific gene expression [4], and androgen responsiveness [10]. Also the MFVD cell line is immortalized by expression of a temperature sensitive SV 40 large T-antigen but of mesenchymal origin. MFVD cells were derived from fetal (18 *d.p.f.*) mouse vas deferens and show features of Wolffian duct mesenchymal cells and androgen responsiveness [5]. Both cell lines were continuously cultured under conditions given elsewhere [4, 5] in the presence of 5 nM mibolerone, a synthetic androgen.

2.4. Establishment of Stable Cell Lines. The control cell lines PC1 and MFVD were transfected with the ScaI (unique site in the bacterial ampicillin resistance) linearised N-TAP-mAR vector using Lipofectamine 2000 (Invitrogen). After 48 hours, transfected cells were replated in dilutions from 10^5–10^3 cells/14 cm diameter dish and G418 resistant clones were selected with 750 μg/mL G418 (PC1) and 250 ug/mL G418 (MFVD). Single colonies coming up were picked with cloning rings, grown up and tested for N-TAP-mAR expression by Western blot analysis and immunocytochemistry using the FLAG M2 antibody (Sigma) and the AR-N20 antibody (Santa Cruz).

2.5. Growth of Proliferating and Nonproliferating PC1 and P17 Cells. PC1 cells, derived from the mouse proximal caput epididymus and immortalized by expression of a temperature sensitive SV 40 large T-antigen, were continuously cultured at 33°C, the permissive temperature of large T in the presence of 5 nM mibolerone. At physiological temperature (37°C), cell growth is partly inhibited and T-antigen-expressing cells can survive [11]. Large T is however degraded after prolonged exposure of the cells to the nonpermissive temperature (39°C), then significant cell death occurs and the cells do not recover [11]. To prevent cell death but still have an inhibitory effect on proliferation early passage PC1 and P17 cells were cultured to near confluence at 33°C and kept then for 1 week at 37°C in growth medium containing 5 nM mibolerone before preparing cytoplasmic and nuclear extracts of the still healthy looking cells. For extracts made from proliferating PC1 and P17 cells, the cells were grown at 33°C in growth medium containing 5 nM mibolerone. Similar amounts of cell pellets from proliferating and nonproliferating cells were processed for protein extraction.

2.6. Immunoblotting. Cells from subconfluent cultures were washed, trypsinized, and pelleted by centrifugation and washed 3x in cold PBS. For whole cell lysates, cells were lysed in SDS loading buffer (0.03125 M Tris pH 6.8, 5% glycerol, 0.001% bromphenol blue 3, 1% SDS, 2.5% β-mercaptoethanol). Samples were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride membrane, blocked and probed in phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 10 mM phosphate salts (mono and dibasic) (pH 7.3), 0.05% (vol/vol) Tween 20) containing 10% nonfat dry milk. Primary antibodies were used at recommended dilutions and horseradish peroxidase-conjugated secondary antibodies (Dako) and ECL Plus Blotting Detection Reagents (Amersham) or SuperSignal West Femto (Thermo Scientific) were used as described in the manufacturer's instructions. SRC-1 (128E7) and CTNNB1 (9587) antibodies were purchased from NEB, FLAG M2 (F3165) from Sigma, and N20-AR (Sc-816), NR3C1 (Sc-8992), SMARCC1 (Sc-9748), ACTB (Sc-81178) from Santa Cruz.

2.7. Cytoplasmic and Nuclear Protein Extraction and Purification of N-TAP-mAR. The protein extraction protocol is a modified version of a chromatin extraction protocol for co-purification of histones by Saade et al., 2009 [12]. Cells were grown on 14 cm diameter dishes to confluence (10 plates PC1 or 20 plates P17 gave about 700 μL cell volume), washed 3x with prewarmed PBS, trypsinised with 1 mL trypsin-EDTA (Sigma)/plate 5 minutes 37°C, inactivated with medium, pooled in 50 mL Falcon, spun1200 rpm 5 mins RT, and washed 3x with cold (4°C) PBS. Cells were transferred to a 2 mL microfuge tube and pelleted by centrifugation at 6500 rpm 2-3 minutes at 4°C. Pellets (2 $\times$ 200 μL) were resuspended in 2 $\times$ 1.8 mL hypotonic buffer (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM MgCl, 0.1% Triton, 4.5 mM β-mercaptoethanol, protease inhibitor cocktail (Roche), 5 nM mibolerone, 1 mM Pefabloc (Roche), and Phosstop (Roche)) by pipetting up and down and vortexing vigorously on highest setting for 15 seconds followed by 45 minute incubation on ice vortexing every 10 minutes. Nuclei were spun down at 4°C 13.000 RPM for 5 minutes, the supernatant, called here cytosol fraction, transferred to a clean tube and NaCl was added to 15 mM. This cytosol fraction was added to FLAG-M2 coupled magnetic beads (ca. 5 mg) after keeping an aliquot as cytosol input fraction for PAGE. Coupling of the magnetic beads (Dynabeads M-270 Epoxy from Invitrogen) to the FLAG M2 antibody (Sigma F3165) was performed according to manufacturer's instructions. Cytosol extracts on beads rotated for 1 hour minimum at 4°C. Nuclei were taken up in sucrose buffer (0.34 M sucrose, 10 mM Tris pH8, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 150 mM NaCl, 1 mM DTT, protease inhibitor cocktail (Roche), 5 nM mibolerone, 1 mM Pefabloc (Roche), Phosstop (Roche)) 150 μL/100 μL cell pellet and resuspended. 0.3 units micrococcal nuclease from Sigma (0.1 U/μL in 10 mM Tris/0.1 mM $CaCl_2$ pH8) for 100 μL nuclear extract were added and incubated for 30 min at 37°C. Extracts were then diluted with 1 volume of sucrose buffer and sonicated 6 $\times$ 10 seconds on ice with

10 sec bursts and 10 sec cool downs alternating. Extracts were finally loaded onto FLAG M2-coupled magnetic beads and rotated for at least 1 hour at 4°C in cold room. Supernatants were kept as flow through and aliquots before loading on to the beads as nuclear input. After the incubation period, beads loaded with cytosol preparations were washed 3x with hypotonic wash buffer (10 mM Tris pH 7.5, 10 mM KCl, 1.5 mM MgCl, 0.1% Triton, 4.5 mM β-mercaptoethanol, protease inhibitor cocktail (Roche), 5 nM mibolerone, 1 mM Pefabloc (Roche) and Phosstop (Roche), 0.5% NP40, 0.05% sodium deoxycholate, 0.005% SDS, 15 mM NaCl) or sucrose buffer (nuclear preps) followed by 3 washes with TEV buffer (0.05 M Tris pH 8, 1 mM DTT, 0.5 mM EDTA) supplemented with Protease Inhibitor Cocktail (Roche), mibolerone (5 nM), and phosphatase inhibitors (Phosstop and Pefabloc (Roche), and NaCl to15 mM NaCl in cytosolic and 150 mM NaCl in nuclear TEV buffer. The beads were finally taken up in 100 uL TEV buffer and bound protein complexes were eluted with 10 units of TEV protease (GST-tag) (TEVP US Biological) at RT for 1 hour. Protein concentration in the eluted fraction was determined by Bradford assay. The beads were kept in PBS for recycling.

2.8. Mass Spectrometry. Colloidal coomassie stained protein bands (Colloidal Blue Staining Kit, Invitrogen) were excised out of a 5% PAGE after separation in 7 slices covering a size spectrum of 48 kDa to the top of the gel. Gel slices were briefly washed in MilliQ water and kept in water at −80°C until submitted to "Cambridge Proteomics Services" where all LC-MS/MS experiments were performed using an Eksigent NanoLC-1D Plus (Eksigent Technologies, Dublin, CA) HPLC system and an LTQ Orbitrap Velos mass spectrometer (ThermoFisher, Waltham, MA). Separation of peptides was performed by reverse-phase chromatography used at a flow rate of 300 nL/min and an LC-Packings (Dionex, Sunnyvale, CA) PepMap 100 column (C18, 75 μM i.d. × 150 mm, 3 μM particle size). Peptides were loaded onto a precolumn (Dionex Acclaim PepMap 100 C18, 5 μM particle size, 100A, 300 μM i.d. × 5 mm) from the autosampler with 0.1% formic acid for 5 minutes at a flow rate of 10 μL/min. After this period, the valve was switched to allow elution of peptides from the precolumn onto the analytical column. Solvent A was water + 0.1% formic acid and solvent B was acetonitrile + 0.1% formic acid. The gradient employed was 5–50% B in 45 minutes. The LC eluant was sprayed into the mass spectrometer by means of a new objective nanospray source. All *m/z* values of eluting ions were measured in an Orbitrap Velos mass analyzer, set at a resolution of 30000. Data dependent scans (Top 20) were employed to automatically isolate and generate fragment ions by collision-induced dissociation in the linear ion trap, resulting in the generation of MS/MS spectra. Ions with charge states of 2+ and above were selected for fragmentation.

2.9. Data Processing and Database Searching. After run, the data were processed using Protein Discoverer (version 1.2., ThermoFisher). Briefly, all MS/MS data were converted to mgf (text) files. These files were then submitted to the Mascot search algorithm (Matrix Science, London UK) and searched against Uniprot Mouse database, using a fixed modification of carbamidomethyl and variable modifications of oxidation (M).

3. Results

3.1. Isolation of N-TAP-mAR Complex in MFVD and PC1 Cell Lines. Stable MFVD and PC1 clones expressing N-TAP-mAR were developed as described in Section 2. Total protein was extracted from several clones and analyzed for N-TAP-mAR expression by Western blot using FLAG and AR-specific antibodies (data not shown). A second criteria was the nuclear localization of N-TAP-mAR, which was examined in several clones by immunofluorescence using FLAG and AR-specific antibodies. One MFVD cell line (M7) and one PC1 cell line (P17) were selected on the basis that they expressed similar levels of stably integrated N-TAP-mAR and endogenous wild-type AR (WT AR). Both were predominantly located in the nucleus when grown in medium supplemented with 5 nM mibolerone (Figure 2(a)). The transactivation properties of N-TAP-mAR were tested in COS cells by cotransfection of a GRE reporter construct, with expression levels confirmed by Western blot (Figures 2(b) and 2(c)). Levels of expression are stable, allowing considerable grow up of cells.

Different protein purification protocols for producing nuclear and cytosol cell extracts were tested. Commercially available purification kits and a modified version of a chromatin purification protocol (FLAG-antibody capture) were compared and the latter was chosen. FLAG-antibody capture gave a good recovery of N-TAP-mAR when preparing extracts from M7 (Figure 3(a)) or P17 cells (Figure 3(b)). The known coregulators SRC-1 and CTNNB1 were copurified in P17 extracts but not in M7 extracts. Attempts to optimize His-tag purification conditions were unsuccessful; Nickel affinity purification of the PC1 (N-TAP-mAR negative control) chromatin extracts gave high background with unspecific protein binding to the Nickel resin. The cell lines proliferate at 33°C with growth driven by temperature sensitive SV40 T-antigen; and proliferation stops at 37°C. Figure 3(c) illustrates a preparative gel of TEV protease-eluted proteins purified from the nuclei of P17 cells cultured at 33°C and 37°C. The gel slices were selected for LC- MS/MS, and data processed using the Mascot Search engine at the Cambridge Centre for Proteomics.

The results from the LC-MS/MS search are portrayed as peptide matches and grouped as protein hits using a simple parsimony algorithm (http://www.matrixscience.com/). Only those ions scores that exceed a significance threshold of 0.05 (1 in a 20 chance of being a false positive) contributed to the score. This would translate into 1500 peptides falling within the mass tolerance window to have a score of ≥45. Known AR-interacting proteins (http://androgendb.mcgill.ca) were identified among the protein hits and are listed in Table 1 along with their respective peptide scores. In this first mass spec analysis, we identified 1196 AR associated proteins in FLAG purified

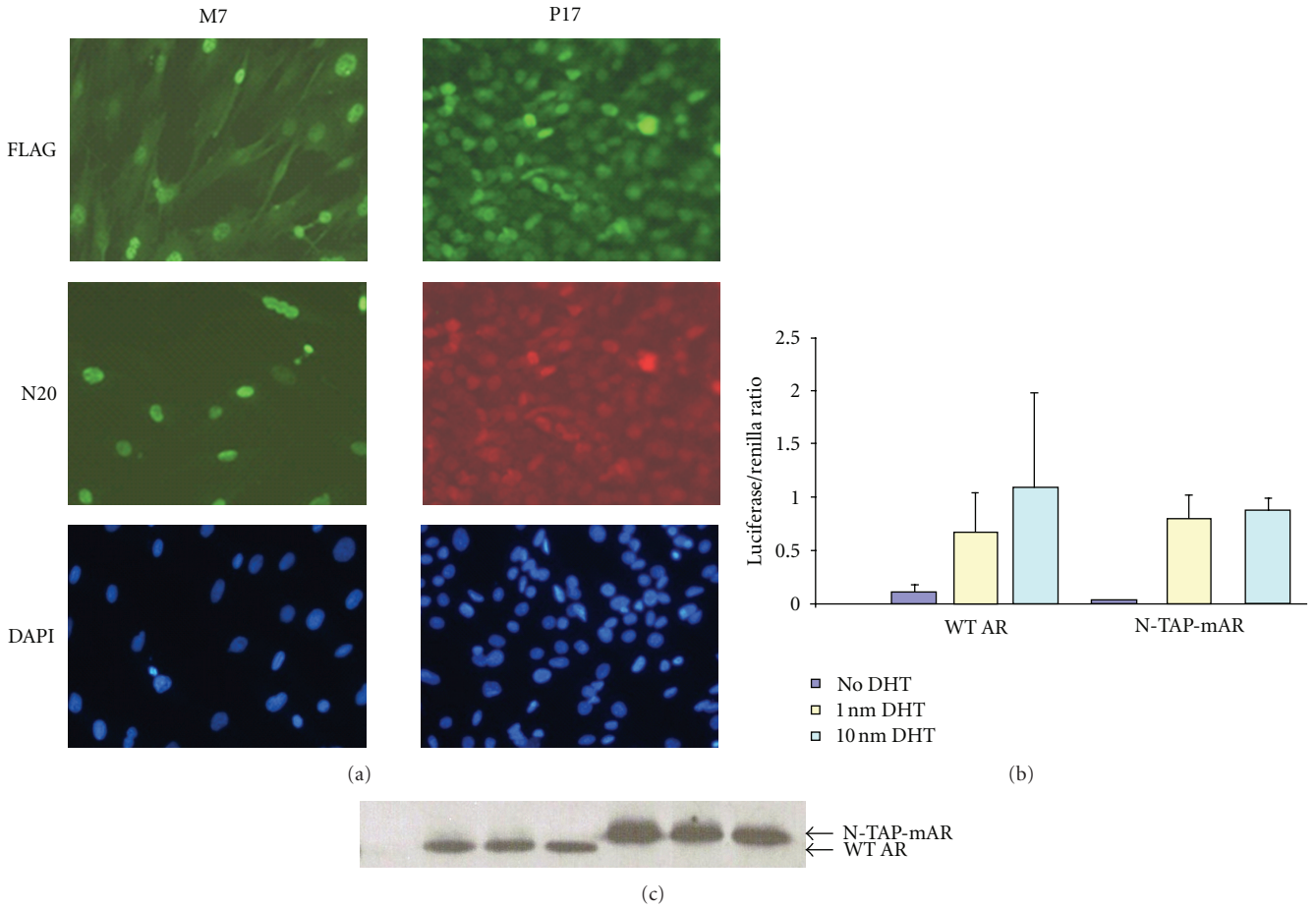

Figure 2: Nuclear localization and transactivation ability of N-TAP-mAR. (a) Immunostaining of a stable mesenchymal cell line M7 and a stable epithelial cell line P17 expressing N-TAP-mAR. The FLAG antibody detects tagged androgen receptor only. N20 antibody recognises both endogenous AR and N-TAP-mAR. Nuclei staining with DAPI indicates the percentage of cells expressing NTAP-mAR. (b) The ability of the modified N-TAP-mAR and WT-AR to activate a glucocorticoid response element (GRE) was assayed in COS-1 cells in a transient transfection luciferase assay. GRE promoter activation, shown here on the *y*-axis as luciferase/renilla ratio, was similar for the tagged and nontagged androgen receptor. (c) Tagged androgen receptor expression (N-TAP-mAR) was elevated in the Western blot done with protein extracts from the transactivation samples relative to WT.

extract from proliferating cells and 1456 from nonproliferating cells. Of those 882 were common between those two groups, 314 were only picked up in FLAG purifications from proliferating cells and 574 were only picked up in FLAG purifications from nonproliferating cells. Functional profiling was carried out using web tools "DAVID" [6, 7] and "g : profiler" [8], providing functional enrichments in the form of pathways, biological processes, molecular functions, metabolic functions, cellular localization, protein-protein interactions, and shared transcription factor binding sites. Only pathways and biological processes which received the highest scores are utilized.

3.2. Analysis of Gene Lists with DAVID Bioinformatics Resources [6, 7].

37°C. The gene list of known AR-interacting proteins identified in the nuclear FLAG purifications of the 37°C samples (37 only + common, Table 1) was accepted as 65 DAVID ID's using DAVID bioinformatics resources [6, 7]. Gene ontology tool "GOTERM_BP_FAT" gives the highest score to the biological process (BP) "regulation of transcription" with 40 contributing genes (Table 2). Another high scoring biological process "chordate embryonic development" listed 11 genes: NCOR2, SMARCA4, AR, PSMC3, PRKDC, KDM1A, TRP53, and EP300 common to both temperatures and MED1, NF1, and SP1 specific to the 37°C samples (Table 4). When analyzing the gene list for pathways, 10 genes are components of the Kegg Pathway: pathways in cancer: EP300, AR, CTNNB1, DAPK3, HSP90, PIAS1, RB1, STAT3, HDAC1, and TRP53. Of these 10 genes only STAT3, RB1, and DAPK3 have inhibitory function in this pathway. 6 of those 10 genes (EP300, AR, CTNNB1, Hsp90, RB1, and TRP53) have been associated with prostate cancer (Table 3).

The BIOCARTA Chart revealed overrepresented pathways: "telomeres, telomerase, cellular aging, and immortality", with 5 genes (XRCC5, XRCC6, HSP90, RB1, and

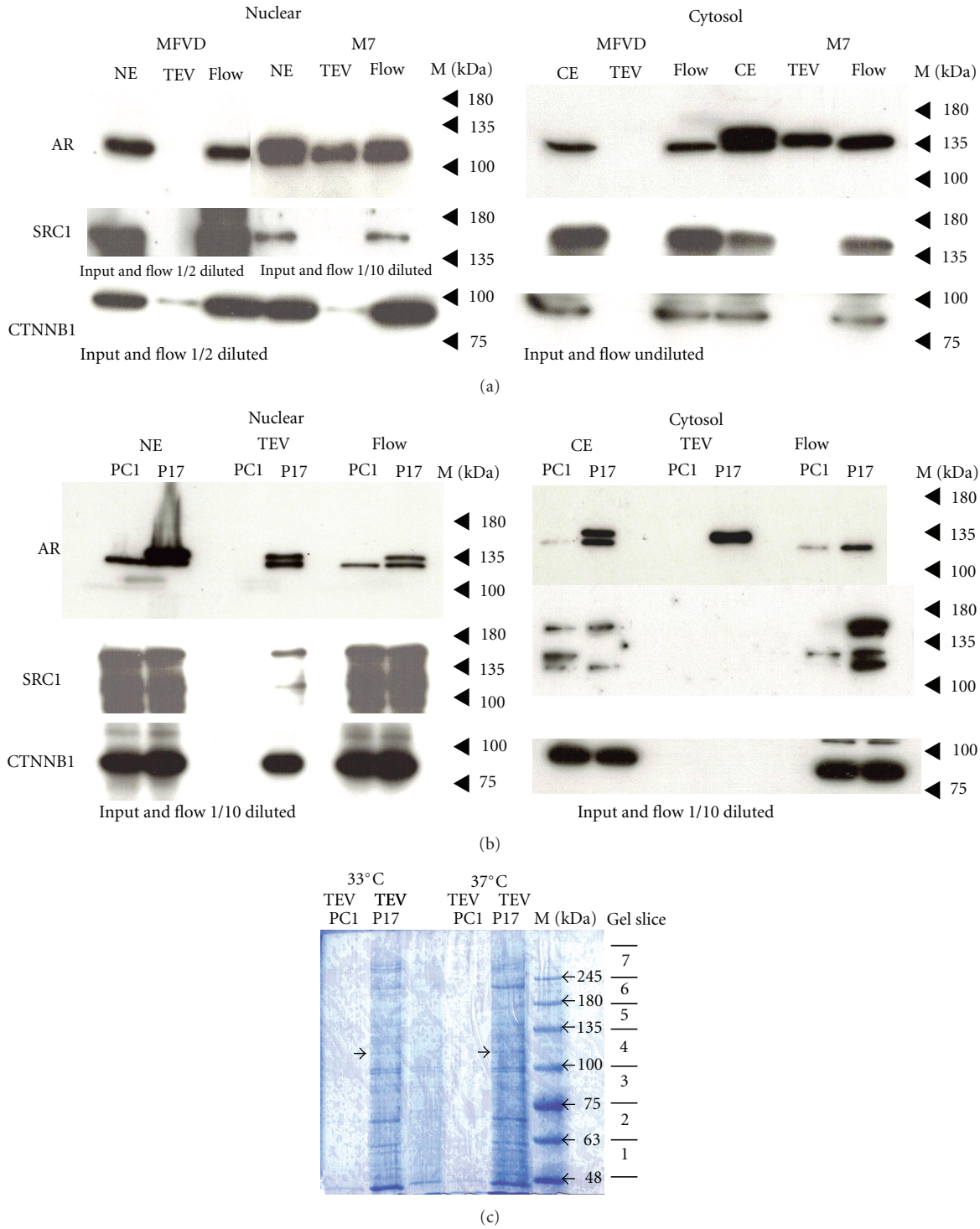

Figure 3: FLAG purifications. (a) Western blot analysis of FLAG-purified M7 nuclear and cytosol extracts using TAP negative MFVD cells as control. Compared are SRC1, beta catenin (CTNNB1), and AR expression in extracts before (NE/CE) and after (FLOW) purification and in the TEV-eluted fractions. AR (WT and N-TAP-mAR comigrate) is recovered in TEV eluate from the M7 but not from the MFVD control purifications. (b) Western blot analysis of FLAG-purified P17 nuclear and cytosol extracts using TAP negative PC1 cells as control. Compared are SRC1, beta catenin, and AR expression in extracts before (NE/CE) and after (FLOW) purification and in the TEV-eluted fractions. AR (WT and N-TAP-mAR are here distinguishable) is recovered in TEV eluate from the P17 but not from the PC1 control purifications. (c) Coomassie stained 5% PAGE loaded with TEV-eluted fractions from the control cell line PC1 and the NTAP-mAR expressing cell line P17 grown at 33°C and 37°C after FLAG purification. This gel was prepared for cutting out gel slices (1–7), which were then submitted for mass spectrometry. The arrows indicate the N-TAP-mAR migration. M is the size estimate by a prestained protein ladder in kilo Daltons (kDa).

Table 1: List of only the known androgen-receptor-interacting proteins and coregulators (http://androgendb.mcgill.ca) identified after mass spec analysis of all gel fractions (Figure 3(a)) from FLAG purifications of nuclear extracts from P17 cells grown under proliferating and nonproliferating conditions (see Section 2). (a) Listed are the interacting proteins that were unique for the proliferating cells (33 only), interacting proteins that were unique for the nonproliferating cells (37 only), and (b) interacting proteins common to both (common). The AR is highlighted. Given are the Uniprot accession numbers identified by the database Mascot search (Uniprot), the score achieved in the peptide summary report (score), the gel slice number (), and information whether the protein had been identified as a coactivator (CoA), corepressor (coR), or the effect has not been reported (—). Activating function has been allocated to GSN [13, 14], without distinguishing between the 2 isoforms [15].

(a)

33 only	coA/coR	Score: P17/*PC1* (slice)	Uniprot	37 only	coA/coR	Score: P17 (slice)	Uniprot
KDM3A	CoA	52 (3)	Q6PCM1	KDM5B	coA	93 (5)	Q80Y84
WHSC1	CoA	252 (5)	Q8BVE8	DAPK3	coA	67 (1)	O54784
MED17	CoA	66 (2)	Q8VCD5	PIAS1	coA/CoR	38 (2)	O88907
MED24	CoA	156 (3)	A6PW47	MED1	coA	44 (6)	Q925J9
ARID1B	CoA	38 (6)	E9Q4N7	GATA3	coA	55 (1)	P23772
SRC	—	57 (1)	Q2M4I4	SP1	coA	81 (3)	O89090
SMARCC1	*CoA*	*1112/60* (5)	Q3UNN4	TGFBI1	coA	32 (1)	Q62219
SMARCD1	CoA	39 (1)	Q61466	TRIM24	coA	165 (4)	Q64127
NPM1	CoA	438 (6)	Q5SQB0	FKBP5	coA	29 (1)	Q64378
GTF2F1	—	89 (2)	Q3THK3	RANBP10	coA	53 (2)	Q6VN19
				CALCOCO1	coA	156 (3)	Q8CGU1
				PSPC1	coA	35 (2)	Q8R326
				GAK	coA	397 (5)	Q99KY4
				NCOA2	coA	68 (5)	Q61026
				NR3C1	coR	97 (3)	Q06VW2
				AP-1	coA/CoR	56 (2)	Q3TXG4
				RB1	coA	55 (5)	Q3URY9
				NF1	CoA	46 (7)	Q04690-1

(b)

Common	coA/coR	Score 33°C: P17/*PC1* (slice)	Uniprot	Score 37°C: P17 (slice)
STAT3	CoA	41 (3)	P42227	90 (3)
DAXX	CoR	66 (4)	Q3UIV3	70 (4)
ARID1A	CoA	107 (5)	A2BH40	78 (5)
HDAC1	*CoR*	*428/52* (2)	*O09106*	251 (2)
BRD7	CoR	169 (3)	O88665	50 (3)
HSP90	*CoR*	*719/250* (3)	*P07901*	1089 (3)
GSN isoform1 [15]	CoA ?	542 (4)	P13020-1	516 (4)
GSN isoform2 [15]	*CoA ?*	*1879/624* (3)	*P13020-2*	1688 (3)
CALR	CoR	148 (1)	P14211	612 (1)
HSPA1B	CoA	434 (2)	P17879	582 (2)
AR	CoA	115 (4)	P19091	200 (4)
XRCC6	*CoA*	*1208/85* (2)	*P23475*	1380 (2)
MCM3	CoA	728 (3)	P25206	1476 (3)
XRCC5	*CoA*	*1727/49* (3)	*P27641*	1717 (3)
PA2G4	*CoR*	*89/257* (1)	*P50580*	54 (1)
ACTN4	*CoA/CoR*	*3147/3229* (3)	*P57780*	3112 (3)
ACTB	CoA	1386 (1)	P60710	1106 (1)
DNAJA1	CoR	173 (1)	P63037	371 (1)
PRKDC	CoA	1134 (7)	P97313	739 (7)
CTNNB1	*CoA*	*242/44* (3)	*Q02248*	242 (3)
SMARCA4	*CoA*	*1841/73* (6)	*Q3TKT4*	1449 (6)

(b) Continued.

Common	coA/coR	Score 33°C: P17/*PC1* (slice)	Uniprot	Score 37°C: P17 (slice)
MYST2	CoR	437 (2)	Q5SVQ0	36 (2)
KHDRBS1	*CoR*	*137/100* (2)	*Q60749*	274 (2)
DDX5	*CoA*	*1099/753* (2)	*Q61656*	1150 (2)
SMARCA2	CoA	727 (6)	Q6DIC0	789 (6)
PPP2R1A	CoR	433 (1)	Q76MZ3	97 (1)
RBM14	—	*232/201* (2)	*Q8C2Q3*	373 (2)
COBRA1	CoR	50 (6)	Q8C4Y3	40 (6)
ATAD2	CoA	405 (5)	Q8CDM1	2221 (5)
KIAA1967	CoA	39 (4)	Q8VDP4	92 (4)
SFPQ	*CoA/CoR*	*766/446* (3)	*Q8VIJ6*	490 (3)
PRPF6	CoA	259 (3)	Q91YR7	218 (3)
NONO	*CoA/CoR*	*625/197* (1)	*Q99K48*	472 (1)
PELP1	CoA	261 (5)	Q9DBD5	504 (5)
SART3	CoR	286 (4)	Q9JLI8	863 (4)
NCOR2	CoR	107 (7)	Q9WU42	95 (7)
PSMC3	CoA	148 (1)	A2AGN7	247 (1)
EHMT2	CoA	66 (5)	A2CG76	282 (5)
KDM1A	CoA	210 (4)	A3KG93	100 (4)
ZFP318	CoR	35 (3)	B0V2M3	30 (3)
EP300	CoA	31 (7)	B2RWS6	107 (7)
FLNA	*CoA/CoR*	*6598/1307* (7)	*B7FAU9*	6972 (7)
BRD8	CoA	62 (5)	Q8R3B7	164 (5)
SUPERVILLIN	*CoA*	*53/44* (6)	*Q8K4L3*	439 (6)
DDX17	—	*584/336* (2)	*Q3U741*	645 (2)
HDAC6	CoA	99 (4)	Q3UG37	142 (4)
TRP53	*CoA*	*1026/71* (1)	*Q80ZA1*	1090 (1)
SRCAP	CoA	57 (7)	Q8BKT0	82 (7)

TRP53) involved. Another overrepresented pathway "chromatin remodeling by hSWI/SNF ATP-dependent complexes" involves genes SMARCA4, ACTB, NR3C1, NF1, ARID1A. A third overrepresented BIOCARTA pathway is "control of gene expression by vitamin D receptor" with EP300, SMARCA4, MED1, NCOA2, and ARID1A involved (Table 3). All 3 pathways do not include the androgen receptor.

33°C. The Gene list of known AR-interacting proteins identified in the nuclear FLAG purifications of the 33°C samples (33 only + common, Table 1) was accepted as 56 DAVID ID's using DAVID bioinformatics resources [6, 7]. ARID1B was not detected as DAVID ID and therefore not included in the analysis. As with the samples from 37°C,the gene ontology tool "GOTERM_BP_FAT" gives the highest score to the biological process (BP) "regulation of transcription" with 34 contributing genes (Table 2). Looking at overrepresented pathways only 7 AR-interacting proteins are components of the Kegg pathway "pathways in cancer": EP300, AR, CTNNB1, HSP90, STAT3, HDAC1, and TRP53. Proteins RB1, DAPK3, and PIAS1 are not present in the 33°C samples (Table 3). Following on from that, only 5 of those genes have been associated with prostate cancer (EP300, AR, CTNNB1, HSP90, and TRP53) and the inhibitory function of RB1 is missing (Table 3).

Again the BIOCARTA Chart brings up as overrepresented pathways: "telomeres, telomerase, cellular aging, and immortality", but here with only 4 genes being involved (XRCC5, XRCC6, HSP90, and TRP53) and RB1 missing. "Chromatin remodeling by hSWI/SNF ATP-dependent complexes" has NR3C1 and NF1 missing and gained 3 new components with ARID1B, SMARCC1, and SMARCD1. SMARCA4, ACTB, and ARID1A are present. The pathway "control of gene expression by vitamin D receptor" has now 5 components present EP300, SMARCA4, but not MED1 and NCOA2 anymore. ARID1A is still present and the 2 new components SMARCC1 and SMARCD1.

3.3. Analysis of Gene Lists with g:Profiler [8]. Identical gene lists submitted to DAVID were also submitted to the gene ontology online tool g:Profiler for functional characterization. "Regulation of transcription" was not a high scoring biological process" on this occasion, whereas "gene

Table 2: Gene lists representing the biological processes (BP) "regulation of transcription" and "gene expression" identified by the bioinformatics tools "DAVID" and "g-profiler" as being overrepresented among the known AR-interacting proteins of the N-TAP-mAR purification. Listed are the interacting proteins, the AR is in bold face, that were unique for the proliferating cells (33), interacting proteins that were unique for the nonproliferating cells (37), and interacting proteins common to both (common).

Regulation of transcription (BP) DAVID			Gene expression (BP) g-profiler		
33	Common	37	33	Common	37
KDM3A	EP300			EP300	
	SMARCA4			SMARCA4	
	AR			**AR**	
WHSC1		MED1	WHSC1		MED1
	CTNNB1			CTNNB1	
MED17		NR3C1	MED17		NR3C1
	PRPF6			PRPF6	
	KDM1A			KDM1A	
MED24		SP1	MED24		SP1
	TRP53			TRP53	
	MYST2			MYST2	
	DDX5		SRC	DDX5	
	DAXX			DAXX	
SMARCC1		GATA3	SMARCC1		GATA3
	RBM14		SMARCD1	RBM14	
	BRD7		NPM1	BRD7	
GTF2F1		CALCOCO1	GTF2F1		CALCOCO1
	EHMT2			EHMT2	
	FLNA			FLNA	
		KDM5B			KDM5B
	NONO			NONO	
	NCOR2			NCOR2	
		NCOA2			NCOA2
	PA2G4			PA2G4	
		RB1			RB1
	STAT3			STAT3	
	HDAC1			HDAC1	
	SFPQ			SFPQ	
		TRIM24			TRIM24
	HDAC6			PRKDC	
	COBRA1			CALR	
	ATAD2			XRCC6	
	MCM3			SART3	
	BRD8				
	SMARCA2				
	KHDRBS1				
	ZFP318				
		TGFB1l			
		PSPC1			
		PIAS1			

Table 3: Top half: gene lists representing the BIOCARTA pathways "telomeres, telomerase, cellular aging, and immortality", "chromatin remodeling by hSWI/SNF ATP-dependent complexes" and "control of gene expression by vitamin D receptor" found to be overrepresented among the known AR-interacting proteins identified in the N-TAP purifications by the bioinformatics tool DAVID. Bottom half: gene lists representing the "Kegg pathway" "pathways in cancer", and "prostate cancer" identified as being overrepresented among the known AR-interacting proteins identified in the N-TAP purifications by the bioinformatics tool "DAVID" and "g-profiler". Listed are the interacting proteins that were unique for the proliferating cells (33), interacting proteins that were unique for the nonproliferating cells (37), and interacting proteins common to both (common). The AR is bolded.

Cellular aging, telomeres, immortality (DAVID)			Chromatin remodelling by SWI/SNF ATP-dependent complexes (DAVID)			Control of gene expression by vitamin D receptor (DAVID)		
33	Common	37	33	Common	37	33	Common	37
							EP300	
					NR3C1			MED1
				ARID1A			ARID1A	
	HSP90		ARID1B					
			SMARCD1			SMARCD1		
		RB1	SMARCC1			SMARCC1		
	XRCC5			SMARCA4			SMARCA4	
	XRCC6			ACTB				NCOA2
	TRP53				NF1			

pathways in cancer DAVID			pathways in cancer (g-profiler)			prostate cancer (DAVID)			prostate cancer (g-profiler)		
33	common	37	33	common	37	33	common	37	33	common	37
	EP300			EP300			EP300				
	AR			**AR**			**AR**			**AR**	
	CTNNB1			CTNNB1			CTNNB1			CTNNB1	
		DAPK3			DAPK3						
	HSP90			HSP90			HSP90			HSP90	
		PIAS1			PIAS1						
		RB1						RB1			
	STAT3			STAT3							
	HDAC1			HDAC1							
	TRP53			TRP53			TRP53			TRP53	

expression" scored high at both temperatures, with many genes overlapping in both categories (Table 2). Also here the Kegg pathway components for prostate cancer are revealed for both temperatures, but only 4 (HSP90, AR, CTNNB1, and TRP53) as shown in Table 3.

37°C. Kegg pathway: "pathways in cancer" is only detected in the 37°C samples, with 9 components represented (EP300, STAT3, HDAC1, HSP90, AR, CTNNB1, TRP53, DAPK3, and PIAS1). The gene ontology subgroups for "biological processes" (BPs), "anatomical structure morphogenesis", and "embryo development" are overrepresented in the 37°C samples and overlapping components are involved (Table 4). "Embryo development" components are NCOR2, AR, CTNNB1, KDM1A, PRKDC, SP1, MED1, SMARCA4, HDAC1, TRP53, TGFB1l, PSMC3, and NF1. "Anatomical structure morphogenesis" components are NCOR2, AR, CTNNB1, ACTB, PRKDC, SP1, MED1, SMARCA4, HDAC1, TRP53, TGFB1l, GSN, NF1, GATA3, STAT3, NR3C1, and STAT3. Comparison of these 2 groups suggests a more specific function in morphogenesis during embryo development for NR3C1, GATA3, and STAT3 (strong evidence) GSN and ACTB (weak evidence).

33°C. Among cellular component profiling, the nBAF complex is solely detected in the 33°C samples with SMRCC1, SMARCD1, SMRCA4, and ARID1A (not listed in tables). These proteins are also components of the SWI/SNF chromatin remodelling complex.

3.4. Western Blot Analysis of Cofactors Identified Only in Proliferating or Only in Nonproliferating PC1 Cells. The observation that some AR-interacting proteins were only detectable in proliferating cells and others only in nonproliferating cells (Table 1) could simply mean that the quality of the cell extracts varies and much less protein is present in one of the extracts. Another reason might be that expression levels of those proteins alter during cell proliferation, and less or more protein is available for interaction with AR. A third option is that the binding affinity of AR to the interacting proteins changes with the proliferation status of the cells. To address these questions, we carried out Western

Table 4: Gene lists for the biological processes (BP) "chordate embryonic development" and "embryo development" found to be overrepresented among the known AR-interacting proteins identified in the N-TAP purifications from the 37°C samples but not the 33°C samples by the bioinformatics tool "DAVID" and "g-profiler". Listed are the interacting proteins that were unique for the nonproliferating cells (37) and interacting proteins common to both (common). The AR is bolded. AR-interacting proteins identified only in the 33°C samples are not involved in biological processes addressed here. No counterpart for the biological process "anatomical structure morphogenesis" could be identified by DAVID.

Chordate embryonic Development (BP) DAVID			Embryo development (BP) g-profiler			Anatomical structure morphogenesis (BP) g-profiler		
33	Common	37	33	Common	37	33	Common	37
	NCOR2			NCOR2			NCOR2	
	SMARCA4			SMARCA4			SMARCA4	
	AR			**AR**			**AR**	
		MED1			MED1			MED1
		NF1			NF1			NF1
		SP1			SP1			SP1
	PSMC3			PSMC3				
	PRKDC			PRKDC			PRKDC	
	KDM1A			KDM1A				
	TRP53			TRP53			TRP53	
				CTNNB1			CTNNB1	
					TGFBI1			TGFBI1
								NR3C1
				HDAC1			HDAC1	
							GSN	
							GATA3	
	EP300						ACTB	
							STAT3	

blots, probing those AR-interacting proteins which were differentially expressed (Figure 4).

No major differences in expression were observed when extracts were probed with AR-N20 and ACTB control antibodies, but differences were found elsewhere. NR3C1 is expressed at much higher levels in nonproliferating versus proliferating P17 cells, and expression levels of SMARCC1 were slightly lower in nonproliferating (37°C) PC1/P17 when compared to proliferating cells (33°C). The difference in the amounts of AR copurified SMARCC1 in proliferating versus nonproliferating is not as striking as it is for NR3C1, and the amounts at "low expression" temperatures are probably too small to be detected by mass spectroscopy. The proliferation status of P17 cells seems to affect SMARCC1 and NR3C1 expression levels per se as SMARCC1 expression is up and NR3C1 expression is down in proliferating cells. However, the loss of NR3C1 expression in proliferating cells is unlikely to account for all the loss in AR binding observed here. Similar loss of SMARCC1 expression in nonproliferating cells does not account for all the loss in AR binding detected. Therefore, expression levels of coregulators and binding affinity to AR may contribute to the differences observed in the 33°C and 37°C coregulator profiles. The N-TAP-mAR itself appears to increase GR expression levels by at least 3 fold under nonproliferating conditions comparing GR expression in PC1 and P17 input samples (Figure 4).

4. Discussion

We have developed the epithelial and mesenchymal mouse cell lines P17 and M7 for copurification of AR-associated protein complexes under native conditions. The two cell lineages are derived from the proximal caput epididymus (P17) and the mesenchyme of the fetal vas deferens (M7) of the mouse. The decision to carry out preparative scale N-TAP-mAR purification on PC1 cells rather than MFVD cells was based on the observation that CTNNB1 and SRC-1 could not be copurified from MFVD cells with our extraction protocol, whereas in P17 copurifications both coactivators were easily detected. Another advantage of choosing the PC1 cell line was the more abundant AR expression and the rapid growth compared to the mesenchymal cells. One confluent dish of PC1 gave about 5–10 times as much cell pellet than 1 confluent dish of MFVD.

To demonstrate proof of principle we used the newly developed purification protocol to detect differences in AR cofactor binding in cells grown under proliferating (33°C) and nonproliferating (37°C) conditions. As expected, many of the copurified proteins confirm the role of the AR in chromatin remodelling machinery, transcriptional complexes and associated with the cytoskeleton. The purification protocol we use is relatively crude and results in enrichment of 200–500 bp DNA fragments after a micrococcal nuclease

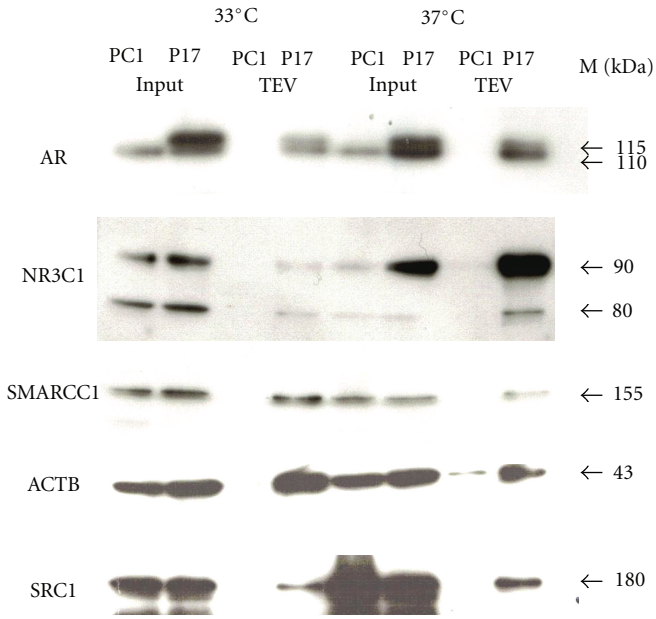

Figure 4: AR interacting proteins SMARCC1 and NR3C1 are differentially expressed in proliferating versus nonproliferating P17 cells. Western blot analysis of known AR coregulators identified by mass spectrometry only in AR FLAG copurifications from proliferating P17 cells (SMARCC1) and of a known coregulator identified by mass spectrometry only in AR FLAG copurifications from nonproliferating P17 cells (NR3C1). SRC1 was not identified at all, neither in purifications from proliferating nor nonproliferating P17 cells. AR and ACTB were identified in both FLAG copurifications from P17 cells by mass spectrometry. Compared to the other identified known AR interacting proteins NR3C1, SMARCC1, and ACTB, a smaller portion of the total SRC1 present in the nuclear extract (P17 Input) interacts with AR (P17 TEV). M is the size estimate by a prestained protein ladder in kilo Daltons (kDa).

digestion. The isolation protocol is nondenaturing and keeps the chromatin fraction as intact as possible. No size fractionation step is included, which could allow isolation of larger chromatin fractions, especially heterochromatic fractions which are not degraded by micrococcal nuclease. This fraction could contribute to a background of unspecific binding, which is difficult to control for as this might not occur in the PC1 control sample, where the "anchor" in form of the N-TAP-mAR is missing. On the other hand, the purification protocol is selecting for stable interactions, because no cross-linking step is included in our purification procedure.

We have not tested whether novel interacting proteins identified with this approach are indeed associated with AR or whether they are just contaminants. These potential interacting proteins could be novel AR-interacting proteins, but could also bind unspecific to the FLAG-M2-coupled magnetic beads. Unspecific binding to the FLAG antibody-coupled magnetic beads is estimated at 5% based on the recovery of protein from the FLAG purification of the PC1 control sample. This would mean that 1 out of 20 identified proteins is not part of the AR-associated complex. Unfortunately we were not able to reduce this background with an additional His purification step, because the FLAG purified and protease (TEV) eluted fraction was not able to specifically bind Nickel resin, probably caused by complex components covering up the His tag. In this study we concentrate only on a small proportion of all the potential AR-binding partners identified, representing the already known AR-interacting proteins. We show that our approach has the potential to differentiate between proteins which preferably form part of the AR complexes in proliferating or nonproliferating conditions, and those proteins where interaction is independent of the proliferation status of the cells.

4.1. Functional Enrichments among the Known AR Cofactors Identified with Two Different Bioinformatics Resources. Common pathways enriched for or overrepresented in proliferating and nonproliferating AR FLAG purifications were the biological processes "transcriptional regulation" and "gene expression". Considering that the androgen receptor is classified as a transcription factor, high scores in those categories are expected. Scoring surprisingly high were BIOCARTA pathways not involving AR at all such as "chromatin remodelling by hSWI/SNF ATP-dependent complexes" and "control of gene expression by vitamin D receptor (VDR)" (Figure 5), which involves the WINAC chromatin-remodelling complex. Both WINAC and SWI/SNF complexes have BAF components (Brg1-associated factors). The requirement of the BAF complexes has been shown *in vitro* for ligand-dependent transactivation by nuclear hormone receptors, such as vitamin D3 receptor, retinoid X receptor, and peroxisome proliferator-activated receptor PPAR-γ [16]. It has also been shown *in vivo* for reconstitution of glucocorticoid-receptor-(NR3C1-)

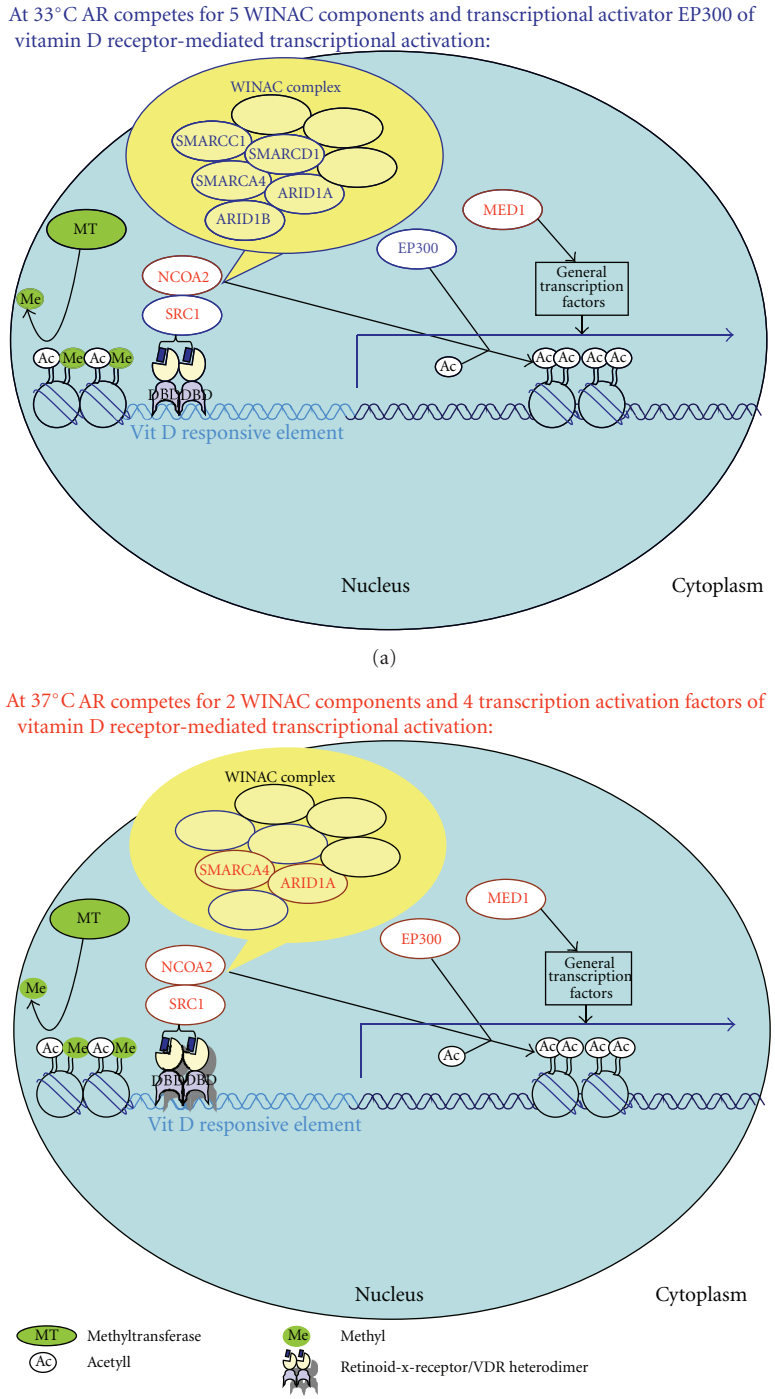

Figure 5: AR-interacting proteins are WINAC complex components and transcriptional activators of VDR-mediated gene expression. Presented is a simplified version of the BIOCARTA pathway "control of gene expression by Vitamin D receptor (VDR)" illustrating how AR and VDR may compete for shared coregulators. (a) In AR copurifications from proliferating P17 cells (33°C), the transcriptional activators EP300, SRC1*, and 5 proteins from the ATP, dependent chromatin remodelling complex WINAC: SMARCA4, ARID1A, ARID1B, SMARCC1, and SMARCD1 were identified. Under proliferating conditions, AR would therefore compete with VDR for 5 WINAC components and for 2 transcriptional activators. (b) In copurifications from nonproliferating P17 cells (37°C) only 2 known AR-interacting proteins identified were components from the WINAC complex: SMARCA4 and ARID1A. However, 4 proteins identified: EP300, NCOA2, MED1, and SRC1* act as transcriptional activators in VDR, mediated gene expression. Under nonproliferating conditions, AR would therefore compete with VDR for 2 WINAC components and 4 transcriptional activators. *SRC1 is a general transcription activator for steroid receptors and also component of this pathway. SRC1 was not identified by mass spectrometry, but AR-associated in Western blots of FLAG-purifications from nuclear extracts of proliferating and nonproliferating P17 cells (Figures 4 and 3(b)).

dependent transcription [17], chromatin remodelling on interferon and virus inducible genes [18] and in neural development with a subunit switch in the npBAF (neural progenitors-specific) chromatin remodelling complex, essential for the transition from neural stem/progenitors to postmitotic neurons [19] but has never been associated with AR. Also the Vitamin D3 receptor has not been identified as AR-interacting protein neither in our purification (data not shown) nor by others. It is however possible that AR transactivation might be stimulated by components of the WINAC complex. The VDR and the AR could therefore compete for shared coregulators, which would explain the observation that AR stimulation by androgens suppresses VDR [20, 21], while AR downregulation by siRNA stimulates VDR levels in LnCAP cells [21]. WINAC complex components might be potential coplayers in AR transactivation, which could be tested with siRNA cotransfection experiments in our cell line. G-profiler, which does not offer a tool such as the BIOCARTA, also identified BAF components as being overrepresented. Another overrepresented pathway is the prostate cancer pathway, which is picked up by both bioinformatics tools. G-profiler does not identify EP300 as a gene involved in prostate cancer as it is done by DAVID (Table 3 bottom). Also the "Kegg cancer pathway" is picked up by both bioinformatics tools: g-profiler identifies the same list of genes among the known AR-interacting proteins as "DAVID", only that RB1 is not included in the g-profiler gene list (Table 3 bottom).

For male genital development and PAIS relevant biological processes are "embryo development", which is here represented with 13 genes identified by g-profiler and 11 identified by DAVID. Of those 10 are overlapping (Table 4). The embryonic genes identified by DAVID are restricted to chordate development and do not include CTNNB1, HDAC1 and TGFBI1, although CTNNB1 and HDAC1 "knock outs" in mouse have been shown to result in developmental phenotypes [22, 23]. G-profiler did not pick up EP300. A role of EP300 in patterning and development was suggested by studies in mice in which EP300 expression was disrupted [24]. Both bioinformatic tools David and g-profiler complemented each other in this study in identifying androgen receptor regulated pathways and biological processes.

In future we aim to replace the endogenous AR with N-TAP-mAR. We will hopefully be able to apply the FLAG purification protocol tested in this study to identify differences in the proteome caused by the respective AR mutation. The protein purification approach taken here and shown to identify differences in co-factor recruitment of AR under proliferating and nonproliferating conditions is encouraging to undertake further experiments aiming to identify specific interaction protein profiles for AR mutants associated with PAIS.

Conflict of Interests

The authors declare that they have no competing interests.

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Research Article

Checklist for the Structural Description of the Deep Phenotype in Disorders of Sexual Development

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This paper addresses the question, how the variations of the deep phenotype in disorders of sex development (DSD) are appropriately described. This is a relevant question, because extensive phenotypic variability occurs in gonads and sex ducts. With the advance of video endoscopy and laparoscopy, fresh insight in gonadal and sex duct anatomy is emerging. So far, an attempt to standardize the diagnostic approach and, in particular, how to document these findings has not been published. We propose a standardized examination schedule for these procedures. It consists of 5 pictures of relevant anatomic features. For laparoscopy, it includes two pictures each of gonads and sex ducts on either side and an image of the retrovesical space. For endoscopy, the examination of the ureteric orifices, the posterior urethra, and the urogenital sinus derivates is recommended. Adherence of a standardized schedule and image storing enhances patient autonomy, because they can carry their examination for a second opinion without need for repeated examination. Physicians and scientists create a structured image library that facilitates the comparison of clinical outcomes, research on genotype phenotype associations and may lead to better classifications.

1. Introduction

Most disorders of sexual development are characterised by a particular phenotype. Phenotypic variations of the external genitalia have stimulated numerous classification systems. The "deep" phenotype of gonads and sex ducts has not been addressed systematically. Variations of these organs can be detected only by imaging procedures or direct vision. The knowledge of these details is relevant for the clinical management. It is of diagnostic importance when mutational analysis and hormone levels are inconclusive. Gonadal anatomy may also influence the decision of taking a biopsy or performing gonadectomy. Disorders of sex development are rare, making the development of detailed classifications difficult.

Videoendoscopic examination techniques are now widely available. They permit the inspection and imaging of structures that are barely visible with noninvasive imaging procedures. Moreover, images can be stored easily and copied for the patients own purposes. These images may also facilitate the communication among members of the interdisciplinary DSD team. Informed decision making for genital surgery or gonadectomy critically depends on an understanding of how the individual's anatomy deviates from what is defined as "normal."

Furthermore, there may be a scientific interest in unravelling genotype-phenotype relations. Many new mutations leading to DSD are discovered each year. Relating these new findings to a detailed examination of the "deep" phenotypic variations may foster insight of gene function during urogenital development.

In spite of the highly variable anatomy, affected individuals and their families ask the same questions. Can the gonads be safely retained? Is there a need for surgery and what will be the result?

Gonadectomy and genital surgery are controversial issues, and part of the controversy is about diagnostic uncertainty and lack of standardized procedures. This articles offers a concept for the standardized storage of images taken during endoscopy and laparoscopy.

2. Patients and Methods

During 2002 through 2011, 68 patients with DSD underwent diagnostic evaluation at our institution.

Table 1: Patients, diagnosis, and procedures.

Diagnosis	No. of patients	Procedures
Complete androgen insensitivity	8	Genitoscopy, laparoscopy, herniorrhaphy
Complete gonadal dysgenesis	12	Genitoscopy, laparoscopy, gonadectomy
Partial gonadal dysgenesis	12	Genitoscopy, laparoscopy, genitoplasty
Defects of androgen synthesis	6	Genitoscopy, laparoscopy, genitoplasty
Ovotesticular DSD	3	Genitoscopy, laparoscopy
Congenital adrenal hyperplasia	17	Genitoscopy
Turner syndrome	3	Genitoscopy, laparoscopy
Partial androgen insensitivity	1	Genitoscopy, laparoscopy
Frazier syndrome	1	Genitoscopy, laparoscopy
Unclear diagnosis	5	Genitoscopy, laparoscopy

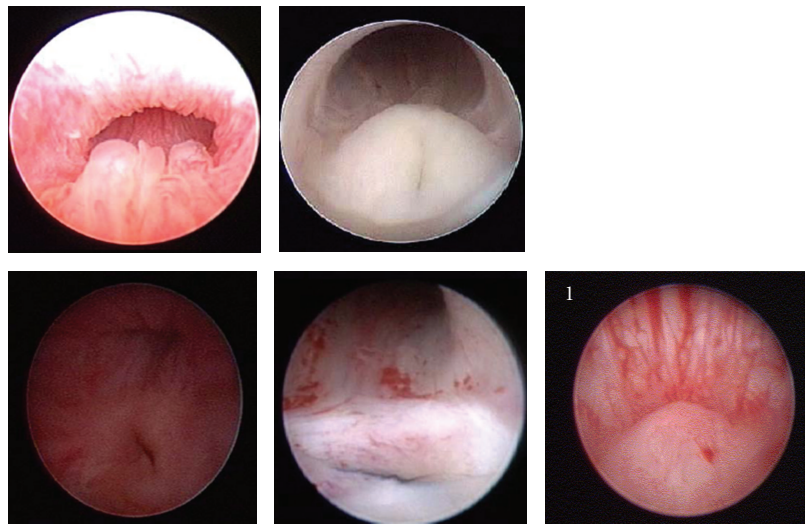


Figure 1: Posterior urethra and utriculus in patients with severe hypospadias and partial gonadal dysgenesis. Size and shape of the colliculus vary from patient to patient. The last image shows a normal colliculus for comparison. Note the different backing of the urethral wall and the difference in shape.

Our reports, images, and videos obtained during examinations of various disorders of sex development were reviewed. All patients who had either laparoscopy, endoscopy or both were included.

Cystoscopies and genitoscopies were reexamined with a focus on urethral anatomy, in particular of the posterior urethra.

Laparoscopic images were reviewed with a focus on gonadal and sex duct variations within the same disorder. Diagnosis and procedures are reassumed in Table 1.

3. Results

The review of our cystoscopies and genitoscopies showed that a different number of images were stored for each examination. Comparing the images of the posterior urethra in patients with partial gonadal dysgenesis and hypospadias, many variations in the utricular region were found. Figure 1 shows examples of these variations.

In patients with congenital adrenal hyperplasia, a similar variability was found at the entrance of the vagina to the urogenital sinus (Figure 2). In patients affected by total gonadal dysgenesis, variations of gonadal development are of particular interest. Size and shape of the gonad varied from true streaks to ovoid shape. Examples of this variability and an example of a tumour arising in a gonad are depicted in Figure 3.

While gonads in androgen insensitivity are very similar to normal testis, a marked variability in their localization and the paramesonephric (Wolffian) structures and even Mullerian structures was evident. Figure 4 shows examples of this.

These examples show convincingly that very different "deep phenotypes" may exist within a given disorder. These

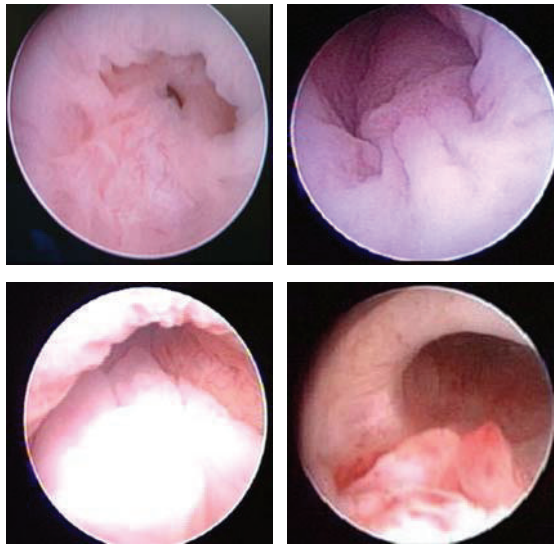


Figure 2: Posterior urethra in congenital adrenal hyperplasia. The area around the vaginal opening is represented. Mucosal folds resemble the hypoplastic verum montanum seen in patients with partial gonadal dysgenesis.

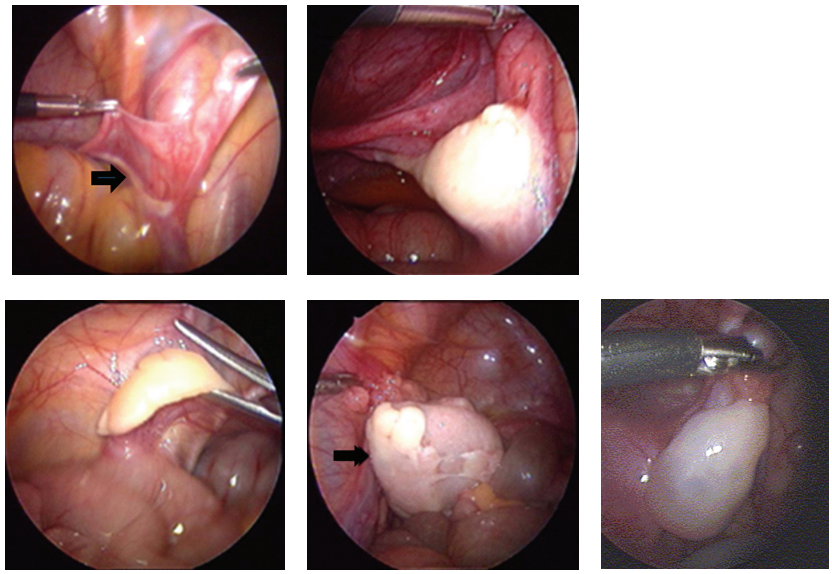


Figure 3: Variations in gonadal shape and size observed in patients with gonadal dysgenesis. The image in the top row on the left shows a streak gonad (arrow). The right lower images show a germ-cell tumour replacing a dysgenetic gonad (arrow). For comparison, a normal postpubertal ovary is shown.

observations prompted us to recommend a standardized examination schedule with a precise proposal on what images to take. Photographic views are referred to defined anatomic landmarks as shown in Figures 5 and 6.

4. Discussion

Our own experience and a review of the literature showed a wide variability of diagnostic procedures and terminology that was used to describe the deep phenotype in DSD. We focussed on endoscopy and laparoscopy, because compared to the noninvasive investigations they allow the best visualisation. However, techniques and reporting of findings are also subject to variation and we concluded that a standardization of the surgical evaluation and defining what images to store would be most useful. Based on the reevaluation of 66 patients with various DSD treated at our institution we propose the examination schedule shown above. All the

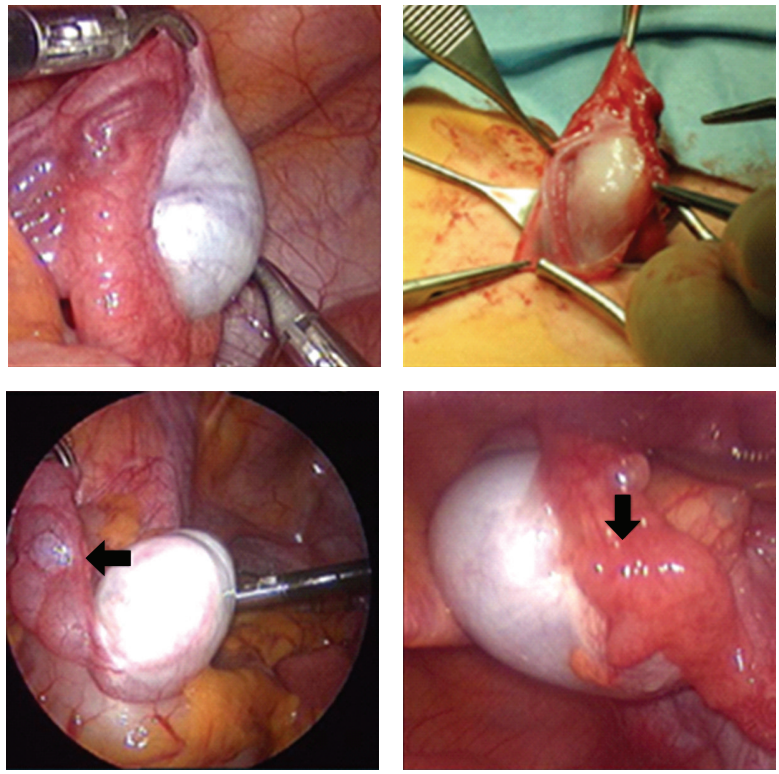
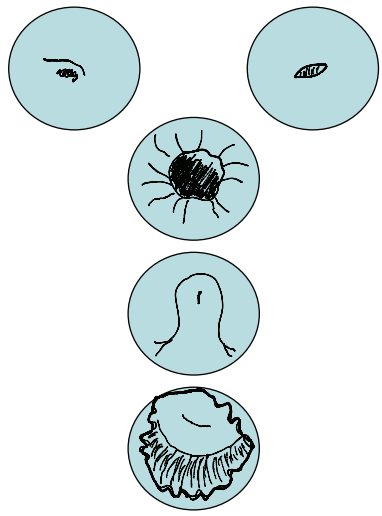


Figure 4: Patients with complete androgene insensitivity have normal appearing gonads in the abdomen or the inguinal canal. Sex duct development is highly variable. The lower panel shows cystic dilatation of an epidydimis, the right lower panel a fallopian tube close to a testis (arrow).



View centered on the ureteric orifices in the bladder. On the right, a normally shaped and placed ostium is represented. On the left, the orifice is abnormally wide.
This view should encompass the bladder neck.

Below the bladder neck, a view should be centered on the posterior urethra in virilized patients, encompassing the verum montanum.

Vaginal view centered on the cervix.

Figure 5: Standard views recommended for cystourethroscopy/genitoscopy. Two images should be taken of the ureteral orifices and one of the bladder neck. Moving downward, the bladder neck, the posterior urethra with the colliculus and the vagina/cervix should be documented.

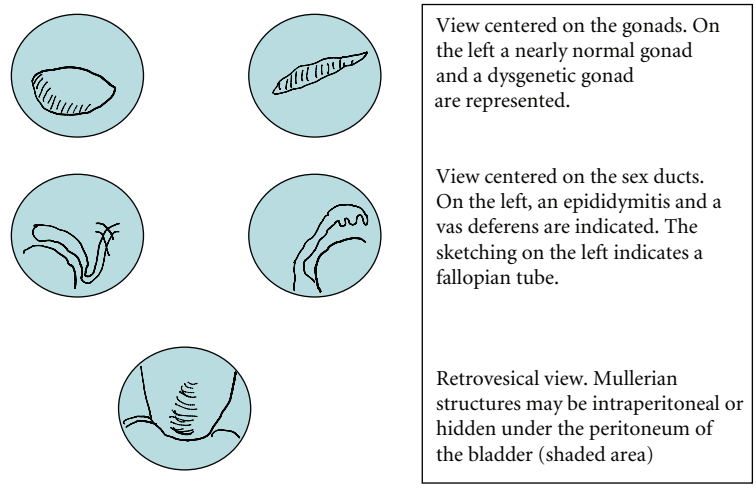


Figure 6: At laparoscopy, 2 images should be taken of each gonad and the attached sex duct. The retrovesical space should be explored for the presence an uterus or vas deferens.

views are clearly defined, refer to stable anatomic landmarks, and are easily applicable.

Storing of images is helpful for the interested individual and those who offer counselling or care. DSD requires a holistic approach, and the communication between team members is likely to be more efficient, when relevant anatomic details can be shared easily [1]. Both patients and doctors can obtain second opinions for rare or controversial clinical situations.

Documenting anatomical findings in detail and high quality may improve future outcome studies. Size, shape, and surface anatomy of the gonads are highly variable, and these factors may be relevant for the individual tumour risk. The endoscopic findings are important for the planning of genital reconstruction. The distance between bladder neck and vaginal confluens is important for vaginal reconstruction and continence [2]. Anomalies of the verum montanum are common and contribute to the influx of urine. The vaginal dimensions and relations to urethra are important for sexual function. Several noninvasive imaging procedures have been described [3], but the quality of endoscopy and laparoscopy for the visualisation of small mobile structures is unparalled [4].

For most rare conditions, outcome research is badly needed but difficult to obtain. Images taken in a standardized fashion can be pooled to create bigger patient populations. They can be analyzed applying checklists, and interobserver bias and observer bias can be addressed. Phenotypic features can be also evaluated retrospectively. This may facilitate clinical decision making, create a comparable data base for future investigations, and improve quality by eliminating ambivalent terminology. Furthermore, the definition of the normal phenotype is in continuous evolution [5]. Endoscopy and imaging techniques not only delineate pathologic findings, but also contribute a comprehensive definition of the normal phenotype and its variations.

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Review Article

Molecular Bases and Phenotypic Determinants of Aromatase Excess Syndrome

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Aromatase excess syndrome (AEXS) is a rare autosomal dominant disorder characterized by gynecomastia. This condition is caused by overexpression of *CYP19A1* encoding aromatase, and three types of cryptic genomic rearrangement around *CYP19A1*, that is, duplications, deletions, and inversions, have been identified in AEXS. Duplications appear to have caused *CYP19A1* overexpression because of an increased number of physiological promoters, whereas deletions and inversions would have induced wide *CYP19A1* expression due to the formation of chimeric genes consisting of a noncoding exon(s) of a neighboring gene and *CYP19A1* coding exons. Genotype-phenotype analysis implies that phenotypic severity of AEXS is primarily determined by the expression pattern of *CYP19A1* and the chimeric genes and by the structural property of the fused exons with a promoter function (i.e., the presence or the absence of a natural translation start codon). These results provide novel information about molecular mechanisms of human genetic disorders and biological function of estrogens.

1. Introduction

Aromatase encoded by *CYP19A1* is a cytochrome P450 enzyme that plays a key role in estrogen biosynthesis [1]. It catalyzes the conversion of Δ^4-androstendione into estrone (E_1) and that of testosterone (T) into estradiol (E_2) in the placenta and ovary as well as in other tissues such as the fat, skin, bone, and brain [1].

Overexpression of *CYP19A1* causes a rare autosomal dominant disorder referred to as aromatase excess syndrome (AEXS, OMIM no. 139300) [2–8]. AEXS is characterized by pre- or peripubertal onset gynecomastia, gonadal dysfunction, advanced bone age from childhood to pubertal period, and short adult height in affected males [2–8]. In particular, gynecomastia is a salient feature in AEXS, and, therefore, this condition is also known as hereditary gynecomastia or familial gynecomastia [5]. Affected females may also show several clinical features such as macromastia, precocious puberty, irregular menses, and short adult height [5, 6, 8].

Recently, three types of cryptic genomic rearrangements around *CYP19A1* have been identified in 23 male patients with AEXS [2–4]. The results provide useful implications not only for the clarification of underlying mechanisms but also for the identification of phenotypic determinants. Here, we review the current knowledge about AEXS.

2. The Aromatase Gene (*CYP19A1*)

CYP19A1 encoding aromatase is located on 15q21.2 adjacent to *DMXL2* and *GLDN* (Figure 1) [3, 9]. It spans ~123 kb and consists of at least 11 noncoding exons 1 and nine coding exons 2–10 [9–12]. Each exon 1 is accompanied by a tissue-specific promoter and is spliced alternatively onto a common splice acceptor site at exon 2, although some transcripts are known to contain two of the exons 1 probably due to a splice error [9–11]. Transcription of *CYP19A1* appears to be tightly regulated by alternative usage of the multiple

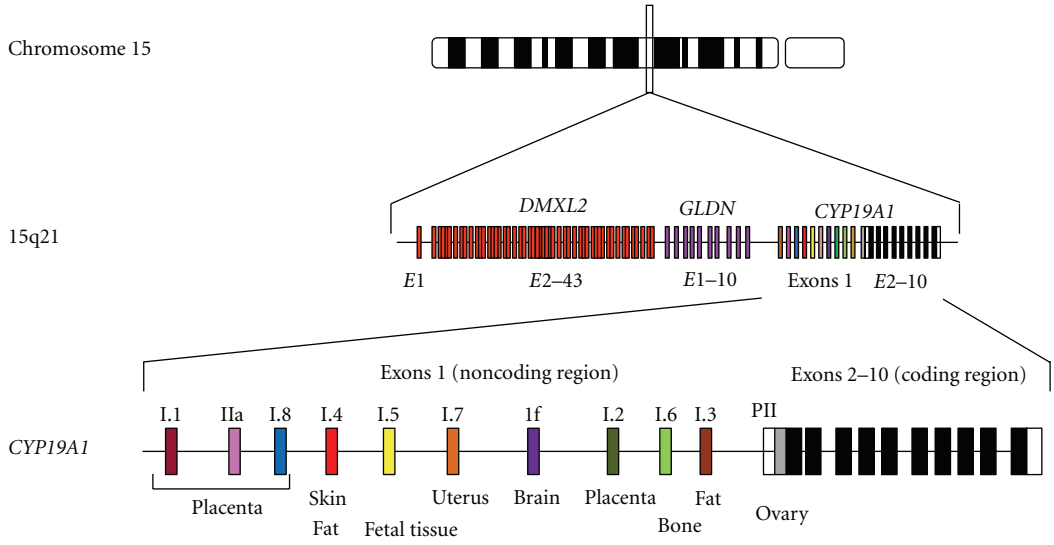

Figure 1: Simplified schematic representation indicating the genomic structure of *CYP19A1*. *CYP19A1* is located on 15q21.2 adjacent to *DMXL2* and *GLDN* and consists of at least 11 noncoding exons 1 and nine coding exons 2–10 [9, 10]. Each exon 1 is accompanied by a tissue-specific promoter and is spliced alternatively onto a common splice acceptor site at exon 2 [9–13].

promoters [9–13]. Actually, *CYP19A1* is strongly expressed in the placenta and moderately expressed in the ovary, whereas it is only weakly expressed in a rather limited number of tissues including skin, fat, and hypothalamus [4, 13]. Of the 11 noncoding exons 1, exon I.4 seems to play a critical role in the regulation of estrogen biosynthesis in males, because this exon contains the major promoter for extragonadal tissues [9, 10].

3. Molecular Bases of AEXS

A family with dominantly transmitted gynecomastia of prepubertal onset was first described in 1962 by Wallach and Garcia [14]. After this initial report, several cases have been described [5–8, 15]. Laboratory examinations of the affected males revealed markedly elevated serum estrogen values and estrogen/androgen ratios and significantly increased aromatase activity in fibroblasts and lymphocytes [5–8, 15]. Linkage analyses in two families indicated a close association between *CYP19A1*-flanking polymorphic markers and the disease phenotype [5, 6]. Thus, the condition was assumed to be caused by gain-of-function mutations of *CYP19A1,* and, therefore, the name of AEXS was coined for this condition [7, 8]. However, since direct sequencing and Southern blotting analysis failed to detect mutations or copy number abnormalities in the coding region of *CYP19A1* [5, 6], the molecular basis of this entity remained elusive until recently.

In 2003, Shozu et al. reported a father-son pair and a sporadic case with AEXS in whom they identified heterozygous chromosomal inversions of the chromosome 15 [2]. Subsequently, Demura et al. performed detailed molecular studies for these cases and additional two cases and characterized four types of inversions affecting the 5′ region of *CYP19A1* [3]. Each inversion has resulted in the formation of a chimeric gene consisting of *CYP19A1* coding exons and exon 1 of the widely expressed neighboring genes, that is, *CGNL1*, *TMOD3*, *MAPK6,* and *TLN2*. These data imply that overexpression of *CYP19A1* in the inversion-positive cases are caused by cryptic usage of constitutively active promoters. Consistent with this, *in silico* analysis revealed the presence of promoter-compatible sequences around exon 1 of *CGN1*, *TMOD3*, and *MAPK6* in multiple cell types, although such sequences remain to be identified for noncoding exons of *TLN2* [4].

We recently studied 18 males from six families with AEXS (families A–F) and identified three types of heterozygous cryptic genomic rearrangements in the upstream region of the *CYP19A1* coding exons (Figure 2) [4]. In families A and B, we identified the same 79,156 bp tandem duplication encompassing seven of the 11 noncoding exons 1 of *CYP19A1*. Notably, this duplication includes exon I.4 that functions as a major promoter for extragonadal tissues such as fat and skin; therefore, *CYP19A1* overexpression in these families would be explained by increasing the number of this promoter. Indeed, RT-PCR analysis detected a splice variant consisting of exon I.4 at the 5′ side and exon I.8 at the 3′ side in lymphoblastoid cell lines and skin fibroblasts of the patients, indicating that the duplicated exon I.4 at the distal nonphysiological position actually functions as transcription start sites. In family C, we identified a 211,631 bp deletion affecting exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*. This deletion appears to have caused *CYP19A1* overexpression because of cryptic usage of *DMXL2* exon 1 as an extra transcription start site for *CYP19A1*. Indeed, RT-PCR revealed the presence of chimeric mRNA clones consisting of *DMXL2* exon 1 and *CYP19A1* exon 2, supporting the notion that aberrant splicing has occurred between these two exons. Such *DMXL2*/*CYP19A1* chimeric mRNA accounted for 2–5% of *CYP19A1*-containing transcripts from skin fibroblasts. In families D–F, we identified

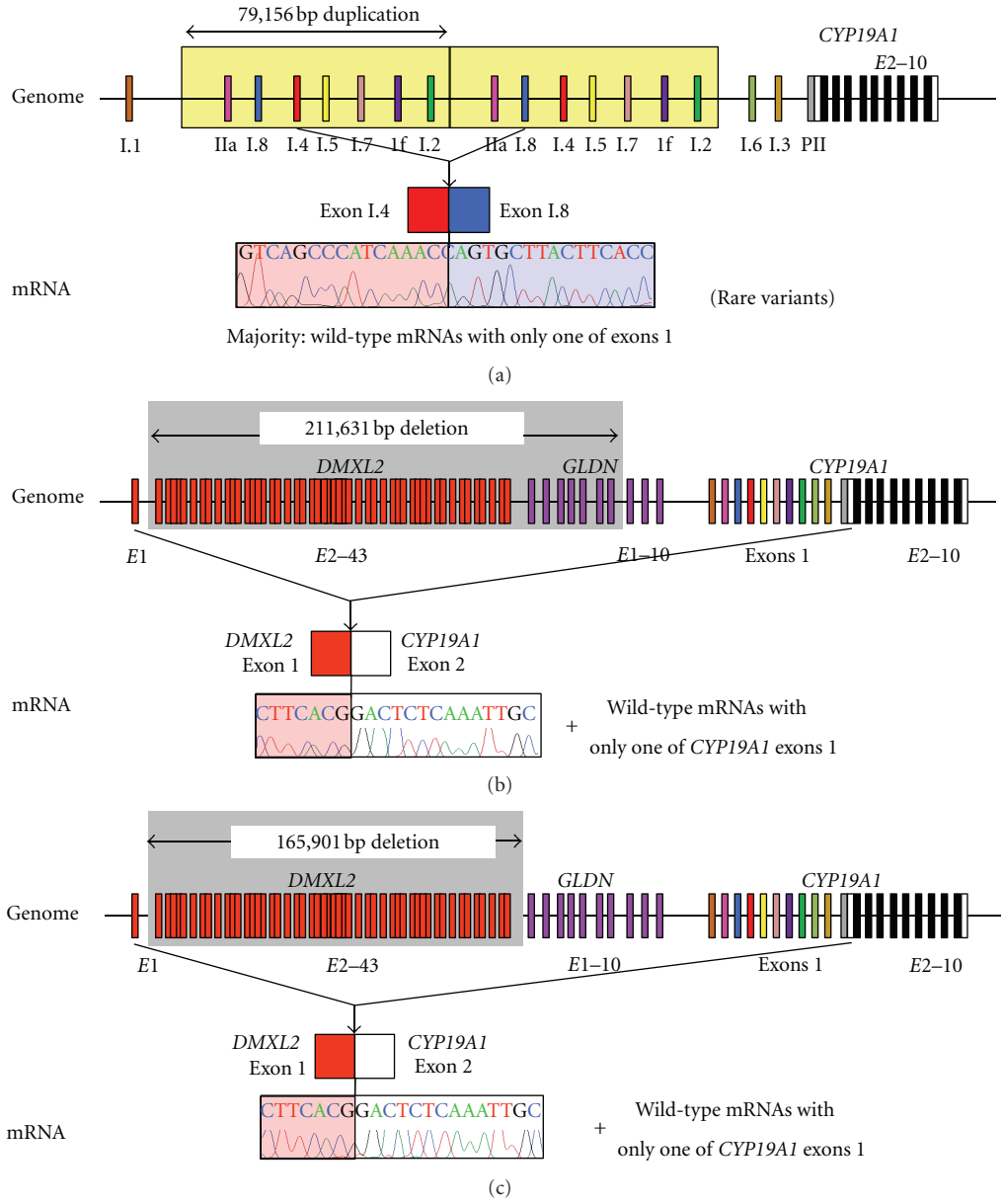

Figure 2: Schematic representation of duplications and deletions identified in patients with AEXS. (a) the tandem duplication of families A and B [4]. Genome: the duplication (yellow boxes) includes seven of the 11 noncoding exons 1 of *CYP19A1*. mRNA: the sequence of a rare transcript is shown. The 3′-end of exon I.4 is connected with the 5′-end of exon I.8. (b) The deletion of family C [4]. Genome: the deletion (a gray area) includes exons 2–43 of *DMXL2* and exons 5–10 of *GLDN*. mRNA: The sequence of a rare chimeric gene transcript is shown. *DMXL2* exon 1 consisting of a noncoding region and a coding region is spliced onto the common acceptor site of *CYP19A1* exon 2. (c) The deletion of families D–F [4]. Genome: the deletion (a gray area) includes exons 2–43 of *DMXL2*. mRNA: the sequence of a rare chimeric gene transcript is delineated. The mRNA structure is the same as that detected in family C.

an identical 165,901 bp deletion including exons 2–43 of *DMXL2*. RT-PCR identified the same chimeric mRNA as that detected in family C.

Collectively, three types of genomic rearrangements on 15q21 have been identified in AEXS to date, namely, inversion type (four subtypes), duplication type, and deletion type (two subtypes) (Figure 3(a)) [2–4]. In this regard, sequence analyses for the breakpoints have indicated that (1) inversion types are formed by a repeat sequence-mediated nonallelic intrachromosomal or interchromosomal recombination or by a replication-based mechanism of fork stalling and template switching (FoSTeS) that occurs in the absence of repeat sequences and is often associated with microhomology [16], (2) duplication type is generated by FoSTeS, and (3) deletions are produced by nonhomologous end joining that takes place between nonhomologous sequences and is frequently accompanied by an insertion of a short segment at the fusion point or by a nonallelic recombination [16].

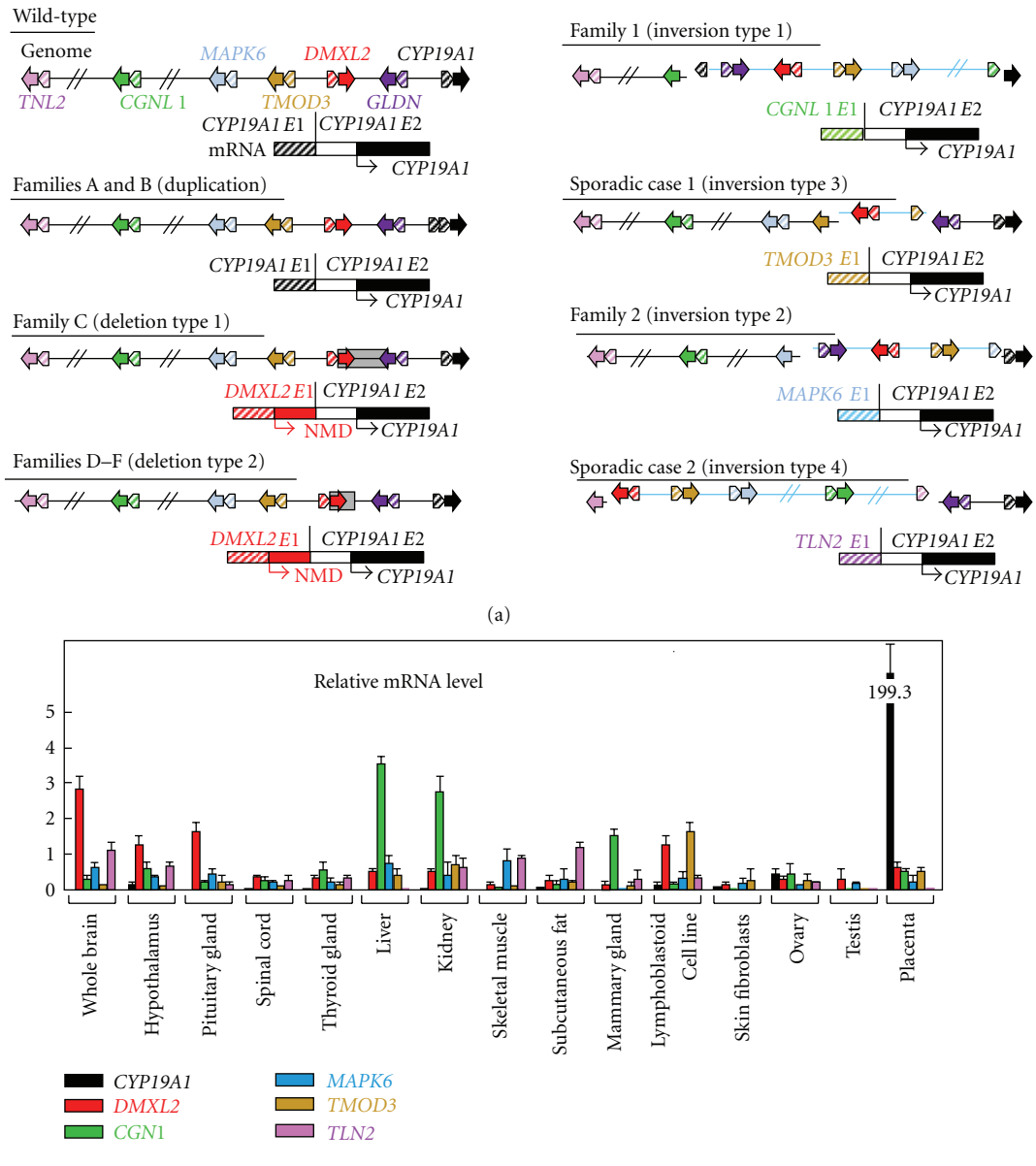

Figure 3: Structural and functional properties of the fused exons. (a) Schematic representation of the rearranged genome and mRNA structures. The white and the black boxes of *CYP19A1* exon 2 show untranslated region and coding region, respectively. For genome, the striped and the painted arrows indicate noncoding and coding exons, respectively (5′ → 3′). The inverted genomic regions are delineated in blue lines. For mRNA, colored striped boxes represent noncoding regions of each gene. The *DMXL2-CYP19A1* chimeric mRNA has two translation initiation codons and therefore is destined to produce not only *CYP19A1* protein but also a 47 amino acid protein which is predicted to undergo nonsense-mediated mRNA decay (NMD). The deletion and the inversion types are associated with heterozygous impairment of neighboring genes (deletion or disconnection between noncoding exon(s) and the following coding exons). The inversion subtype 1 is accompanied by inversion of eight of the 11 *CYP19A1* exons 1, and the inversion subtype 2 is associated with inversion of the placenta-specific *CYP19A1* exon I.1. (b) Expression patterns of *CYP19A1* and the five neighboring genes involved in the chimeric gene formation [4]. Relative mRNA levels against *TBP* in normal human tissues are shown.

Thus, it appears that genomic sequence around *CYP19A1* harbors particular motifs that are vulnerable to replication- and recombination-mediated errors. The results provide novel mechanisms of gain-of-function mutations leading to human diseases.

4. Clinical Features of AEXS

To date, a total of 23 male cases from 10 families have been reported to have molecularly confirmed AEXS (Table 1, Figure 3(a)) [2–4]. They exhibited pre- or peripubertal onset

Table 1: Summary of clinical studies in male patients with aromatase excess syndrome (modified from [4]).

(a)

Family			Family A			Family B		Family C				Family D			Family E		
Mutation types			Duplication			Duplication		Deletion				Deletion			Deletion		
The promoter involved in *CYP19A1* overexpression			*CYP19A1*			*CYP19A1*		*CYP19A1*				*DMXL2*			*DMXL2*		
Case		Case 1	Case 2		Case 3		Case 4	Case 5		Case 6		Case 7	Case 8		Case 9		Case 10	
Age at examination (year)		66	15		20		15	15		13		42	9		12		13	
<Phenotypic findings>																		
Gynecomastia (tanner breast stage)		**2**	**2**		**2**		**3**	**4**		**4**		**4**	**3**		**4**		**4**	
Onset of gynecomastia (year)		**13**	**13**		**10**		**11**	**12**		**11**		**11**	**7**		**9**		**10**	
Mastectomy (year)		No	**Yes (15)**		No		**Yes (15)**	**Yes (15)**		**Yes (13)**		No	No		**Yes (12)**		**Yes (13)**	
Testis (ml)		N.E.	**12**		**12**		**12**	**12**		12		N.E.	3		12		20	
Pubic hair (tanner stage)		N.E.	2-3		4		5	4		3		N.E.	1		3		4	
Facial hair		Normal	Scarce		**Scarce**		Normal	Absent		Absent		N.E.	Absent		Absent		Absent	
Height (SDS)[a]		−1.2	−0.3		+0.4		+0.8	−2.0		−1.0		−1.6	**+2.7**		±0		+1.8	
Bone age (year)[b]		N.E.	N.E.		N.E.		16.0	16.0		13.5		N.E.	**13.0**		**15.0**		**17.0**	
Fertility (spermatogenesis)		Yes	?		(Yes)[h]		?	?		?		Yes	?		?		?	
<Endocrine findings>[c]		B	B	S	B	S	B S	B	S	B	S	B	B	S	B	S	B	S
<At Dx>	Stimulus																	
LH (mIU/mL)	GnRH[e]	3.8	2.3	**14.3**	2.1	**17.0**	2.4 29.4	1.9	40.6	1.8	69.2		1.1	11.5	**0.6**	39.5	6.7	14.8
LH (mIU/mL)	GnRH (after priming)[f]		1.8	**9.5**	**1.3**	**10.7**												
FSH (mIU/mL)	GnRH[e]	**1.7**	**3.1**	**5.3**	**<0.5**	**1.2**	**0.9 2.4**	**1.4**	**4.2**	**2.0**	**7.8**		**3.2**	**6.6**	**0.6**	**2.9**	**0.7**	**1.0**
FSH (mIU/mL)	GnRH (after priming)[f]		**2.6**	**3.2**	**<0.5**	**0.9**												
Prolactin (ng/ml)			4.3		5.3			8.2		9.1			11.3		18.8			
Δ^4A (ng/mL)		**0.5**			**1.1**		**1.2**					**0.6**			**0.7**		**2.4**	2.9
T (ng/mL)	hCG[g]	2.9	**1.6**		**2.2**		4.0	**2.6**	7.2	**1.4**	7.9		**0.6**	3.6	**2.4**		3.2	9.7
DHT (ng/mL)		0.4			0.2												0.4	1.2
Inhibin B (pg/mL)		**61.6**			**74.6**		**83.5**	**75.2**										
E_1 (pg/mL)		157			120		124					57			63		53	
E_2 (pg/mL)		29	15		22		59	56		38		24	19		25		58	
E_2/T ratio ($\times 10^3$)		10.0	9.4		10.0		14.8	21.5		27.1			31.7		10.4		18.1	

(b)

Family		Family F								Family G		Family H		Sporadic	
Mutation types		Deletion								Inversion		Inversion		Inversion	
The promoter involved in *CYP19A1* overexpression		*DMXL2*								*CGNL1*		*MAPK6*		*TMOD3*	*TLN2*
Case		Case 11	Case 12	Case 13	Case 14	Case 15	Case 16	Case 17		Case 18	Case 19	Case 20	Case 21[j]		Case 22	Case 23
Age at examination (year)		69	35	44	45	9	8	13		10	35	7	13		17	36
<Phenotypic findings>																
Gynecomastia (tanner breast stage)		**Yes**[i]	**Yes**[i]	**Yes**[i]	**Yes**[i]	**2**	**3**	**3**		**3**	**Yes**	**3**	**5**		N.E.	**Yes**
Onset of gynecomastia (year)		?	?	?	?	**8**	**8**	**11**		**10**	**5**	**5**	**8**		**7**	?
Mastectomy (year)		**Yes**[i]	**Yes**[i]	**Yes**[i]	**Yes**[i]	No	No	**Yes (?)**		**Yes (?)**	**Yes (16)**	No	**Yes (?)**		**Yes (?)**	**Yes (19)**
Testis (ml)		N.E.	N.E.	N.E.	N.E.	2	1.5	**2**		2	N.E.	N.E.	N.E.		Normal	N.E.
Pubic hair (tanner stage)		N.E.	N.E.	N.E.	N.E.	1	1	2		1	Normal	1	**2-3** (at 21.0)		N.E.	N.E.
Facial hair		N.E.	N.E.	N.E.	N.E.	Absent	Absent	Absent		Absent	**Absent**	Absent	N.E.		**Scarce**	N.E.
Height (SDS)[a]		N.E.	~ −1.5	~ −1.5	~ −1.5	+1.4	N.E.	**+2.0**		**+2.4**	**Short**	**>+2.5**	−1.6 (at 21.0)		**Short**	N.E.
Bone age (year)[b]		N.E.	N.E.	N.E.	N.E.	**12.5**	**13.0**	**15.0**		**14.5 (at 12.5)**	N.E.	**13.0 (at 5.5)**	**17.0**		N.E.	N.E.
Fertility (spermatogenesis)		Yes	Yes	Yes	Yes	?	?	?		?	Yes	?	?		?	?
<Endocrine findings>[c]		B	B	B	B	B	B	B	S	B	B	B	B	S	B	
<At Dx>	Stimulus															
LH (mIU/mL)	GnRH[e]	**0.2**	3.5	1.7	3.0	0.2	<0.1	2.6	6.3	1.5	1.7	0.1	2.6	**10.0**	4.3	
LH (mIU/mL)	GnRH (after priming)[f]															
FSH (mIU/mL)	GnRH[e]	**1.4**	**2.3**	**0.8**	**0.8**	1.4	0.5	**0.8**	**1.2**	**1.2**	**1.5**	0.3	**<0.1**	**<0.1**	**2.7**	
FSH (mIU/mL)	GnRH (after priming)[f]															
Prolactin (ng/ml)																
Δ^4A (ng/mL)		1.4	**0.4**	1.7	**0.5**	0.3	<0.3	**0.9**	1.5	1.3	**0.8**	0.3	2.4	**0.9**		
T (ng/mL)	hCG[g]	**2.6**	**2.5**	**2.1**	**2.5**	<0.1	<0.1	**2.7**	9.2	**2.7**	3.2	<0.1	**1.2**	3.8	**2.3**	
DHT (ng/mL)													0.2	0.5		
Inhibin B (pg/mL)																
E_1 (pg/mL)		32	34	59	34	26	41	77		86	903	119	544		556	
E_2 (pg/mL)		10	19	24	31	11	7	25		40	223	15	178		392	
E_2/T ratio ($\times 10^3$)		3.8	7.6	11.4	12.4			9.3		14.8	69.6		148.3		170.4	

SDS: standard deviation score; Dx: diagnosis; Tx: therapy; LH: luteinizing hormone; FSH: follicle stimulating hormone; Δ^4A: androstenedione; T: testosterone; DHT: dihydrotestosterone; E_1: estrone; E_2: estradiol; GnRH: gonadotropin-releasing hormone; hCG: human chorionic gonadotropin; N.E.: not examined; B: basal; and S: stimulated.

Abnormal clinical findings are boldfaced.

Abnormally low hormone values are boldfaced, and abnormally high hormone values are underlined.

[a]Evaluated by age- and ethnicity-matched growth references; heights ≥+2.0 SD or below ≤ −2.0 SD were regarded as abnormal.

[b]Assessed by the Tanner-Whitehouse 2 method standardized for Japanese or by the Greulich-Pyle method for Caucasians; bone age was assessed as advanced when it was accelerated a year or more.

[c]Evaluated by age-matched male reference data, except for inhibin B and E_1 that have been compared with data from 19 adult males.

[d]Treated with aromatase inhibitors (anastrozole).

[e]GnRH 100 μg/m^2 (max. 100 μg) bolus i.v.; blood sampling at 0, 30, 60, 90, and 120 minutes.

[f]GnRH test after priming with GnRH 100 μg i.m. for 5 consecutive days.

[g]hCG 3000 IU/m^2 (max 5000 IU) i.m. for 3 consecutive days; blood sampling on days 1 and 4.

[h]Although Case 3 has not yet fathered a child, he has normal spermatogenesis with semen volume of 2.5 ml (reference value: >2 ml), sperm count of 105 × 10^6/ml (>20 × 10^6/ml), total sperm count of 262.5 × 10^6 (>40 × 10^6), motile cells of 70% (>50%), and normal morphological sperms 77% (>30%).

[i]These four patients allegedly had gynecomastia that required mastectomy (age unknown).

[j]The sister has macromastia, large uterus, and irregular menses; the parental phenotype has not been described.

The conversion factor to the SI unit: LH 1.0 (IU/L), FSH 1.0 (IU/L), E_1 3.699 (pmol/L), E_2 3.671 (pmol/L), Δ^4A 3.492 (nmol/L), and T 3.467 (nmol/L).

gynecomastia, small testes with fairly preserved masculinization, obvious or relative tall stature in childhood and grossly normal or apparent short stature in adulthood, and age-appropriate or variably advanced bone ages. Blood endocrine studies revealed markedly elevated E_1 values and E_2/T ratios in all cases examined and normal or variably elevated E_2 values. In addition, Δ^4-androstenedione, T, and dihydrotestosterone values were low or normal, and human chorionic gonadotropin (hCG) test indicated normal T responses. Notably, LH values were grossly normal at the baseline and variably responded to GnRH stimulation, whereas FSH values were low at the baseline and poorly responded to GnRH stimulation even after preceding GnRH priming, in all cases examined.

The severity of such clinical phenotypes is primarily dependent on the underlying mechanisms (Table 1). They are obviously mild in the duplication type, moderate in the deletion type, and severe in the inversion type, except for serum FSH values that remain suppressed irrespective of the underlying mechanisms. Likewise, gynecomastia has been reported to be ameliorated with 1 mg/day of aromatase inhibitor (anastrozole) in the duplication and the deletion types and with 2–4 mg/day of anastrozole in the inversion type [4].

5. Expression Pattern of *CYP19A1* and the Chimeric Genes as One Phenotypic Determinant

Phenotypic severity is much milder in the duplication type than in the deletion and the inversion types. This would be explained by the tissue expression pattern of *CYP19A1* and the chimeric genes. Indeed, RT-PCR analysis using normal human tissue samples revealed that *CYP19A1* is expressed only in a limited number of tissues such as placenta, ovary, skin, and fat, while the five genes involved in the formation of chimeric genes are widely expressed with some degree of variation (Figure 3(b)). Therefore, it is likely that the duplication types would simply increase *CYP19A1* transcription in native *CYP19A1*-expressing tissues, whereas the deletion and the inversion types lead to *CYP19A1* overexpression in a range of tissues, because expression patterns of chimeric genes are predicted to follow those of the original genes. Furthermore, it is also likely that the native *CYP19A1* promoter is subject to negative feedback by elevated estrogens [17], whereas such negative feedback effect by estrogen is weak or even absent for the chimeric genes in the deletion and the inversion types.

6. Structural Property of the Fused Exons as Another Phenotypic Determinant

Phenotypic severity is also milder in the deletion type than in the inversion types, despite a similar wide expression pattern of genes involved in the chimeric gene formation (Table 1, Figure 3(b)). In this context, it is noteworthy that a translation start codon and a following coding region are present on exon 1 of *DMXL2* of the deletion type but not on exons 1 of the chimeric genes of the inversion types (Figure 3(a)). Thus, it is likely that *DMXL2*/*CYP19A1* chimeric mRNAs transcribed by the *DMXL2* promoter preferentially recognize the natural start codon on *DMXL2* exon 1 and undergo nonsense-mediated mRNA decay and that rather exceptional chimeric mRNAs, which recognize the start codon on *CYP19A1* exon 2, are transcribed into CYP19A1 protein. By contrast, such a phenomenon would not be postulated for the inversion-mediated chimeric mRNAs. Consistent with this, it has been shown that the *DMXL2*/*CYP19A1* chimeric mRNA is present only in 2–5% of *CYP19A1*-containing transcripts from skin fibroblasts, whereas the *CGNL1*/*CYP19A1* chimeric mRNA and the *TMOD3*/*CYP19A1* chimeric mRNA account for 89–100% and 80% of transcripts from skin fibroblasts, respectively [2, 4].

In addition, the genomic structure caused by the rearrangements would affect efficiency of splicing between noncoding exon(s) of neighboring genes and *CYP19A1* exon 2. For example, in the inversion subtype 1, the physical distance between *CGNL1* exon 1 and *CYP19A1* exon 2 is short, and, while a splice competition may be possible between exon 1 of neighboring genes and original *CYP19A1* exons 1, eight of 11 *CYP19A1* exons 1 including exon I.4 have been disconnected from *CYP19A1* coding exons by inversion (Figure 3(a)). This may also enhance the splicing efficiency between *CGNL1* exon 1 and *CYP19A1* exon 2 and thereby lead to relatively severe overexpression of the *CGNL1-CYP19A1* chimeric gene, although this hypothesis would not be applicable for other chimeric genes.

7. Implication for the Hypothalamus-Pituitary-Gonadal Axis Function

It is notable that a similar degree of FSH-dominant hypogonadotropic hypogonadism is observed in the three types, although E_1 and E_2 values and E_2/T ratios are much higher in the inversion type than in the duplication and deletion types (Table 1). In particular, FSH was severely suppressed even after GnRH priming in the duplication type [4]. This implies that a relatively mild excess of circulatory estrogens can exert a strong negative feedback effect on FSH secretion primarily at the pituitary. This would be consistent with the results of animal studies that show strong inhibitory effect of E_2 on transcription of FSH beta-subunit gene in the pituitary cells and almost negligible effect on synthesis of LH beta-subunit and secretion of LH [18, 19]. In this regard, while T responses to hCG stimulation are normal in the duplication and the deletion types and somewhat low in the inversion type, this would be consistent with fairly preserved LH secretion in the three types and markedly increased estrogen values in the inversion type. In addition, whereas fertility and spermatogenesis are normally preserved in the three types, this would be explained by the FSH-dominant hypogonadotropic hypogonadism, because FSH plays only a minor role in male fertility (spermatogenesis) [20].

8. Conclusions

Current studies argue that AEXS is caused by overexpression of *CYP19A1* due to three different types of cryptic genomic rearrangements including duplications, deletions, and inversions. It seems that transcriptional activity and structural property of the fused promoter constitutes the underlying factor for the clinical variability in most features of AEXS except for FSH-dominant hypogonadotropic hypogonadism. Thus, AEXS represents a novel model for gain-of-function mutation leading to human genetic disorders.

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Research Article

Delayed Recognition of Disorders of Sex Development (DSD): A Missed Opportunity for Early Diagnosis of Malignant Germ Cell Tumors

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Disorders of sex development (DSD) are defined as a congenital condition in which development of chromosomal, gonadal or anatomical sex is atypical. DSD patients with gonadal dysgenesis or hypovirilization, containing part of the Y chromosome (GBY), have an increased risk for malignant type II germ cell tumors (GCTs: seminomas and nonseminomas). DSD may be diagnosed in newborns (e.g., ambiguous genitalia), or later in life, even at or after puberty. Here we describe three independent male patients with a GCT; two were retrospectively recognized as DSD, based on the histological identification of both carcinoma *in situ* and gonadoblastoma in a single gonad as the cancer precursor. Hypospadias and cryptorchidism in their history are consistent with this conclusion. The power of recognition of these parameters is demonstrated by the third patient, in which the precursor lesion was diagnosed before progression to invasiveness. Early recognition based on these clinical parameters could have prevented development of (metastatic) cancer, to be treated by systemic therapy. All three patients showed a normal male 46,XY karyotype, without obvious genetic rearrangements by high-resolution whole-genome copy number analysis. These cases demonstrate overlap between DSD and the so-called testicular dysgenesis syndrome (TDS), of significant relevance for identification of individuals at increased risk for development of a malignant GCT.

1. Introduction

Congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical are termed "Disorders of Sex Development" (DSD) [1] and have replaced the formerly used "intersex" term. It is estimated that DSD affects 1 in 4,500 to 5,000 live births in the general population, although with variability regarding the various DSD subtypes [1]. DSD patients are subdivided into different entities: 46,XY DSD, 46,XX DSD, and sex chromosomal DSD. Within these subgroups, patients, with gonadal dysgenesis (GD) and hypovirilization with presence of part of the Y chromosome (i.e., GBY), are known to have an increased risk to develop carcinoma *in situ* (CIS) or gonadoblastoma (GB), the precursor lesions of seminoma(SE)/dysgerminoma(DG) and nonseminoma, referred to as malignant type II germ cell tumors (GCTs) ([2–4], for review). In GD migration of the germ cells and/or their organization in the gonads is disturbed, leading to incomplete formation of the gonads. Hypovirilization is caused by defects in androgen-dependent target tissues, errors in testosterone biosynthesis, and testicular unresponsiveness to stimulation from the pituitary

[5], leading to underdevelopment of the male differentiation lineage.

GB is the *in situ* germ cell malignancy of the ovary and dysgenetic gonad which, in a significant number of cases, will develop into an invasive dysgerminoma or, less often, nondysgerminoma, being histologically and genetically counterparts of testicular seminoma and nonseminoma [6]. GB is composed of a mixture of embryonic germ cells (OCT3/4 and SCF (official term: KITLG) positive, amongst others) and supportive cells, with characteristics of granulosa cells (FOXL2 positive) [7]. GB can be found in undifferentiated gonadal tissue and in gonadal tissue with immature testis differentiation [8], overall related to a low level of testicularization (i.e., level of testis formation). CIS (cells also positive for OCT3/4 and SCF, amongst others), on the other hand, being the precursor of the similar types of cancer (SE and nonseminoma) of the testis, is associated with SOX9-positive Sertoli cells [7] and is found in well-differentiated testicular tissue [9].

For malignant transformation of embryonic germ cells in the context of type II GCTs, presence of part of the Y chromosome is crucial, referred to as gonadoblastoma on the Y chromosome (GBY) region by Page in 1987 [2]. *TSPY* is currently considered to be the most likely candidate gene for this genomic region [10, 11], and of diagnostic value, because both CIS and GB show coexpression of OCT3/4, SCF, and TSPY.

In spite of the overall low incidence in the general population, type II testicular GCTs are the most common malignancy in Caucasian males aged between 15 and 45 years, the incidence of which is still rising [12]. It has been suggested that the so-called testicular dysgenesis syndrome (TDS) is the underlying reason [13], estimated to affect 1 in 500 live births. However, existence of TDS is also questioned [14]. TDS links various clinical observations like cryptorchidism, subfertility/infertility, and hypospadias with exposure to certain environmental factors, with either a xenoestrogen or antiandrogen function. However, genetic factors, especially a limited number of single nucleotide polymorphisms (SNPs) are also recognized to play a role in development of this type of cancer [15, 16]. Most likely, the pathogenesis is a close and subtle interplay between both genetic and environmental factors, referred by us to as "Genvironment."

Here three unique unrelated male patients are presented demonstrating the relevance of TDS and DSD recognition for early diagnosis of malignant type II GCTs, possibly preventing progression to metastasized disease.

2. Materials and Methods

2.1. Patients. Three unrelated male patients, all with hypospadias and cryptorchidism in their clinical history, are described. All patients underwent hypospadias corrections, and two patients had orchidopexy early in life. Two of the patients were only retrospectively recognized as having DSD based on the presence in a single gonad of GB next to CIS as precursor lesions. The third patient described, having been recognized early in life as having DSD/TDS (i.e., hypospadias and cryptorchidism), shows that early identification of the condition can lead to early detection of the cancer precursor lesion before progression to invasiveness occurs. Detailed description is presented in Section 3.

2.2. Tissue Samples. Collected tissue samples were diagnosed according to WHO standards [17] by an experienced pathologist in gonadal pathology, including GCTs (JWO). The use of tissue samples for scientific reasons was approved by an institutional review board (MEC 02.981 and CCR2041). Samples were used according to the "Code for Proper Secondary Use of Human Tissue in The Netherlands" as developed by the Dutch Federation of Medical Scientific Societies (FMWV, Version 2002, updated 2011). Fresh tissue material was fixed in 10% buffered formalin for 24 hrs and paraffin embedded according to standard protocols.

2.3. Immunohistochemical Staining. Immunohistochemistry was performed on paraffin-embedded tissue sections of 3-μm thickness. Hematoxylin (Klinipath, Duiven, The Netherlands) and eosin (Klinipath) counterstaining was performed according to standard procedures. After deparaffinization and 5 min incubation in 3% H_2O_2 to inactivate endogenous peroxidase activity, antigen retrieval was carried out by heating under pressure of up to 1.2 bar in an appropriate buffer; 0.01 M sodium citrate (pH 6) or 0.01 M EGTA, 0.01 M TRIS (pH 9). After blocking endogenous biotin using the avidin/biotin blocking kit (SP-2001, Vector Laboratories, Burlingame, CA, USA), the sections were incubated for either 2 hrs at room temperature (OCT3/4, SOX9) or overnight at 4°C (TSPY, FOXL2, SCF). Appropriate biotinylated secondary antibodies were used for detection and were visualized using the avidin-biotin detection and substrate kits (Vector Laboratories). The antibodies used directed against OCT3/4, TSPY, SCF, SOX9, and FOXL2 have been described before [7, 18–20].

2.4. Fluorescent In Situ Hybridization. Slides of 5 μm thickness were deparaffinized and heated under pressure of up to 1.2 bar in appropriate buffer; 0.01 M sodium citrate (pH 6). Slides were digested using 0.01% pepsin (Sigma Aldrich, St. Louis, MO, USA) in 0.02 M HCl at 37°C, with an optimal digestion time of 2.5 min. Slides were rinsed and dehydrated, and the probes dissolved in hybridization mixture were applied. Probes for centromere X (BamHI) and centromere Y (DYZ3) were used, labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) using a nick-translation kit (Gibco BRL, Paisley, UK). After denaturizing (80°C for 10 min), hybridization overnight (37°C), and washing steps, probes were visualized using Cy3-conjugated avidin (1 : 100, Jackson ImmunoResearch, West Grove, PA, USA) and Sheep-anti-dig FITC (1 : 50, Roche Diagnostics) and analyzed using a fluorescent microscope (Leica Microsystems, Rijswijk, The Netherlands).

2.5. Copy Number Analysis. Genomic DNA was isolated from peripheral blood (patient 1 and 3) and frozen gonadal tissue without presence of malignant cells (patient 2) using

standard procedures. For each sample, 200 ng of DNA was labelled and hybridized onto the Human OmniExpress microarray (Illumina, San Diego, CA, USA) at the Australian Genome Research Facility (Melbourne, Australia) following manufacturer's instructions. Data was analyzed with Genome Studio (Illumina) and cnvPartition, using default settings.

3. Results

3.1. Clinical History, Hormonal and Genetic Data, and Immunohistochemical Analyses

Patient 1. Review of the existing clinical data was prompted by the histological evaluation of the right testis at the age of 26 years (showing dysgenetic characteristics, see below). It was found that the patient had multiple surgical corrections of proximal hypospadias between his second and tenth year of age, because of the severity of this anomaly. Orchidopexy of the left testis by an inguinal approach was performed at three years of age, while no right gonad was found during inguinal exploration on the right side at that time. At 26 years of age, the patient underwent surgery for a left-sided inguinal hernia. During the procedure, the right testis (inguinal position) was identified at the left hand side (i.e., crossed testicular ectopia) and removed because of a macroscopically abnormal/tumor-like appearance.

Histological examination of this gonad showed dysgenetic characteristics, containing CIS, GB, DG, and SE (representative hematoxylin and eosin (H&E) staining shown in Figure 1(a)). The CIS- and GB-germ cells showed a positive staining for OCT3/4 (Figure 1(b), brown), TSPY (Figure 1(c), red), and SCF (Figure 1(d), brown). The supportive cells in context of CIS stained predominantly positive for SOX9 (Figure 1(e), brown), while those in the context of GB stained predominantly for FOXL2 (Figure 1(f), brown). Coexpression is, however, observed, suggesting an issue of balance. In line with current treatment options, the patient received prophylactic radiotherapy according to standard guidelines. During close followup (3 years), the patient showed no relapse of the disease.

Genetic analysis by karyotyping of peripheral blood lymphocytes, and FISH using X and Y centromeric probes on gonadal tissue (representative FISH shown in Figure 1(g)) indicated a normal male 46,XY constitution. Hormonal data analysis at the age of 24 years indicated a suboptimal testicular function (hypergonadotropic hypogonadism), FSH 12 and 17.5 U/L (normal 2.0–7.0 U/L), LH 5.6 and 8.4 U/L (normal 1.5–8.0 U/L), testosterone 13.2 and 16.2 (normal 10–30 nmol/L), Inhibin B 119 and 74 ng/L (normal 150–400 ng/L). Tumor markers measured after the removal of the affected gonad with the cancer showed a slightly elevated level of AFP 15–19 μg/L (normal < 10–15 μg/L), normal levels of β-HCG < 0.5 IU/L (normal < 0.5 IU/L), and LDH 152–314 U/L (normal < 450 U/L).

Taken together, the histological observations, clinical history, karyotyping, and hormonal data support the diagnosis of the patient as a 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations is schematically shown in Figure 1(h).

Patient 2. Review of the (limited) clinical history was provoked by the histological evaluation of the left testis at the age of 21 years, showing dysgenetic characteristics (see below). It revealed presence of bilateral intra-abdominal testes, while the male patient also showed hypospadias, as well as presence of a uterus. At 20 years of age, the patient was diagnosed with a right intra-abdominal testicular SE (of which no material or further information could be retrieved). During surgical removal of the affected gonad, the left sided intra-abdominal testis was positioned at an inguinal site. This remaining testis was biopsied six months later because of unexplained enlargement and was subsequently removed because of presence of CIS and GB (see below).

Histological evaluation of the left biopsy showed the presence of GB and CIS, which was followed by orchidectomy. Further histological examination indicated the presence of dysgenetic characteristics, CIS and GB (representative H&E shown in Figure 2(a)), supported by staining for OCT3/4 (Figure 2(b), brown), TSPY (Figure 2(c), red), and SCF (Figure 2(d), brown), next to SE and DG. The supportive cells in GB stained again positive for FOXL2 (Figure 2(e), brown) and for SOX9 in CIS (Figure 2(f), brown). Because of proven metastasized disease, the patient received chemotherapy following standard procedures. No follow-up information is available.

Genetic analysis by karyotyping of peripheral blood lymphocytes and FISH using X and Y centromeric probes on gonadal tissue indicated a normal male 46,XY karyotype (data not shown). No hormonal or tumor marker data was available.

In summary, histological evaluation, review of clinical history, and karyotyping indicate that the patient must be diagnosed as a 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations is schematically shown in Figure 2(g).

Patient 3. The male patient showed multiple congenital anomalies at birth, amongst other penoscrotal hypospadias and bilaterally cryptorchid testes. He underwent multiple hypospadias corrections at one and two years of age. Orchidopexy of the right testis to a high scrotal position was performed at two years of age, and a herniotomy and orchidopexy, to a high scrotal position, of the left testis was carried out at 3 years of age. Overall appearance of the left testis together with total dissociation of epididymis and testis prompted a biopsy to be taken at that time (representative H&E shown in Figure 3(a)). It was diagnosed as prepubertal testicular parenchyma with seminiferous tubules containing Sertoli cells and germ cells, without indication for malignancy. The patient was lost to followup until 12 years of age at which time he was examined because of incontinence problems and came under attention of the initial clinician treating the hypospadias by coincidence. Physical examination showed a pubertal boy (Tanner stage P4G3) with a scrotal localization of the right testis, while

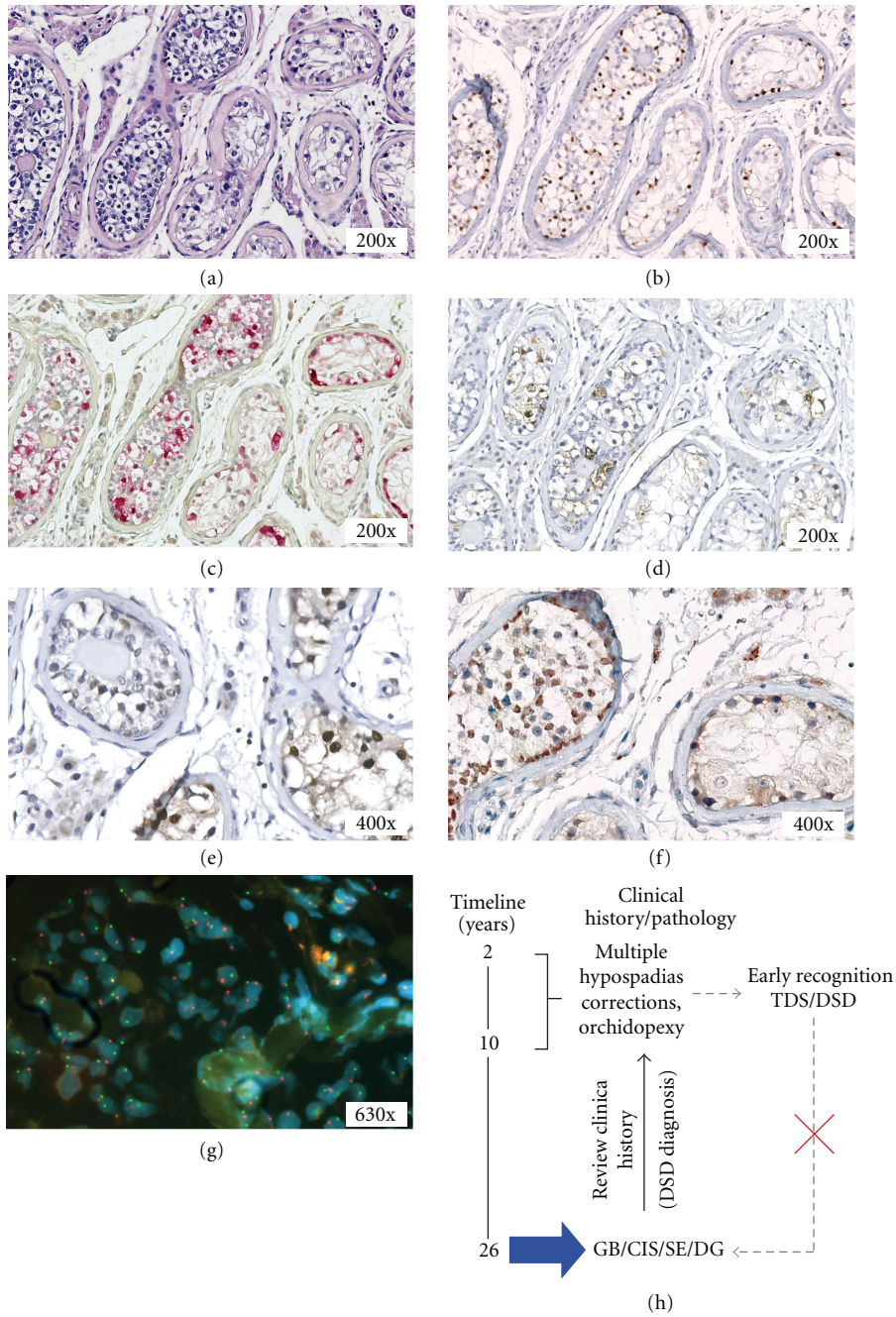

Figure 1: Immunohistochemical staining and fluorescent *in situ* hybridization (FISH) of the gonadoblastoma and carcinoma *in situ* lesions of patient 1. (a) Representative hematoxylin and eosin staining. The germ cells present in the GB and CIS stain positive for (b) OCT3/4 (brown), (c) TSPY (red), and (d) SCF (brown). (e) The supportive cells in the CIS lesion are SOX9 positive (brown staining) and are negative for FOXL2. (f) In the GB, the supportive cells stain positive for FOXL2 (brown staining) and are negative for SOX9. (a–f) In every image the GB lesion is shown on the left side (embryonic germ cells intermixed with granulose-like supportive cells), CIS containing seminiferous tubules on the right side (CIS cells associated with Sertoli cells on the basal lamina). Magnification 200x and 400x for all. Slides (b)–(f) are counterstained with hematoxylin. (g) Representative FISH with Y-centromere-specific probe (shown in red) and X-centomere-specific probe (shown in green). Magnification 630x. (h) Schematic representation of the different moments in time of clinical intervention, blue arrow, identification of a malignant type II germ cell tumor, together with GB and CIS as precursor lesions at the age of 26 years. Review of the clinical history showed hypospadias and cryptorchid testes, signs of TDS/DSD which were not recognized at an early age. Grey-dashed arrows; early recognition of TDS/DSD could have allowed early detection and treatment of the malignancy, thereby, preventing the need for additional systemic treatment.

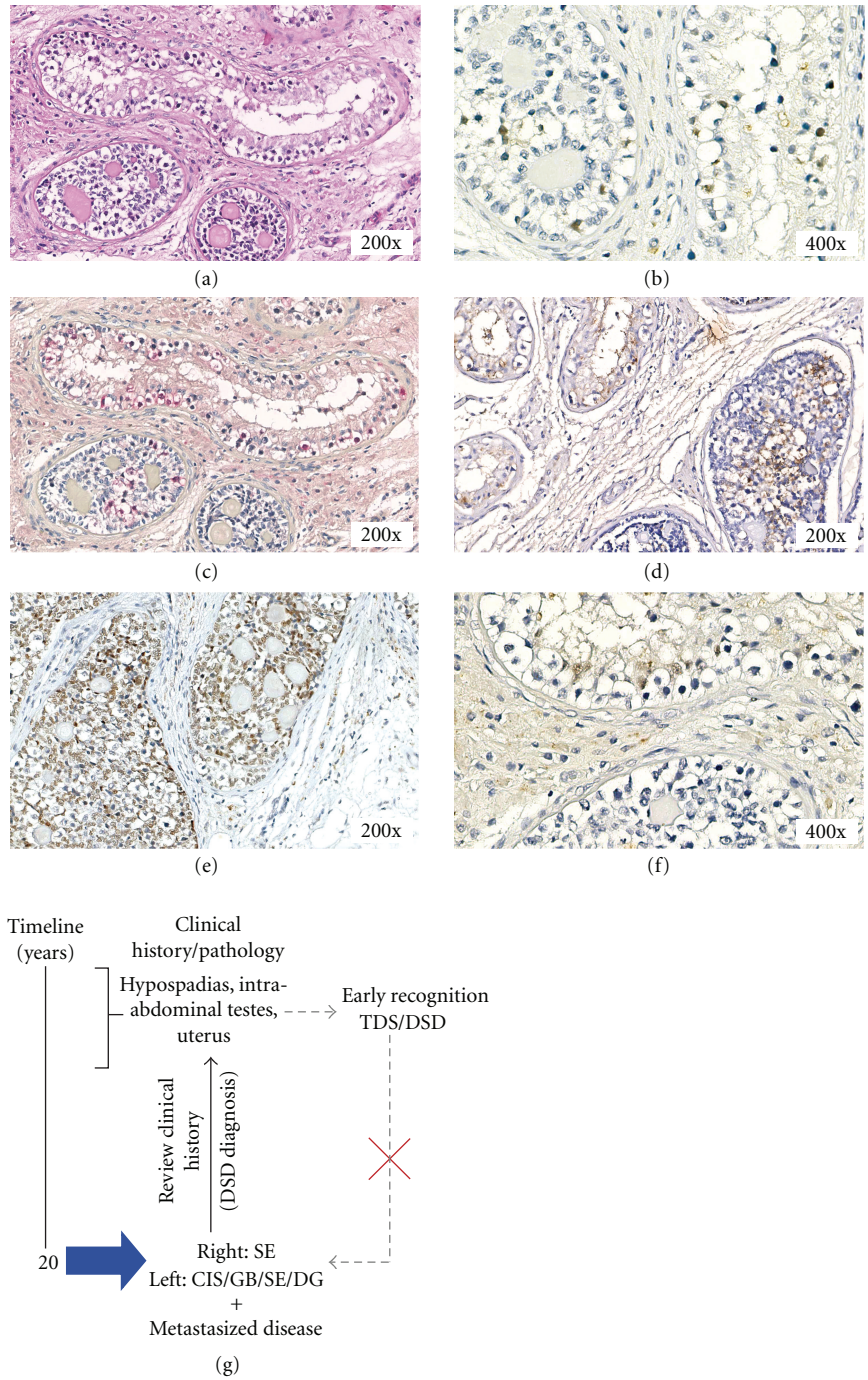

Figure 2: Immunohistochemical staining of the gonadoblastoma and carcinoma *in situ* lesions of patient 2. (a) Representative hematoxylin and eosin staining. Positive staining for (b) OCT3/4 (brown), (c) TSPY (red), and (d) SCF (brown) of the germ cells present in the GB and CIS. (e) In the GB the supportive cells stain positive for FOXL2 (brown). (f) The supportive cells in the CIS lesion are SOX9 positive (brown staining) and are negative for FOXL2. (a–d), (f) Again, both GB (embryonic germ cells intermixed with granulose-like supportive cells) and CIS (associated with Sertoli cells on the basal membrane of the tubules) are shown. Magnification 200x and 400x for all. Slides (b)–(f) are counterstained with hematoxylin. (g) Timeline showing the clinical history, histology, and actions taken.

the left testis was not palpable. Further examination using ultrasound showed an inguinal position of the left testis (ascending testis) and bilateral testicular microcalcifications (microlithiasis). Because of the inability to position the left testis in the scrotum, and the knowledge about the increased risk for development of a malignant GCT based on the clinical characteristics, the left testicle was removed, and the right testis was biopsied.

Histological examination of the left testis (representative H&E staining shown in Figure 3(d)) showed seminiferous

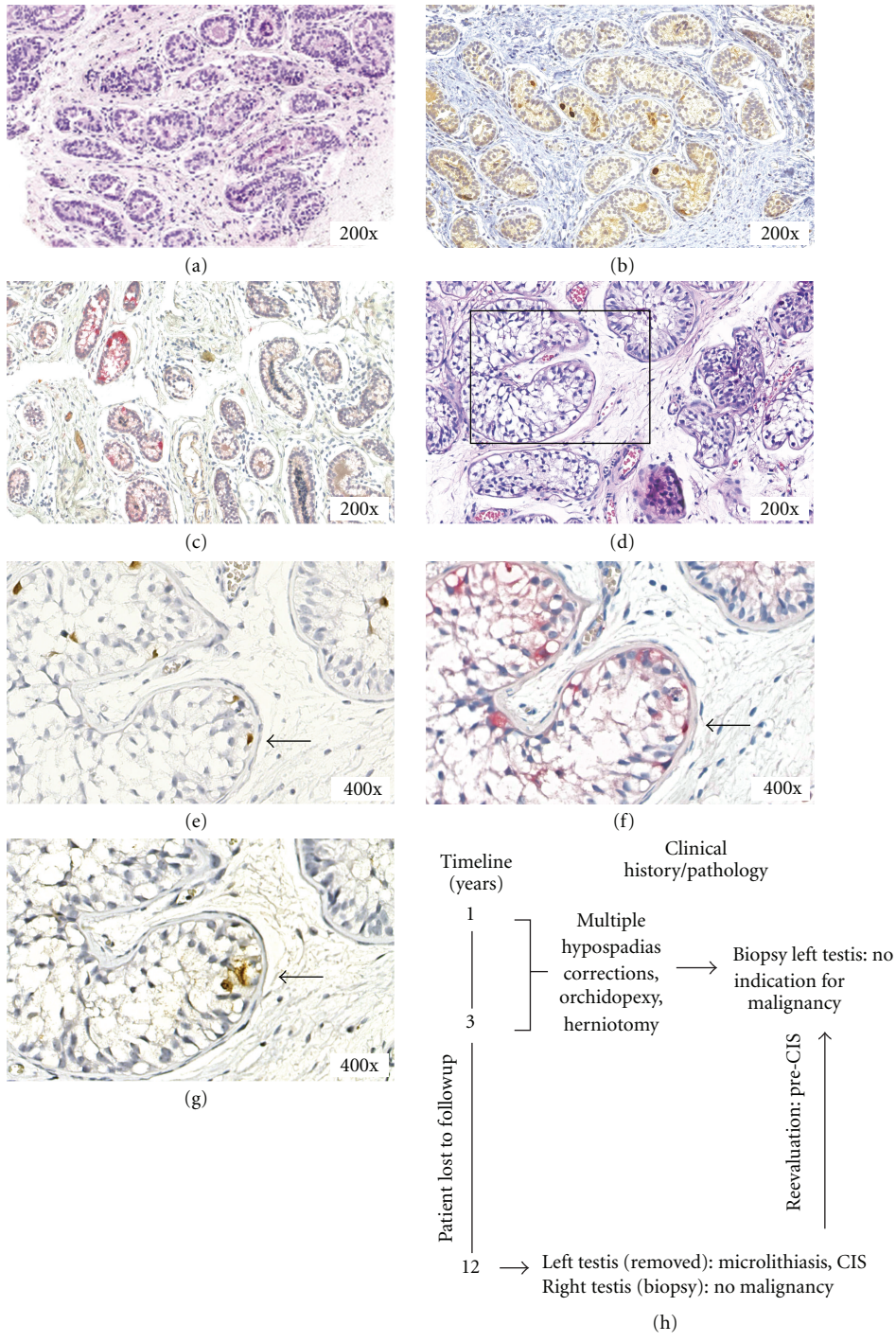

Figure 3: Immunohistochemical staining of the carcinoma *in situ* lesion of patient 3 at three and twelve years of age. (a) Representative hematoxylin and eosin staining. Positive staining for (b) OCT3/4 (brown), (c) TSPY (red) of the germ cells present in the CIS. (a–c) Biopsy tissue at 3 years of age. (d) Representative hematoxylin and eosin staining. Positive staining for (e) OCT3/4 (brown), (f) TSPY (red), and (g) SCF (brown) of the CIS cells. (d–g) Gonadal tissue at 12 years of age. (e–g) Region indicated with a square in (d) is shown. Note the expression of OCT3/4, TSPY, and SCF in the CIS cell indicated by the arrow. Magnification 200x and 400x for all. Slides (b)–(g) are counterstained with hematoxylin. (h) Timeline showing the clinical history, histology, and actions taken.

tubules containing CIS, supported by staining for OCT3/4 (Figure 3(e), brown), TSPY (Figure 3(f), red), and SCF (Figure 3(g), brown). Costaining of these markers in single CIS cells was identified (indicated by the arrow). The presence of CIS initiated reexamination of the biopsy taken at the age of three years. Because of limited material available, only staining for OCT3/4 (Figure 3(b), brown) and TSPY (Figure 3(c), red) could be done, showing the presence of premalignant germ cells, referred to as pre-CIS. This conclusion was not made at the time of original sampling because of lack of appropriate markers. The biopsy taken from the right testis showed normal testicular parenchyma without malignancy (negative OCT3/4 staining, data not shown).

Genetic analysis by karyotyping of peripheral blood lymphocytes and FISH using X and Y centromeric probes on gonadal tissue indicated a normal male 46,XY karyotype (data not shown). Hormonal data at the age of 12 years were as follows: FSH 1.8 and 3.1 U/L (normal < 6.0 U/L), LH 0.4 and 1.0 U/L (normal < 2.5 U/L), testosterone 1.6 and 6.7 nmol/L (normal 3.0–6.5 nmol/L), and AMH 18.8 μg/L (normal 30–200 μg/L). The tumor markers tested were within the normal range: AFP < 1 μg/L (normal < 10 μg/L) and β-HCG 0.1 IU/L (normal < 0.5 IU/L).

Taken together, histological evaluation, review of clinical history, and karyotyping indicate that the patient must be diagnosed as 46,XY DSD, type A: disorder of testicular development, 1: partial gonadal dysgenesis [1]. A summary of the various actions and observations in time is schematically shown in Figure 3(h).

3.2. Copy Number Analysis of Known DSD Genes. A peripheral blood DNA sample from patient 1 and 3 and a DNA sample isolated from frozen gonadal tissue from patient 2 (as no peripheral blood was available) were checked for copy number changes by genome-wide SNP analysis using Illumina OmniExpress Beadchips. This supported the 46,XY karyotype and showed no aberrations affecting known DSD genes (data not shown).

4. Discussion

Here, two unrelated male patients are presented, both diagnosed with an invasive malignant type II GCT. One was prophylactically treated with irradiation for a stage I seminoma, and the other received chemotherapy for proven metastasized disease. These treatment protocols have been found to increase the risk for long-term sequelae [21]. Presence of GB, known to be associated with DSD [8], besides CIS, as precursor in these patients, triggered review of their clinical history. Both cases showed severe hypospadias and cryptorchidism. These are identifiers of DSD, as well as TDS, both conditions known for their increased risk of malignant type II GCTs [3, 13]. In addition, patient 1 had crossed testicular ectopia, a very rare anomaly, reported to be associated with TDS and DSD [22]. No genetic confirmation of an underlying DSD was found in any of the patients, even using high-resolution genome wide analysis. In spite of this lack of identification of the molecular basis of the underlying disorder, the observations have significant implications regarding development of strategies for early diagnosis of type II GCTs, as well as understanding the biology of the disease.

DSD patients can be diagnosed early in life based on various characteristics, including sexual ambiguity, family history, discordant karyotype and genital appearance, and aberrant male and female genitalia. In children and young adults, however, DSD can present as an inguinal hernia in a girl, incomplete or delayed puberty, virilization in a girl, primary amenorrhea, breast development in a boy, and a previously unrecognized genital ambiguity [1].

When sex determination is disrupted in an early stage of Sertoli cell differentiation, a high risk for GB is found [9]. The GB lesion is composed of immature germ cells intermixed with supportive cells classified as granulosa [23]. The GB lesions found in the two presented patients showed these characteristics as well, based on immunohistochemical finding using OCT3/4, TSPY, SCF, SOX9, and FOXL2. Next to the GB component, CIS was also present in both. This in fact triggered the search for additional clinical arguments in line with the diagnose of these patients as DSD. The findings presented indicate that, by proper application of the current knowledge of risk factors for type II GCTs, these patients could have been diagnosed earlier, thereby, possibly preventing the use of irradiation and chemotherapy. That this is in fact a feasible option is demonstrated by the third patient presented. It demonstrates the power of applying the current markers for diagnosis of the premalignant lesions of type II GCTs. In fact, reevaluation of the biopsy of this patient, taken at three years of age, showed coexpression of OCT3/4 and TSPY in germ cells located on the basal lamina. These cells are referred to as pre-CIS, from which CIS will develop. Proper identification of the risk factors for type II GCTs, in particular related to DSD and TDS, will increase the possibility to identify patients at risk for malignancy at an early age, allowing application of limited-harmful treatment protocols.

OCT3/4 expression is most likely related to the survival of the germ cells [24], while the role of TSPY is less clear. It has been suggested to be related to cell-cycle regulation [25–27]. In addition, SCF is informative to diagnose CIS and GB, especially to distinguish CIS from germ cells delayed in their maturation [28]. Of interest in this context is the linkage of specific single nucleotide polymorphisms with development of type II GCTs in the general Caucasian population, including involvement of SCF [15, 16]. However, the impact of these risk alleles in the DSD populations remains to be investigated.

The left testis of patient 1 and the right testis of patient 3 are still *in situ* at a scrotal localization. For the first patient available hormonal data indicated suboptimal testicular function (high FSH, low inhibin, testosterone low normal range). In spite of treatment by prophylactic irradiation, and absence of metastasis (based on routine examinations), the patient is under close surveillance because of a minor elevated AFP level. No hormonal indications for testicular dysfunction could be observed in patient 3, while no data were available for patient 2.

Recently two families have been independently reported showing an overlap between DSD and TDS. One family showed two sisters with XY sex reversal, gonadal dysgenesis, and GB, and the other family included one daughter with a mosaic karyotype and GB. All patients showed a *SRY* mutation, inherited from the father, being mosaic. The fathers of both families presented with TDS, one with oligoasthenozoospermia, and a testicular SE, the other with hypospadias, cryptorchidism, oligoasthenozoospermia, and a testicular SE as well [29, 30]. In other words, TDS and DSD form a continuum, which is informative to identify individuals at risk for type II GCTs.

As indicated, the two patients demonstrated here with a type II GCT, and proven metastasized cancer in one, at time of diagnosis, also show the value of identification of parameters known to be related to TDS and DSD, including cryptorchidism, hypospadias, and the presence of GB (in the latter). Based on these characteristics, these two patients would have been diagnosed as 46,XY DSD, disorder of testicular development, 1: partial gonadal dysgenesis [1], being at increased risk for development of a malignant type II GCT. Feasibility of early diagnosis, leading to the prevention of development of an invasive, and possibly even a metastatic cancer, is clearly demonstrated by the third patient.

Conflict of Interests

The authors state no conflict of interests.

Acknowledgments

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Research Article

Clinical Presentation of Klinefelter's Syndrome: Differences According to Age

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The aim of the study was to establish the characteristics of presentation of 94 patients with Kinelfelter's syndrome (KS) referred to the endocrinologist at different ages. The diagnosis of KS was more frequent in the age group between 11 and 20 years (46.8%). Most of the patients (83.7%) showed the classic 47,XXY karyotype and 7.1% showed a 47,XXY/46,XY mosaicism. Half of the patients younger than 18 years presented mild neurodevelopmental disorders. The most frequent clinical findings were cryptorchidism in prepubertal patients, and small testes, cryptorchidism, and gynecomastia in pubertal patients. FSH, LH, AMH, and inhibin B levels were normal in prepubertal patients and became abnormal from midpuberty. Most adults were referred for small testes, infertility, and gynecomastia; 43.6% had sexual dysfunction. Testosterone levels were low in 45%. Mean stature was above the 50th percentile, and 62.5% had BMI $\geq$ 25.0 kg/m^2. In conclusion, the diagnosis of Klinefelter syndrome seems to be made earlier nowadays probably because pediatricians are more aware that boys and adolescents with neuro-developmental disorders and cryptorchidism are at increased risk. The increasing use of prenatal diagnosis has also decreased the mean age at diagnosis and allowed to get insight into the evolution of previously undiagnosed cases, which probably represent the mildest forms. In adults average height and weight are slightly higher than those in the normal population. Bone mineral density is mildly affected, more at the spine than at the femoral neck level, in less than half of cases.

1. Introduction

The original clinical presentation of Klinefelter's syndrome was described by Klinefelter's, Reifenstein, and Albright in 1942, who reported 9 adult males with gynecomastia, small and firm testes, azoospermia, and elevation of serum FSH with functional Leydig, cells [1]. In 1959, using cytogenetics, Jacobs and Strong described the existence of an extra X chromosome, that is, 47,XXY karyotype, in a male with Klinefelter's syndrome [2]. This classical karyotype is present in approximately 90% of the cases. Patients with mosaicisms or with pure cell lineages carrying one or more extra X chromosomes and at least one Y chromosome (e.g., 47,XXY/46,XY, 47,XXY/45,X/46,XY, 48,XXXY, 49,XXXXY, 48,XXYY, etc.) are also considered as Klinefelter's syndrome variants, although the clinical presentation may be quite different in some cases [3].

Because chromosomal and gonadal features are atypical in Klinefelter's syndrome, the condition has been included as a disorder of sex development by the International Consensus Conference organized by the Lawson Wilkins Pediatric

Endocrine Society and the European Society for Paediatric Endocrinology in 2006 [4, 5]. Furthermore, Klinefelter's syndrome is characterized by a genetic, whole gonadal dysfunction [6], affecting germ cells from early fetal life [7, 8] and Sertoli's and Leydig's cells from mid-puberty [9].

From an endocrinological standpoint, patient's complaints may vary, according to gonadal dysfunction, from signs of sex hormone deficiency in young adults to infertility in a male without other signs of hypogonadism. Before puberty, the condition is usually underdiagnosed due to the fact that childhood is a period characterized by physiologically low androgen levels and no sperm production [3]. Cryptorchidism and mild developmental disorders may be the alerting signs.

The main objective of this study was to establish the frequency and clinical characteristics of the different forms of presentation of Klinefelter's syndrome at different ages in a large cohort of patients.

2. Patients and Methods

We performed a retrospective chart analysis that included all patients with a confirmed diagnosis of Klinefelter's syndrome of 5 different centers in Buenos Aires, Argentina, from 1982 to 2008. The definition of Klinefelter's syndrome, as inclusion criterion for this study, required the availability of a karyotype consisting of an X-chromosome polysomy and at least one Y chromosome, either as a single lineage or as a mosaicism. The karyotype was analyzed in at least 30 metaphases.

The study was approved by the review boards of the participating institutions.

The main outcome measures were age at diagnosis, frequency of history or existence of cryptorchidism, gynecomastia, neuro-developmental disorders and signs of hypogonadism and serum hormone levels (FSH, LH, testosterone, AMH, inhibin B, estradiol, prolactin, TSH, and thyroid hormones), lipid and carbohydrate profiles, sperm analysis, and bone mineral density measurement at referral. Results were expressed as the prevalence (frequency of presentation at referral) or as the mean ± standard deviation or median and range, as applicable. Where necessary, quantitative variables were transformed into qualitative ones. For instance, hormone levels were classified as normal, elevated, or low according to the reference ranges for age. Data were analyzed using Instat Statistical Software (GraphPad Software, Version 3.01). Pearson's correlation coefficients and two-sample t-test for continuous variables were used. P values below 0.05 were considered to indicate statistical significance.

3. Results

Records from 98 patients with Klinefelter's syndrome were analyzed: 44 of them were under 18 yr old at referral (18 were under 10 yr old and 26 were between 10 and 17.9 yr old), and 54 were aged 18 yr or older. The most prevalent age range at diagnosis was 11–20 yr (Figure 1). A prenatal diagnosis was made in 4 cases. Karyotype was 47,XXY in 83.7% and 47,XXY/46,XY in 7.1% of the 98 patients; the other variants were homogeneously infrequent (Table 1).

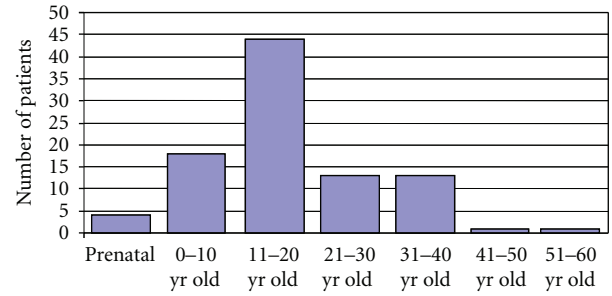

Figure 1: Age at diagnosis of the 94 patients of the present study.

Table 1: Karyotypes found in the 98 patients of this study.

Karyotype	n	%
Pure lineage	*86*	*87.76*
47,XXY	82	83.67
48,XXXY	1	1.02
48,XXYY	3	3.06
Mosaicisms	*12*	*12.24*
47,XXY/46,XY	7	7.14
47,XXY/48,XXYY	2	2.04
47,XXY[4]/48,XXXY[2]/46,XY[44]	1	1.02
49,XXXXY[44]/48,XXXY[6]	1	1.02
47,XXY[36]/48,XXXY[1]/46,XX[1]/46,XY[2]	1	1.02

Table 2: Prevalence of clinical signs in pediatric and adolescent patients with Klinefelter's syndrome.

	Prepubertal ($n = 18$)	Pubertal ($n = 26$)
Neuro-developmental disorders	8 (44.4%)	14 (53.8%)
Small testes	3 (16.7%)	20 (76.9%)
Cryptorchidism	10 (55.5%)	6 (23.0%)
Gynecomastia	0 (0%)	11 (42.3%)
Small penis	3 (16.7%)	3 (11.5%)
Prenatal diagnosis	4 (22.2%)	0 (0%)
Pie bot	1 (5.5%)	0 (0%)
Varicocele	3 (11.5%)	3 (11.5%)
Dysmorphisms	1 (3.8%)	1 (3.8%)

3.1. Pediatric and Adolescent Patients with Klinefelter's Syndrome. The age group below 18 yr old was subclassified according to their pubertal status in prepubertal (median age 5.07 yr, range 0.75–9.28 yr) and pubertal (median age 14.3 yr, range 10.1–17.7 yr). In the prepubertal patients, cryptorchidism and neuro-developmental disorders, including behavior and/or learning difficulties, were the most prevalent findings (Table 2). In pubertal patients, the most frequent findings were small testes, neuro-developmental disorders, and gynecomastia.

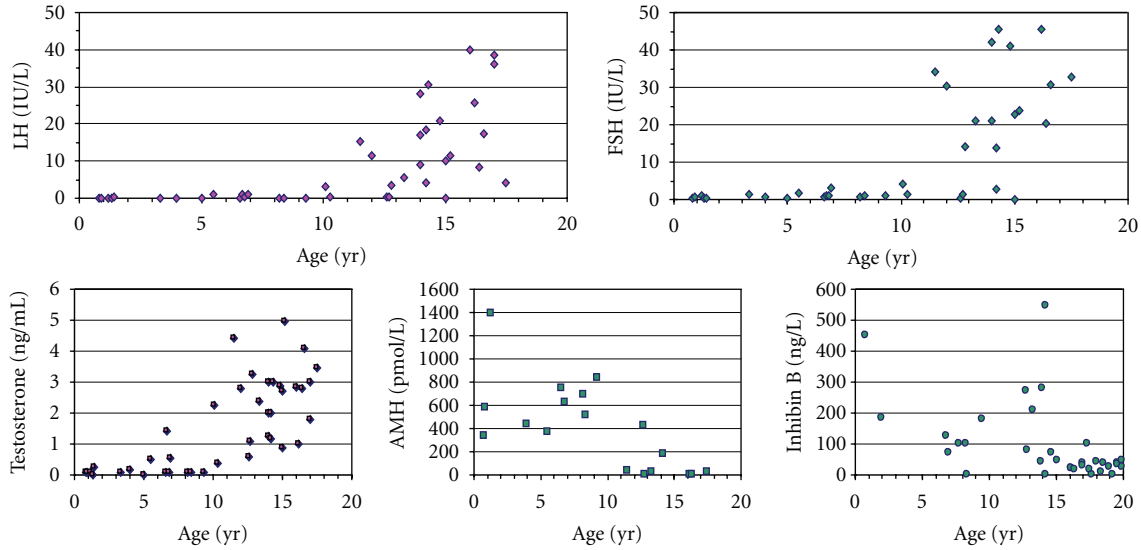

Figure 2: Serum hormone levels in pediatric and adolescent patients with Klinefelter's syndrome.

Table 3: Prevalence of main complaints and clinical signs and symptoms in adult patients with Klinefelter's syndrome.

Main complaints	%
Infertility	34.8
Small testes	34.8
Gynecomastia	10.9
Erectile dysfunction	6.5
Hypergonadotrophic hypogonadism	4.3
Eunuchoid proportions	2.2
Decreased libido	2.2
Signs and symptoms	**%**
Small testes	100
Infertility	100
Eunuchoid proportions	35.2
Gynecomastia	31.3
Erectile dysfunction	29.3
Reduced libido	27.5
Varicocele	23.3
Neurodevelopmental disorders	22.0
Small penis	0

Anthropometric evaluation was performed in boys and adolescents. The mean ± SD height standard deviation score (SDS) was 0.3 ± 1.8 (median 0.7, range −4.1 to 3.6) in prepubertal patients, and 1.1 ± 1.1 (median 1.1, range −1.4 to 2.9) in pubertal patients. SDS 0 represents the 50th percentile for the Argentine population, −2 is the 3rd percentile, and +2 is the 97th percentile. Our data indicate that pubertal patients with Klinefelter's syndrome were significantly higher ($P < 0.0001$). While height SDS above +2 was found in one prepubertal patient, it was above +2 in 5 pubertal patients. There was a significant correlation between height SDS and age ($n = 40$, $r = 0.304$, $P < 0.05$). Weight for height was adequate in most patients, except one prepubertal and 7 pubertal boys, with a body mass index (BMI) above the 95th percentile for age. Evaluation of the genitalia showed the existence of reduced penile size in 16.7% and 11.5% of prepubertal and pubertal patients, respectively, (Table 2). Testes were bilaterally nonpalpable in 22.2% of prepubertal patients. In those with palpable gonads, testicular volume was 1-2 mL in all cases except three (21.4%), who had abnormally small testes (<1 mL). In pubertal patients, testicular volume was below normal in all patients in the Tanner stages 3–5 (median 3.7 mL, range 1–8 mL). Bilateral gynecomastia was observed in 11 cases (42.3%). Varicocele was found in 3 cases (11.5%).

Evaluation of the reproductive axis hormone levels was available during followup in 13 prepubertal and 28 pubertal patients with Klinefelter's syndrome (Figure 2). FSH, LH, testosterone, inhibin B, and AMH levels were normal in all prepubertal boys. In pubertal patients, FSH and LH increased above the normal at 12.8 yr and 13.8 yr, respectively. A negative correlation was found between age and FSH ($r = -0.62$, $P < 0.001$) and age and LH ($r = -0.59$, $P < 0.001$). Serum AMH and inhibin B fell below normal from pubertal Tanner stage 3. Testosterone was below normal, and hormone replacement therapy was indicated, in 7 of 28 pubertal patients (25%) before the age of 18 yr.

3.2. Adult Patients with Klinefelter's Syndrome. Infertility and small testes were the most prevalent complaints in adult patients (Table 3). Sexual function inquiry revealed reduced libido in 27.5% of patients and erectile dysfunction in 29.3% (Table 3). Information on maximal education level was available in 26 patients: 42.3% attained tertiary education.

Anthropometric evaluation revealed a median height above the 50th percentile for the Argentine male population (mean ± SD = 178.8 ± 9.0 cm, median 180 cm, range 161–200 cm) and a slight overweight (mean ± SD weight 83.6 ± 21.0 kg, median 83.5 kg, range 53–171; mean ± SD

Table 4: Serum levels of reproductive axis hormones and carbohydrate and lipid profiles in adult patients with Klinefelter's syndrome.

	Mean ± SD	% Abnormal	Reference levels
FSH (IU/L)	35.4 ± 16.2	100	1–8
LH (IU/L)	22.3 ± 11.6	83.0	2–12
Total testosterone (ng/mL)	2.74 ± 1.65	45.1	2.8–8.8
Estradiol (pg/mL)	26.5 ± 13.2	6.3	18–44
Prolactin (ng/mL)	16.5 ± 11.3	33.3	5–20
TSH (μU/mL)	2.11 ± 0.99	0.0	0.47–4.64
Fasting glucose (mg/dL)	91.5 ± 13.1	0.0	70–110
Total cholesterol (mg/dL)	189 ± 40	40.0	110–200
LDL (mg/dL)	124 ± 36	50.0	<130
HDL (mg/dL)	43 ± 7	47.4	>40
Triglycerides (mg/dL)	115 ± 77	15.0	50–200

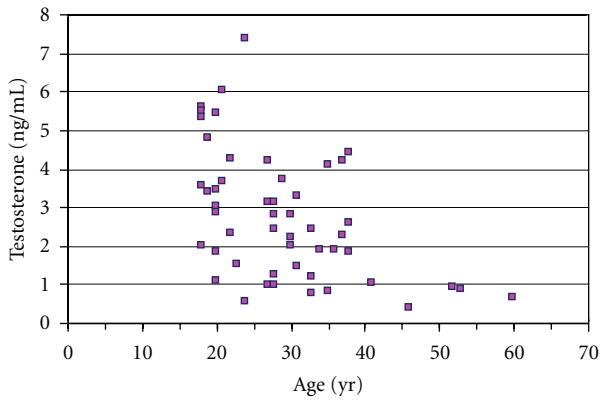

Figure 3: Serum testosterone levels in adult patients with Klinefelter's syndrome. Age versus testosterone: $r : -0.5$, $P < 0.001$.

BMI 26.4 ± 5.5 kg/m^2, median 25.9 kg/m^2, range 17.2–51.1 kg/m^2) 19 patients (47.5%) had a BMI between 25 and 29.9 kg/m^2, and 6 patients (15%) were overtly obese (BMI > 30 kg/m^2). Eunuchoid proportions were observed in 35.2% of patients.

Evaluation of the genitalia showed small testes in all cases (median 3.5 mL, range 1–8 mL), varicocele in 23.3%, and gynecomastia in 31.3% (bilateral in most of the cases).

Laboratory measurements (Table 4) revealed elevated FSH in 100% of cases and LH in 83%. Half of the patients with normal LH had subnormal total testosterone levels. Total testosterone decreased with age (Figure 3), and a negative correlation ($r = -0.50$, $P < 0.001$) was found between these variables. In the adult group as a whole, total testosterone was below normal in 45.1% of the cases. Prolactin was slightly elevated in 33% and estradiol in 6.2% of the cases. Semen analysis showed azoospermia in 89.3% of the cases and oligozoospermia in the remaining cases. No association was found with the karyotype. Total cholesterol was >200 mg/dL in 40%, LDL was >130 mg/dL in 50%, and HDL was <45 mg/dL in 47.4% of the cases. Fasting glucose levels were within the normal range in all patients.

Evaluation of bone mineral density (BMD) by DXA was performed in 27 adult patients. Results of BMD at lumbar spine were normal in 59.3% and showed osteopenia in 33.3% and osteoporosis in 7.4% of the cases. At the femoral neck level, BMD was normal in 72% and showed osteopenia in 28% of the cases, with no cases of osteoporosis.

4. Discussion

Klinefelter's syndrome is the commonest chromosomal abnormality in humans, with an estimated prevalence of 1 in 600 live births in males [3]. However, the condition is usually underdiagnosed, with an estimated 25% of the expected number of patients being ever diagnosed, and only a minority being diagnosed in childhood [10]. This may be due to the fact that Klinefelter's syndrome was initially a clinical diagnosis of adult males classically described as tall, with gynecomastia, small testes, azoospermia, sparse body hair, narrow shoulders, and broad hips. Those XXY males not presenting these typical features are probably missed, as are almost 90% of prepubertal patients in whom the diagnosis of hypogonadism is usually delayed until puberty [6]. Here we report the clinical and biochemical findings in a large cohort of Klinefelter's patients from pediatric age through adulthood. This is the largest series of patients with Klinefelter's syndrome in the literature. We are aware that a selection bias exists in our series, since all patients had been referred to a pediatric or adult endocrinologist. Therefore, the prevalence of clinical and biochemical findings in children, adolescents, and adults with Klinefelter's syndrome we report herein is applicable to those expected in an endocrinology setting.

The most prevalent age at diagnosis in our series was during adolescence, with more than half of our patients being diagnosed before adult age. This may reflect the fact that pediatricians and pediatric neurologists are increasingly aware of the increased prevalence of XXY karyotype in boys with mild neuro-developmental disorders, resulting in a rise in the diagnosis and early referral to the pediatric endocrinologist. Another reason may be the fact that cryptorchidism, a frequent cause of referral to the pediatric endocrinologist, is more frequent in patients with Klinefelter's syndrome [11–14]. In fact, the frequency of cryptorchidism was high in our series, mainly in the pediatric

population. The almost tenfold higher prevalence of Klinefelter's syndrome in cryptorchid boys [11, 12] supports the indication of a karyotype analysis.

In coincidence with the initial characterization of long-term outcomes [15] and, more recently, of longitudinal growth in patients with Klinefelter's syndrome [16], our patients were slightly taller than the general male population. In boys and adolescents, we observed a positive correlation between age and stature. Eunuchoid proportions cannot be ascribed to hypoandrogenism since the increased height is present before pubertal onset, but rather to the existence of an extra copy of the SHOX gene, mapping to X and Y chromosomes and involved in linear growth [17]. It should be noted, however, that the height of 19% of our adult patients fell below the 50th percentile for the Argentine population, indicating that tall stature is not a prerequisite for the diagnosis of Klinefelter's syndrome.

A dissociated gonadal dysfunction with lack of sperm in semen and reduced testis volume, reflecting a severe disorder of the seminiferous tubules, with normal/low testosterone levels, indicating a mild dysfunction of the interstitial compartment, was described in the initial report by Klinefelter's and colleagues [1]. Our results, together with other recent reports [7–9, 18, 19], further indicate that the establishment of the dysfunction of the seminiferous tubule components is also dissociated. In fact, germ cells are already affected in early postnatal life while Sertoli's cells secrete normal levels of AMH and inhibin B until mid-puberty. Similarly, Leydig's cells seem to produce androgens normally until mid-puberty; thereafter, although androgen levels might be within the normal range in a proportion of patients with Klinefelter's syndrome, there is an increase in LH, indicating a suboptimal Leydig cell functional capacity. Our results show that FSH levels are increased approximately one year before LH levels during puberty in patients with Klinefelter's syndrome, which suggests that Sertoli's cell function is affected not only more severely than that of Leydig's cells but also earlier. The age for testosterone replacement in these patients is controversial, yet clear clinical and biochemical signs of hypoandrogenism are an undisputed indication for the initiation of androgen therapy [20]. In our series, 7 patients required testosterone replacement before the age of 18 yr. Decreased Leydig's cell function has also been detected by measuring INSL3 [21, 22] and confirmed by histological studies showing increased interstitial fibrosis and decreased androgen receptor expression with age [7, 23]. Nonetheless, the indication for androgen therapy should not be unnecessarily anticipated, since the likelihood of obtaining sperm seems to decrease once treatment is initiated [24].

Lipid metabolism was abnormal in a variable proportion of our patients, as revealed mainly by the measurement of total cholesterol and its fractions, in coincidence with a recent report [25]. The latter study shows that the disorder in the lipid metabolism could not be modified by androgen therapy. Bone mineral density was mildly decreased in our patients, in line with a recent report in Korean males, which showed no significant correlation with androgen deficiency [26].

In conclusion, the diagnosis of Klinefelter's syndrome was more frequent between 11 and 20 yr of age in our series, probably due to the fact that in the last years pediatricians have become more aware of the likelihood of the diagnosis in boys and adolescents with neuro-developmental disorders, cryptorchidism, and small testes. The increasing use of prenatal diagnosis has also decreased the mean age at diagnosis and allowed to get insight into the evolution of previously undiagnosed cases, which probably represent the mildest forms. The function of the hypothalamic-pituitary-gonadal axis is preserved during infancy and childhood, with a progressive decline of the seminiferous tubule compartment from mid-puberty. The Leydig cell compartment shows a mild dysfunction compensated by an increase in LH during late puberty and early adulthood. As previously described, hypoandrogenism occurs in an increasing proportion of patients with age. Small testes, infertility, gynecomastia. decreased libido, and sexual or ejaculatory dysfunction were associated complaints. In adults average height and weight are slightly higher than in the normal population. Bone mineral density is mildly affected, more at the spine than at the femoral neck level, in less than half of cases.

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Clinical Study

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

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Disorder of sex development (DSD) patients in Indonesia most often do 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 males, and 34 as females. 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), and 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 workup 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.

1. 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.

2. Subject and Methods

2.1. 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 followup in 87 patients, and 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.

2.2. 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 at al. [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 hrs 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.

2.2.1. 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.

2.2.2. 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, Calif) [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].

2.2.3. 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_000044.2 numbering according to Gottlieb et al. [18], *SRY*: X53772.1 and *WNT4*: nm 030761.4).

2.2.4. Pathology. Histopathological assessments were performed by means of hematoxylin and eosin stainings and immunohistochemistry for various markers of germ cells, for example, 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].

3. Results

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

3.1. 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).

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	Consanguinity %	Quigley stage						Sex of rearing		
		1	2	3	4	5	6	Male	Female	NA
46, XX DSD	0		3	5	6	4	4	2	19	1
46 XY DSD	0	4	22	22	6	1	3	45	13	
Chrom. DSD	0	1	3	0	2	0	2	5	3	

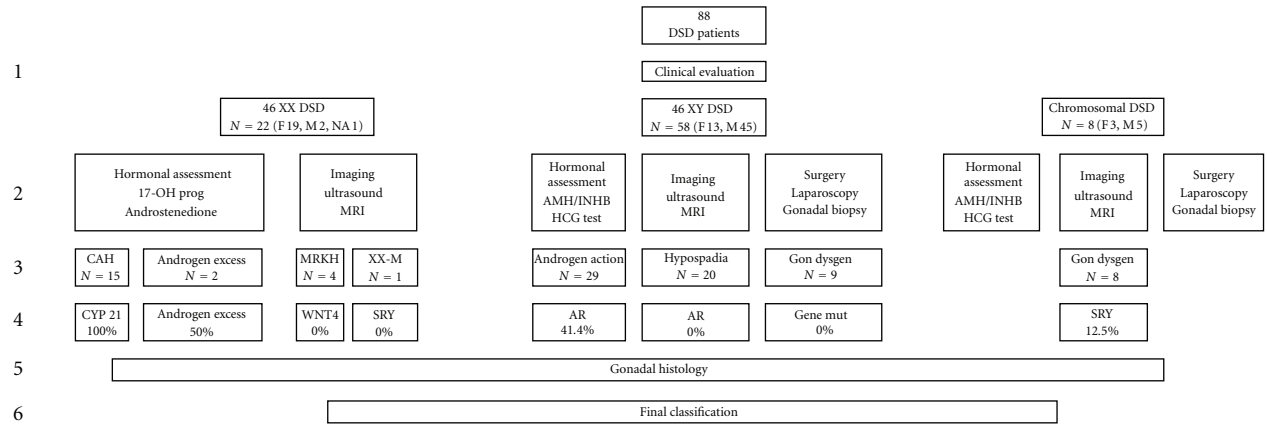

Figure 1: Stepwise diagnostic approach. An algorithm used with the aim of classifying the patients following the new classification system [8]. Row number 1 represents the clinical evaluation of the patients and classification following the primary root; number 2 the hormonal analysis and imaging followed by the secondary root classification (row 3). Row number 4 shows the percentage mutations that were found and number 5 the gonadal histology leading to the tertiary root and final classification of the patients. Explanation of abbreviations: MRKH: Mayer-Rokitansky-Küstner-Hauser Syndrome; XX-M; XX male.

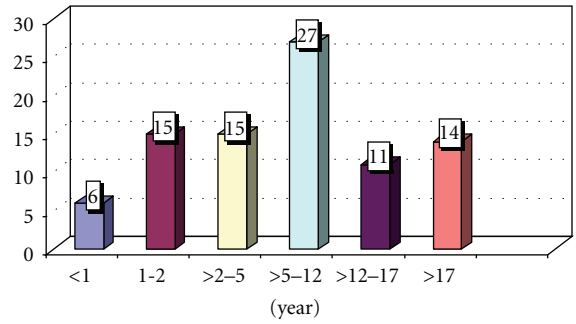

Figure 2: Age distribution of the patients at presentation.

The group of 88 patients contains four families with two or more patients with DSD (a total of nine). There was no known consanguinity of the parents. Fifty-two patients were raised as male and 35 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.

3.2. Hormonal Analysis, Imaging, and Surgery

3.2.1. 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 suppletion 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 gonadotropins (LH: 12.3 IU/L, FSH: 8.1 IU/L) were identified in one 33-year-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-year-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, and 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.

3.2.2. 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), and 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.

3.2.3. 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 followup 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

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

3.3.1. 46, XX DSD. 15 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.

3.3.2. 46, XY DSD. 29 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 5α-dihydrotestosterone (DHT) levels to hCG.

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

3.4. Mutation Analysis. Based on these results, mutation analysis was performed.

3.4.1. 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.

3.4.2. 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; 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.

3.4.3. 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.

3.5. 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.

3.6. 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.

4. 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 socioeconomic 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 undermasculinization of unknown etiology, which was assigned in 20 patients (34%). However, we cannot rule out 5α-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 yrs, 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 loosing 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

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 with slightly increased values of gonadotropins	No abnormalities	Laparoscopy	No	Ovarian Leydig cell tumor lacking histological signs of malignancy	Disorder of androgen 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 androgen action
3	13	Male	46 XY	3	High basal testosterone level	Gonads not in situ* no uterus and adnexa 1/3 vagina present	Laparoscopy gonadectomy	AR neg	CIS	PAIS
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 patients	Total
46, XX DSD		
Disorder of androgen excess, other	2	
Disorder of androgen excess, 21 hydroxylase deficiency	15	
Defect of mullerian development, other	4	
Disorder of gonadal development, ovotesticular DSD	1	22
46, XY DSD		
Disorder of gonadal development, other	9	
Disorder of androgen action, PAIS	12	
Disorder of androgen action, other	17	
Male undermasculinisation of unknown aetiology	20	58
Chromosomal DSD		
46 XX/46 XY DSD, disorder of gonadal development, ovotesticular DSD	1	
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	8

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 is 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 17β-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 prepubertal 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 mosaicism. 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 economical 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 nonscrotal position of the gonad increases this risk. Proactive 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 nonpalpable 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] is needed in the first week of life in order to recognize a salt-loosing 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 lifesaving 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 patients' 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.

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Review Article

Androgens and Adipose Tissue in Males: A Complex and Reciprocal Interplay

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Clinical evidence shows that in males obesity is frequently associated with hypogonadism and vice versa; also, low testosterone levels have been considered a "hallmark" of metabolic syndrome in men. These observations indicate that there is a strict connection between anatomically and functionally distinct cell types such as white adipocytes and Leydig cells, that synthesize testosterone. Adipose tissue is able to control several functions of the testis through its products secreted in the bloodstream. On the other hand, circulating levels of testosterone and estradiol deeply affect adipocyte proliferation, differentiation, and fat mass distribution, hereby controlling critical metabolic functions, such as food intake, insulin sensitivity, vascular reactivity, and immunity. This paper highlights the existing clinical and experimental evidence linking androgens and adipose tissue and illustrates the consequences occurring when the balance between fat mass distribution and eugonadism is lost.

1. Introduction

Adipose tissue, in addition to its role as a storage for triglycerides, can be considered as an active, atypical endocrine organ [1], given its ability to synthesize and secrete into the bloodstream several hormones. Overall, only thirty percent of adipose tissue is represented by mature adipocytes, given that multiple cell types are present in its contest. In fact, the remaining tissue is represented by multipotent stem cells, nerve tissue, small blood vessels, fibroblasts, and preadipocytes in various stages of differentiation [2]. Importantly, adipose tissue contributes to regulate several functions such as energy balance, food intake and appetite, immunity, insulin sensitivity, blood pressure and reproduction [3], releasing adipokines that have both local and systemic biological effects. Dysfunctional secretion of adipokines and free fatty acids contributes to the development of an inflammatory state and has a causal role for the development of the insulin-resistant state of obesity [4].

Two types of adipose tissue are present in mammals: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT stores energy as triglycerides. In case of lack of energy, such as fasting, lipolysis in WAT causes the release of fatty acids into the plasma to provide fuel for energy generation. Indeed lipases contained in adipocytes transform triglycerides into fatty acid and glycerol that are transported via the blood to the muscle, liver, and BAT to fatty acids oxidation [5]. In contrast, BAT plays a physiological function in adaptive thermogenesis storing triglycerides in multilocular adipocytes that serve as energy reserves easily accessible to heat production and restore energy expenditure induced by cold exposure or diet [6].

Also adipose tissue is distributed unevenly through the body and is represented by two major compartments which are different for distribution and metabolism: subcutaneous and visceral depots [7]. Intra-abdominal or visceral fat induces an increased risk of cardiovascular and metabolic complications, whereas subcutaneous fat exerts some still

undefined protective actions [8]. Visceral and subcutaneous adipose tissues express different adipokines. An important role in the pathogenesis of cardiovascular diseases is played by visceral fat, since it expresses many substances strongly involved in cardiovascular diseases, such as leptin, TNFα, IL-6, PAI-1, which have a direct access to the liver via portal vein, with a strong impact on the inflammatory processes [9].

Subcutaneous fat is metabolically less active than visceral fat and produces mostly protective substances such as leptin and adiponectin [10] and is less sensitive to glucocorticoids because it has lower levels of glucocorticoid receptor [11–13], thereby it could exert a protective role on metabolic homeostasis, hence counteracting the dysfunctional adipose tissue in visceral and ectopic compartments [14].

Aim of this paper is to review the most recent clinical and experimental evidence linking androgens and adipose tissue and to discuss the reciprocal interplay between obesity and hypogonadism.

2. Leptin and Testicular Function

The discovery of leptin in 1994 opened an exciting field of intense research, focused on the endocrine function of the adipocyte, and conferred to adipose tissue the attribution of an endocrine organ. Leptin is a pleiotropic cytokine-like hormone that is involved in the regulation of energy homeostasis, neuroendocrine function, immunity, lipid and glucose homeostasis, and fatty acid oxidation [15, 16]. Importantly, in normal condition, circulating levels of leptin are positively correlated with adiposity [17]. On the other hand, leptin-deficient mice carrying an homozigous mutation disrupting leptin gene (*ob/ob* mice) are hyperphagic, show lower energy expenditure at rest, and are less active, and then show a severe form of obesity [18] due to the lack of leptin signalling to the brain. Of course in such condition, leptin levels are virtually absent and do not correlate with fat mass. Furthermore, *ob/ob* mice are sterile and have an abnormal spermatogenesis because of an insufficient hypothalamic-pituitary drive with consequent low circulating gonadal steroids [19, 20]. Leptin treatment normalizes body weight and restore reproduction capacity in *ob/ob* males [20]. Interestingly, it has been shown that obesity *per se* is not the cause of infertility in leptin deficiency because caloric restriction does not restore fertility in the *ob/ob* mouse. This suggests that leptin is directly related to the modifications of reproductive capacity [21]. In line with these studies, in humans, endogenous leptin absence is associated with hypogonadism and absence of pubertal development [22, 23]. Moreover, a mutation in the *db* gene that encodes leptin receptor (Ob-R) leads to the synthesis of truncated leptin receptor that lacks the intracellular domain [24]. The *db/db* mouse has an alterated reproductive function similar to those of the *ob/ob* mouse, but given that the defect is at the receptor level, leptin treatment is unable to either restore fertility or modify the appetite of these animals [25]. Hoggard et al. first identified, by *in situ* hybridization, the expression of Ob-R in the spermatic cells and Leydig cells [26]. It has been shown that leptin enters the testis by a passive, nonsaturable process [27]. A large body of evidence indicates that leptin modulates the paracrine network [28, 29] that controls gonadotropin-stimulated testicular steroidogenesis [30]. Immunohistochemical studies demonstrated that mouse testis germ cells express a functional Ob-R capable of signal transduction [31]. These data suggest that leptin can mediate proliferation and differentiation of germ cells and then might be locally involved in the pathogenesis of infertility observed in leptin-deficient mice [31]. In a recent study, a group of proapoptotic-related genes, that may play an important role in mediating the increased germ cell apoptosis and impaired sperm production, has been identified within the testes of leptin-deficient mice [32]. This study suggests a fundamental role of leptin signalling within the test is in the control of spermatogenesis.

On the other hand, excess of leptin has a negative impact on Leydig cells function [28]. In fact, treatment of cultured rat Leydig cells with leptin strongly inhibits hCG stimulated testosterone production in a dose-dependent manner. Importantly, this effect occurs at concentrations within the range of circulating levels in obese men [25]. Disruption of steroidogenic pathway occurs at the level of 17–20 lyase, as shown by the concomitant accumulation of metabolites upstream of this enzymatic step [28]. Obese patients have reduced androgen concentrations, and this reduction is related to the increase of fat mass [33] and leptin levels [34]. Moreover, the androgen response to human chorionic gonadotropin (hCG) stimulation is impaired in obese men, and leptin is the best hormonal predictor of reduction to androgen response related to obesity [35]. On the other hand, no association between leptin and dihydrotestosterone circulating levels has been observed [36].

These observations suggest that leptin excess might have an important role in the hypogonadism, frequently observed in obese men, through a direct inhibition of Leydig cell steroidogenesis [25]. We have hypothesized that leptin acts through different sites and that there are different concentration thresholds for distinct effects of leptin on reproduction [25]; thus, a narrow range of circulating concentration of leptin are necessary in order to maintain a physiological reproductive function, and concentrations below or above these thresholds have a negative impact on hypothalamus-pituitary axis (lower threshold), or upon Leydig cell steroidogenesis (higher threshold).

3. Androgens, Fat Metabolism, and Adipose Biology

Testosterone is the major circulating androgen and is present in plasma as free or unbound testosterone, albumin-bound, and sex hormone-binding globulin [SHBG]-bound. In lean men, about 50% of testosterone is bound to albumin and other proteins, 44% is bound to SHBG, and 2% is unbound [37]. The biologically active component that is readily available to the tissues (bioavailable testosterone) is the proportion of unbound testosterone together with the albumin-bound fraction. A study showed that bioavailable testosterone is positively related to muscle strength and total body bone mineral density and negatively related to fat mass in healthy elderly men [38]. The fraction of testosterone bound to SHBG in serum is proportional to the SHBG levels.

SHBG production in the liver is regulated by several factors and hormones, and its levels are increased by estrogen and downregulated by obesity and insulin resistance conditions [39].

Androgens influence gene transcription through the activation of the androgen receptor (AR), a ligand-activated transcription factor that binds specific DNA motifs in its target genes [40]. The extension of the polymorphic polyglutamine (CAG repeat number) of the exon 1 of the AR modulates androgen effects: androgen-induced target activities are attenuated according to the length of triplet residues [41]. Such polymorphism can influence the activity and the expression of AR and plasma androgen concentration, directly contributing to the prevalence of central adiposity [42, 43]. In particular, the CAG repeat polymorphism in the androgen receptor gene could modulate body fat mass and serum concentrations of leptin and insulin in men through a direct effect upon adipocyte sensitivity to androgens. Phenotypic effects on body fat mass could be explained by estrogen action more than androgen action, because of the increased estrogen/androgen ratio in the presence of higher CAG length; in fact, in a normal functioning adult hypothalamic—pituitary—gonadal axis, a reduced testosterone feedback, in case of a long AR CAG repeat, is compensated by increased androgen production, because of increased LH stimulation, with subsequent higher conversion to estrogens [44].

Testosterone can act directly or be converted to the more potent androgen 5-dihydrotestosterone (DHT) by 5α-reductase or to estrogens by aromatase (ARO) [45, 46]. ARO activity has been detected in adipose tissue [47], and several studies have demonstrated a potentially important role for this enzyme in obesity, central fat accumulation, and metabolic syndrome (MetS) [48], through estrogen receptors (ERs) and ARs, which are abundantly expressed in the adipocyte and share related functions to suppress adipose tissue accumulation and improve insulin sensitivity [49]. In a recent study, a marked decline in serum leptin levels after short-term aromatase inhibition in healthy young and elderly men has been observed [50].

A line of evidence has been reported that strongly suggests the involvement of estrogen in lipid metabolism in the adipose tissue. Fat mass is increased in male mice with homozygous inactivation of either the estrogen receptor gene or aromatase gene, and estrogen replacement is able to restore normal conditions in these models [51, 52]. At a molecular level, it has been shown that estrogens suppress fat accumulation and lipoprotein lipase (LPL, a key regulating enzyme for energy metabolism, catabolizing plasma triglycerides into free fatty acids and glycerol) mRNA expression in 3T3-L1 cells stably expressing the ER.

Obesity is associated with physiological changes which include important modifications in circulating sex steroids levels. In particular, obese men show increased plasma levels of estrogens and decreased bioavailable levels of androgens. This is due to an increase in ARO activity that mediates peripheral conversion of androgens to estrogens [53]. Circulating values of total testosterone should not be lower than 8 nmol/L (230 ng/dL). Values below this cut-off are associated with severe impairments of body composition and glucose metabolism [54–57].

Finally, adipose tissue is able to affect gonadotropin release by the pituitary, both directly, through increased secretion of cytokines, in particular TNFa [58], or indirectly, by increased conversion of circulating androgens into estrogens, which are known to decrease LH pulse [59].

4. Hypogonadism and Metabolic Syndrome: Clinical Evidence

Excess visceral fat and related comorbidities define a condition named metabolic syndrome, characterized by hypertension, obesity, dyslipidemia, type 2 diabetes, and insulin resistance [60, 61].

It is well established that testosterone deficiency frequently results in loss of libido and erectile dysfunction, which can be easily restored by androgen replacement therapy. Moreover, androgens directly or indirectly affect every body compartment outside reproductive organs including body composition, bone density, physical and cognitive function [62].

Patients with testosterone levels below 8 nmol/L (230 ng/dL) benefit from testosterone replacement, and testosterone administration is considered if total serum testosterone level is between 8 and 12 nmol/L, and symptoms and signs suggestive of testosterone deficiency (obesity, hypertension, dyslipidemia, insulin resistance, erectile dysfunction, decreased muscle mass and strength, decreased bone mineral density, and depressed mood) are present [63].

In recent years, several lines of evidence focused on the frequent association of hypogonadism, obesity, and MetS. Patients with MetS show significantly lower testosterone plasma levels in comparison with healthy individual [54–57, 64, 65]. Furthermore, MetS is associated with low testosterone levels independently from the criteria applied, supporting the concept that MetS can be considered as an independent risk factor for male hypogonadism [66]. In addition, prospective studies have demonstrated that low testosterone levels predict the development of diabetes and MetS [67–69]. In this context, low plasma levels of testosterone and SHBG may be early markers of MetS in nonobese men, providing a warning sign in normal weight men, considered at lower risk of developing MetS [67–69].

On the other hand, low testosterone levels could contribute to the accumulation of excess fat, establishing a vicious cycle. In fact, hypogonadism is known to induce (a) a muscle mass reduction and visceral fat mass increase, (b) insulin resistance, and (c) an increase of the activity of lipoprotein lipase (LPL), the main enzymatic regulator of triglyceride uptake in the fat cell, preferentially in abdominal fat [62].

Interesting studies evaluated body composition changes in men undergoing androgen deprivation therapy for nonmetastatic prostate cancer. 12 and 48 weeks of androgen deprivation determined a significant increase of BMI and fat mass [70] and an increased incidence of diabetes and cardiovascular disease [71].

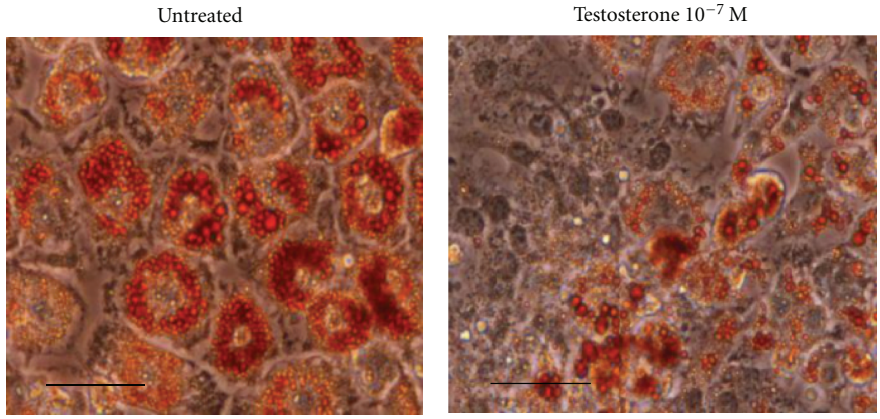

Figure 1: Effect of testosterone on 3T3-L1 adipose differentiation. Red oil staining of mature 3T3-L1 adipocytes (scale bar, 70 μm) (Caprio M. et al., unpublished), classically differentiated in the absence (left) or in the presence (right) of 10^{-7} M testosterone. Testosterone treatment determines a marked reduction in size and number of lipid droplets, in accordance with previous reports [73].

Rapid weight loss with successful weight maintenance in obese men with MetS induced a sustained increase in free testosterone levels [72]. Also, testosterone treatment of obese, insulin-resistant, nondiabetic and diabetic men has been shown to reduce fat mass, increase lean body mass, decrease waist circumference, which represents a valid parameter of the degree of visceral obesity, finally improving HOMA index and glycemic control [58, 66].

The frequent association of hypogonadism and obesity has led a group of experts to formulate the following recommendation: patients with clinical conditions associated with insulin resistance (obesity, type 2 diabetes, MetS) should be screened for testosterone deficiency, given that such conditions often coexist [63].

5. Effects of Low Testosterone Levels on Body Fat Mass

As shown in the previous paragraph, several lines of evidence strongly suggest that androgens influence body fat distribution and accumulation (see Figure 1). Men affected by androgen resistance due to gene inactivation of the AR show high visceral fat [43]. Male mice lacking AR develop obesity with increased lipogenesis in WAT and liver [74, 75]. In males, the antiobesity action of testosterone might be indirectly mediated via AR signalling in skeletal muscle. In fact, testosterone promotes the commitment of pluripotent cells of mesenchymal origin into myogenic lineage *in vitro*, by inhibiting adipogenic differentiation. These effects are mediated through an AR-dependent mechanism [76]. The same authors a few years later demonstrated that in 3T3-L1 preadipocytes, AR modulates adipogenic differentiation by directly activating downstream Wnt effector molecules, including β-catenin, T-cell factor (TCF), and lymphoid-enhancer factor (LEF) [73] (see Figure 1).

Recently, we have demonstrated that Drospirenone (DRSP), a progestogen with a modest antiandrogenic activity, widely used for contraception [77–79], strongly inhibits adipose differentiation both in murine (3T3-L1) as well as in human preadipocytes *ex vivo*. It is important to remark that DRSP is a powerful antagonist of the mineralocorticoid receptor (MR), which is a pivotal factor for the induction of adipogenesis. We have shown that the antiadipogenic effect of DRSP relies on specific antagonism on the MR [80]. Surprisingly, DRSP antiadipogenic effect is blunted in presence of testosterone, whereas we could have predicted a synergic effect on the inhibition of adipogenesis. In order to explain these data, we hypothesize that chronic treatment of preadipocytes *in vitro* with testosterone could upregulate AR, as already shown in different cellular models [81, 82]. As a consequence, increased levels of AR may bind DRSP as an AR antagonist, and the overall availability of DRSP as an anti-MR could result reduced.

AR activation in skeletal muscle might indirectly decrease WAT mass through increased muscle oxidative metabolism or through the release of an unknown circulating factor [49]. Indeed, in muscle cells of transgenic male rats overexpressing AR, increased lean mass with hypertrophy of type IIb fibers, increased oxidative metabolism, and decreased adipocyte size and WAT mass are observed [83]. However, adipose-specific AR knockout mice are not obese and show increased WAT production of leptin without leptin resistance [84]. Authors conclude that in adipocytes AR plays an inhibitory role in leptin production [84], but lack of androgens signalling in the adipocyte is not sufficient to promote obesity. Probably, the adipocyte is not the only player in the complex regulation of fat metabolism, and other cell types in its context could represent important targets of androgens. Tissue-specific inactivation of AR is deemed necessary to clarify these aspects.

6. Future Perspectives and Conclusions

Experimental and clinical studies indicate that adipose tissue and gonads communicate and influence each other, either directly or indirectly, through several circulating factors (see Figures 2(a) and 2(b)). Obesity is often associated with low plasma-testosterone levels and reproductive dysfunction,

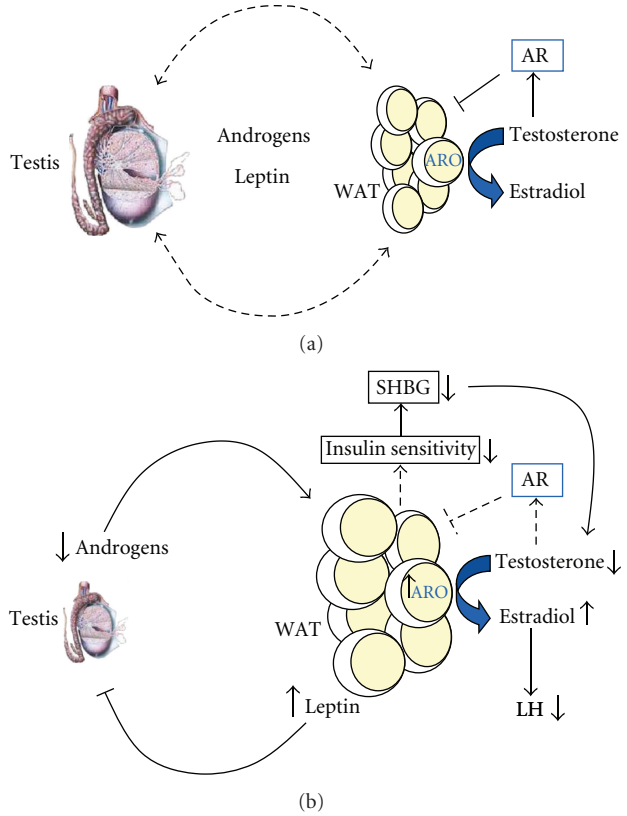

Figure 2: Reciprocal interplay between white adipose tissue (WAT) and testis. (a) In normal conditions, circulating androgens control adipocyte size and adipose mass. On the other hand, plasmatic leptin, mainly produced by adipose tissue, regulate testicular steroidogenesis. Dashed bars indicate the reciprocal interplay between testis (through androgens) and adipose tissue (mainly through leptin). (b) Androgen deficiency induces expansion of fat mass and subsequent dysregulation of several functions controlled by adipose tissue such as insulin sensitivity, blood pressure, vascular reactivity, and immunity. The state of insulin resistance determined by obesity leads to reduced production of SHBG. The consequent reduction in testosterone triggers expansion of adipose mass with subsequent increase in aromatase (ARO) activity, which in turn mediates peripheral conversion of testosterone to estradiol. Increased estrogen levels induce a reduction of LH pulse which contributes to the reduction in androgen production. On the other hand, excess of circulating leptin, due to increased adipose mass, disrupts testicular steroidogenesis, with consequent suppression of androgen production. The vicious cycle is triggered. AR: androgen receptor.

given that low testosterone may not be necessarily observed in human obesity, nor be the only cause of visceral obesity. This can be due to excessive circulating levels of leptin, which have been shown to disrupt the steroidogenic function of Leydig cell, with a subsequent reduction in hCG-driven testosterone production. Other adipokines, together with leptin, could play a direct or indirect role in the alteration of Leydig cell function in obesity.

On the other hand, circulating levels of sex hormones control fat mass distribution and expansion, mainly through activation of estrogen and androgen receptors in adipose tissue. Of interest, a recent work highlighted the profound impact of testosterone on cardiovascular function improving functional capacity, heart rate, muscle strength, and glucose metabolism in elderly patients with coronary heart failure [85]. We hypothesize that the cardiovascular effects of testosterone may be also mediated by adipose tissue, which embeds the heart and the most important vessels (coronaries, carotids, aorta, etc.) and is an active site of conversion of androgens into estrogens, through aromatase activity.

In conclusion, adequate levels and balance of circulating sex hormones are necessary to maintain a correct distribution and size of adipose tissue, which in turn is fundamental to keep a normal reproductive and sexual function. For this reason, screening of obese patients for hypogonadism is deemed necessary in order to better understand the pathophysiology of coexistent metabolic alteration, in order to target it with a replacement therapy. The delicate issue of whether testosterone decline, observed with aging, causes adipose tissue accumulation, or whether weight gain primarily disrupts testicular steroidogenesis, is still unclear and needs further studies.

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Review Article

Genetics of Isolated Hypogonadotropic Hypogonadism: Role of GnRH Receptor and Other Genes

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Hypothalamic gonadotropin releasing hormone (GnRH) is a key player in normal puberty and sexual development and function. Genetic causes of isolated hypogonadotropic hypogonadism (IHH) have been identified during the recent years affecting the synthesis, secretion, or action of GnRH. Developmental defects of GnRH neurons and the olfactory bulb are associated with hyposmia, rarely associated with the clinical phenotypes of synkinesia, cleft palate, ear anomalies, or choanal atresia, and may be due to mutations of KAL1, FGFR1/FGF8, PROKR2/PROK2, or CHD7. Impaired GnRH secretion in normosmic patients with IHH may be caused by deficient hypothalamic GPR54/KISS1, TACR3/TAC3, and leptinR/leptin signalling or mutations within the GNRH1 gene itself. Normosmic IHH is predominantly caused by inactivating mutations in the pituitary GnRH receptor inducing GnRH resistance, while mutations of the β-subunits of LH or FSH are very rare. Inheritance of GnRH deficiency may be oligogenic, explaining variable phenotypes. Future research should identify additional genes involved in the complex network of normal and disturbed puberty and reproduction.

1. Introduction

Normal pubertal development and reproductive function depends on the intact release and action of hypothalamic gonadotropin releasing hormone (GnRH). As a precondition, distinct developmental and functional procedures involving the coordinated action of other hypothalamic hormone-receptor systems are required for GnRH disposal. The detailed diagnostic workup of patients with absent or incomplete pubertal development due to gonadotropin deficiency has recently led to the identification of new genetic causes of isolated hypogonadotropic hypogonadism (IHH) [1–7]. These findings currently improve our understanding of how the onset and course of puberty and reproduction are controlled. The precise classification of the underlying defect in the patient with IHH may, in turn, improve the clinical management including choice and timing of therapeutic intervention.

2. Normal Onset of Puberty

The hypothalamic GnRH pulse generator constitutes the basis of the CNS control of puberty. GnRH secretion is suppressed during childhood via inhibitory neurotransmitters, mainly gamma aminobutyric acid (GABA) and opioid peptides [8]. After a rest period from approximately two until 8 to 9 years of age, declining inhibitory components and amplifying excitatory transmitters including glutamate and kisspeptin enhance GnRH secretion.

The pubertal increase in GnRH secretion is initiated and prompted by changes in transsynaptic and glial inputs to the GnRH neuronal network [8]. Kisspeptins coordinate environmental and metabolic factors for regulation of the hypothalamic-pituitary-gonadal axis through modulation of GnRH, LH, and FSH secretion and steroid feedback [9]. The pulsatile GnRH release from GnRH-containing neurons with frequency and amplitude modulation is the main

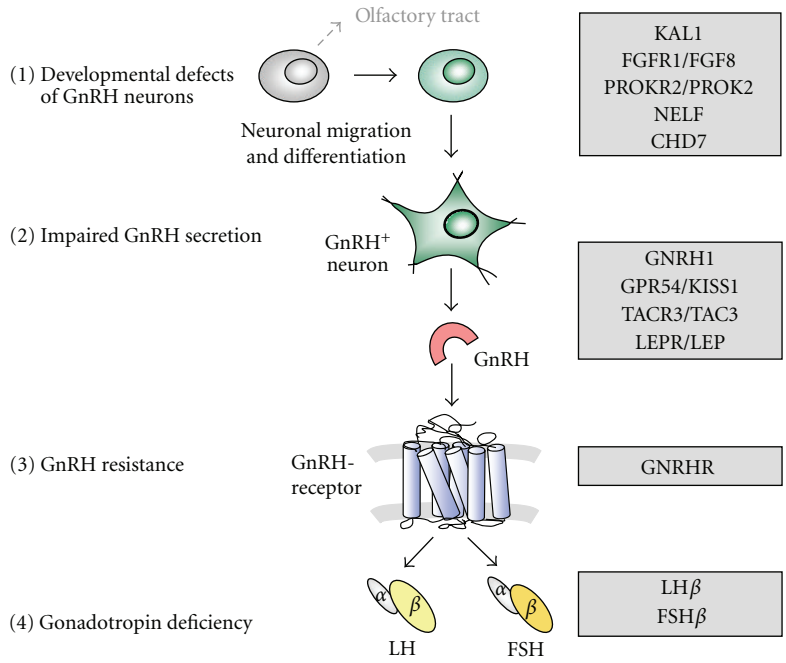

Figure 1: Genetic control of pubertal development. Different levels of GnRH and gonadotropin deficiency due to genetic disorders. (1) Developmental defects of GnRH neurons due to disturbed neuronal migration and differentiation cause aplasia of GnRH neurons and olfactory tract. (2) Impaired GnRH synthesis or secretion is found in the context of functional disorders within the hypothalamus or the GnRH neuron itself. (3) GnRH resistance is caused by inactive GnRH receptor variants localised within the anterior pituitary gland. (4) Gonadotropin deficiency may be due to defect synthesis of LH or FSH β-subunits.

determinant of system activation with progression into and through puberty.

The stimulatory decapeptide GnRH binds in a hairpin structure to its transmembrane receptor expressed in pituitary gonadotrope cells [10]. The amino- and carboxy-terminal domains of GnRH contribute to receptor binding and activation via extracellular and transmembrane domains inducing conformational changes and signal transduction, thereby inducing synthesis and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These gonadotropins bind to their specific receptors in gonads and stimulate synthesis of estrogens and testosterone resulting in clinical signs of puberty. The functional integrity of this hypothalamic-pituitary-gonadal system is the precondition for normal reproductive function [9].

3. Causes of Isolated Normosmic Hypogonadotropic Hypogonadism and Kallmann's Syndrome

IHH is characterised by impaired gonadotropin release in the context of otherwise normal anatomical and functional anterior pituitary function. Serum concentrations for LH, FSH, and sex steroids are inappropriately low in the patient with hypogonadism. Clinical signs and symptoms of hypogonadism include bilateral cryptorchidism in males, absent or incomplete puberty with amenorrhea in females, and infertility. The underlying cause may be due to developmental defects of GnRH neurons, impaired functional activity within GnRH neurons, disturbed interaction between the GnRH ligand and its receptor, or the release of intact gonadotropins (Figure 1).

While aplasia of GnRH neurons occurs in the context of developmental defects of the olfactory bulb, the clinical symptom of anosmia indicates this kind of GnRH deficiency [4, 5, 11–15]. Kallmann's syndrome accounts for 50–52% of cases with IHH, while normosmic IHH is found in 48–50% of cases [16, 17]. Disorders of GnRH release have recently been identified as rare causes of GnRH deficiency in patients with normosmic IHH [2, 3, 7], while inactivating mutations of the GnRH receptor are the most frequent cause for normosmic IHH, especially in familial cases [12, 17–21].

4. Developmental Abnormalities of GnRH Neurons and Anosmia

Developmental defects of the olfactory bulb and GnRH secreting neurons in patients with Kallmann's syndrome are caused by genetic alterations regulating the migration of GnRH neurons from the forebrain to the hypothalamus (Table 1). The KAL1 encoded protein, anosmin-1, is an adhesion protein involved in synaptogenesis, cell adhesion, and olfactory axonal attraction and olfactory bulb morphogenesis [22]. Deletions and mutations of *KAL1* account for approximately 10% of Kallmann's syndrome patients [16, 17, 23]. Individuals with KAL1 mutations may present with additional symptoms such as bimanual synkinesia characterised by involuntary "mirror movements" (Figure 2) and renal

Table 1: Genetic causes of Kallmann's syndrome (KS) and normosmic isolated hypogonadotropic hypogonadism (IHH).

Gene	Gene product	Function	Inheritance	Clinical phenotype	Associated clinical phenotype
KAL1	Anosmin-1	Cell adhesion	X-linked	KS	Anosmia, bimanual synkinesis, renal agenesis
FGFR1	Fibroblast-growth-factor receptor 1	Tyrosine kinase receptor	AD	KS or IHH	Anosmia, cleft lip or palate, ear anomalies, tooth agenesis
FGF8	Fibroblast growth factor 8	Ligand of FGFR1	AD	KS or IHH	
NELF	Nasal embryonic LHRH factor	Neuronal migration	AD	KS	Anosmia
CHD7	Chromodomain-helicase-DNA-binding protein 7	DNA-binding protein, neural crest development	AD	KS or IHH	CHARGE syndrome: anosmia, coloboma, heart anomaly, choanal atresia, retardation, ear abnormalities
PROKR2	Prokineticin receptor 2	GPCR	AD AR	KS or IHH	Anosmia
PROK2	Prokineticin 2	Ligand of PROKR2	AD AR	KS or IHH	Anosmia
WDR11	WD protein	Interaction with EMX1	AD	KS or IHH	Anosmia
GPR54/KISS1R	Kisspeptin-1 receptor	GPCR	AR	IHH	None
TACR3	Neurokinin B receptor	GPCR	AR	IHH	None
TAC3	Neurokinin B	Ligand of TACR3	AR	IHH	None
LEPR	leptin receptor	Single transmembrane-domain receptor	AR	IHH	Obesity
LEP	leptin	Fat-regulating hormone	AR	IHH	Obesity
GNRH1	GnRH	Release of LH and FSH	AR	IHH	None
GNRHR	GnRH receptor	GPCR	AR	IHH	None
LHβ	β-subunit of LH	Ligand of LH/CG receptor	AR	IHH	None
FSHβ	β-subunit of FSH	Ligand of FSH receptor	AR	IHH	None

GPCR: heptahelical transmembrane G-protein-coupled receptor, AD: autosomal dominant.
AR: autosomal recessive.

agenesis [16]. Since *KAL1* is a X-linked gene, familial Kallmann's syndrome occurring only in males suggests a KAL1 defect.

The fibroblast growth factor receptor (FGFR1) gene encodes a tyrosine kinase receptor involved in olfactory bulb development and GnRH neurite outgrowth via FGF signalling and the interaction between FGFR1 and anosmin-1 [22, 24]. Inactivating mutations of this receptor and one of its ligands, fibroblast growth factor 8 (FGF8), have been described in patients with variable degree of hypogonadism mainly with and in few cases without anosmia [4, 14, 15, 22, 25, 26]. In very few subjects with FGFR1 mutations, a complete reversal of GnRH deficiency has been reported [27–29]. Additional clinical signs observed in these individuals include cleft palate or lip, ear anomalies, and tooth agenesis [4, 15, 25, 29]. Heterozygous mutations and deletions of the FGFR1/ FGF8 system account for approximately 10% of Kallmann's syndrome and normosmic idiopathic hypogonadotropic hypogonadism [14, 25, 26].

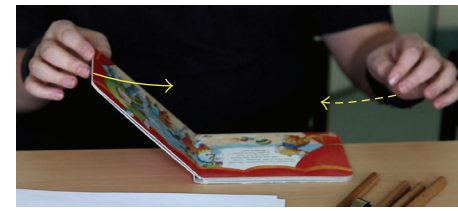

Figure 2: Kallmann's Syndrome. Synkinesia in a patient with KAL1 mutation (c.120_121insC; 122_127del, p.Ala41Glyfs43). When closing a book with one hand (arrow), typical involuntary mirror movement (synkinesia) of the other hand is observed (dotted arrow). This 15-year-old boy presented because of absent puberty. There was orchidopexy at the age of 2 years, disturbed spatial orientation, retarded fine-motor developmental milestones, and anosmia. Tanner P1, G1. LH <0.1 U/L, FSH 0.4 U/L, testosterone 0.2 ng/mL. During GnRH stimulation test, LH 0.5 U/L, FSH 2.1 U/L after 60 minutes. Cranial MRI revealed agenesis of olfactory bulbs and a normal-sized pituitary gland.

The prokineticin receptor 2 (PROKR2), a heptahelical transmembrane G protein-coupled receptor, and its ligand prokineticin 2 (PROK2) are expressed within the CNS including olfactory system, arcuate nucleus, suprachiasmatic nuclei, and median eminence [30, 31]. The PROKR2/PROK2 system is involved in olfactory bulb development and in GnRH neuron migration [32]. Heterozygous, compound heterozygous and homozygous inactivating mutations have been described within the PROKR2/PROK2 system, accounting for less than 10% of individuals with Kallmann's syndrome and normosmic GnRH deficiency [5, 32–34]. One patient with a heterozygous PROKR2 mutation has been reported with reversal of hypogonadism after treatment with testosterone [28, 34].

The human nasal embryonic LHRH factor (NELF) gene is a candidate gene for Kallmann's syndrome because of its association with axonal guidance of olfactory and GnRH neurons in mice [35]. Heterozygous mutations within the NELF gene have been reported in few patients with Kallmann's syndrome [36–38]. So far, the role of NELF in human reproduction is unclear, but NELF may be a critical modifier gene that orchestrates GnRH deficiency in conjunction with other pathogenic genes [36].

Mutations within the chromodomain helicase DNA-binding protein 7 (CHD7) have been identified in patients with CHARGE association, a syndrome in which hypogonadotropic hypogonadism and hyposmia are associated with choanal atresia, coloboma of the iris (Figure 3), cardiovascular malformations, retardation of mental and somatic development, and ear anomalies [13, 39]. Recently, CHD7 heterozygous mutations have been identified in subjects with hypogonadotropic hypogonadism, with and without anosmia [40]. Patients presenting some of the CHARGE syndrome features are more likely to carry CHD7 mutations [41]. CHD7 mutations are found in 5 to 10% of subjects initially classified as Kallmann's syndrome and normosmic IHH patients [40, 41].

Very recently, heterozygous mutations of *WDR11*, encoding a WD protein interacting with the transcription factor EMX1, have been identified in six patients with Kallmann's syndrome or idiopathic hypogonadotropic hypogonadism [42]. The interaction between WDR11 and EMX1 is critical for the development of olfactory neurons while WDR11 missense alterations reduce or abolish this interaction [42]. It was concluded from these results that disturbed pubertal development in these patients is caused by deficient WDR11 protein interaction [42].

5. Defects of GnRH Release and Synthesis

The identification of inactivating mutations within the G-protein-coupled receptor 54 (GPCR54/KISSR) gene has demonstrated the role of kisspeptin, the ligand of GPR54, in the control of GnRH secretion [2, 3, 43–45] (Table 1). GPR54/KISSR is a heptahelical transmembrane receptor, expressed at the surface of GnRH neurons. GPR54 activation via kisspeptin induces GnRH secretion [9]. Neuroendocrine profiles of subjects with GPR54/KISSR mutations revealed low amplitude of LH pulses, suggesting low degree of endogeneous GnRH secretion [3, 46]. Male patients may present at birth with micropenis and cryptorchidism and undetectable gonadotropin levels [43, 46]. GPR54/KISSR mutations account for 2–5% of normosmic IHH [2, 3, 43]. Until now, mutations within the gene of the ligand of GPR54/KISSR, KISS1, have not been described in patients with IHH.

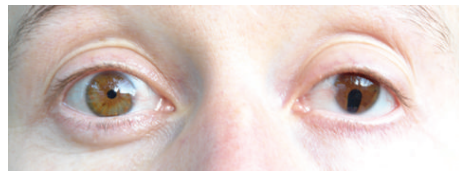

Figure 3: Iris coloboma as a typical characteristic of CHARGE syndrome. A woman with hypogonadotropic hypogonadism and CHARGE syndrome (CHD7 mutation c.4787A>G, p.Asp1596Gly) initially presented at the age of 16 years because of absent puberty. There was a history of choanal atresia, deafness, learning disorders, and anosmia. Tanner B1, P2. LH 0.2 U/L, FSH 0.54 U/L, estradiol 24 pg/mL. During GnRH stimulation test, LH 1.61 U/L, FSH 1.86 U/L.

Very recently, homozygous loss-of-function mutations in TAC3, encoding neurokinin B and its heptahelical transmembrane G-protein-coupled receptor TACR3, have been detected in patients with normosmic IHH [7, 47, 48]. Affected subjects showed very low basal LH secretion with nonpulsatile pattern while pulsatile GnRH treatment normalised LH release and circulating sex steroids [47]. These findings indicate a crucial role of NKB, via its receptor NK3R, in hypothalamic GnRH release [47]. The majority of male patients with TACR3/TAC3 mutations presented with micropenis and lack or pubertal development while recovery of GnRH deficiency was observed in a significant number of male and female adult patients [48]. These observations support the importance of the TACR3/TAC3 signaling during the neonatal period and puberty while its role seems less critical in adulthood [48].

The role of leptin for pubertal development and reproduction has been demonstrated in leptin-null (ob/ob) mice in which leptin administration accelerates puberty and normalises reproductive dysfunction [49]. Leptin, encoded by LEP, is a fat-derived hormone regulating food intake, energy expenditure, and hypothalamic reproductive function. Inactivating mutations in LEP or its receptor LEPR, a single transmembrane-domain receptor of the cytokine receptor family, have been described in patients with hypogonadism and obesity [50–52]. These loss-of-function mutations are rare causes of normosmic IHH. Treatment with recombinant leptin reconstitutes gonadotropin secretion and menstrual cycles in females with amenorrhea due to congenital leptin deficiency [53] or hypothalamic amenorrhea [54].

The most obvious candidate gene for patients with hypogonadotropic hypogonadism was GnRH itself after description of the hypogonadal mouse model with homozygous deletion within the GNRH1 gene [55, 56]. However, several studies initially failed to identify GNRH1 gene mutations in humans with hypogonadotropic hypogonadism [57, 58].

Very recently, homozygous frameshift mutations within the GNRH1 gene, encoding the preprohormone of GnRH, have been identified in patients with IHH [6, 59]. In accordance with the critical role of GnRH, male patients presented with severe hypogonadism including micropenis. *GNRH1* mutations are rare causes of normosmic isolated GnRH deficiency.

6. GnRH Resistance and Gonadotropin Deficiency

Binding of GnRH to its heptahelical transmembrane receptor in the pituitary gland induces receptor activation and signal transduction, finally resulting in secretion of gonadotropins. Since the first description of loss-of-function mutation in the GnRH receptor (GnRHR) [1], many inactivating mutations have been found within the extracellular, transmembrane and intracellular domains of the receptor [11, 19–21] leading to impaired GnRH action (Figure 1). Depending on the degree of functional impairment, these patients present with complete absence of pubertal development or with incomplete puberty [19]. Loss-of-function mutations within the GnRH receptor are the most frequent cause of autosomal-recessive IHH, accounting for 16% to 40% of patients [18, 21, 60]. Since these patients are resistant to GnRH, the effective fertility treatment is achieved with gonadotropins.

Mutations of the β-subunits of luteinizing hormone (LH) or follicle-stimulating hormone (FSH) are rare causes of hypogonadotropic hypogonadism. LH and FSH are glycoprotein hormones, as thyroid-stimulating hormone and human chorionic gonadotropin (hCG). These heterodimeric hormones consist of a common α-subunit and a specific β-subunit, encoded by separate genes. Females with inactivating mutations of the LH β-subunit present with normal puberty, with normal or late menarche followed by oligo- or amenorrhea and infertility due to lack of ovulation [61]. Ovaries in affected women may be enlarged with cysts [62]. Males with inactivating mutations of the LH β-subunit have absent pubertal development due to testosterone deficiency and azoospermia in adulthood because of Leydig-cell hypoplasia [61–63]. Testosterone replacement may result in an increase of testicular volume in the context of high FSH levels [61]. Individuals with inactivating FSHβ mutations present with incomplete pubertal development and primary amenorrhea in females and azoospermia in males [64–66]. Treatment with recombinant FSH induces ovulation but was associated with signs of ovarian hyperstimulation which may be explained by high pretreatment LH levels [67].

7. Clinical Implications

Since pulsatile GnRH secretion is required for descent of the testis in the male fetus, patients with gonadotropin deficiency during fetal life may present with cryptorchidism and variable degree of male undervirilisation. Additional symptoms such as impaired sense of smell, bilateral synkinesia, cleft palate, or choanal atresia are suspicious for specific congenital diseases associated with GnRH deficiency (Table 1). Absent or incomplete pubertal development leading to detailed diagnostic workup may identify congenital GnRH or gonadotropin deficiency.

Hormonal replacement therapy during adolescence is frequently delayed, although earlier signs and symptoms of the patient would have predicted hypogonadotropic hypogonadism. Since hormonal induction of puberty does not always require the definite identification of the underlying cause of GnRH or gonadotropin deficiency, some individuals are investigated only later in life because of infertility. In most cases of IHH, gonadotropin treatment induces ovulation and spermatogenesis [68, 69], while patients with inactive GnRHR variants will not respond to normal doses of GnRH treatment [70, 71]. This GnRH resistance has been overcome with higher GnRH doses in one subject with partially inactivated GnRH receptor mutations [72].

In addition to absent or incomplete pubertal development and infertility, further clinical variants of GnRH and gonadotropin deficiency associated with genetic variants have been recently observed. These variants include adult-onset idiopathic hypogonadotropic hypogonadism [73], functional hypothalamic amenorrhea [74], and spontaneous reversals of well-established GnRH deficiency following long-term therapy with testosterone [28]. Although the mechanisms of reversal of hypogonadotropic hypogonadism are unclear, it is speculated that GnRH neuron plasticity in adults may be modulated by sex steroids [28]. Brief discontinuation of hormonal replacement may, therefore, be reasonable to assess if hypogonadotropic hypogonadism is reversible or persistent [28].

After a detailed individual and family history and physical examination evaluating the degree of hypogonadism and presence of associated clinical symptoms (e.g., Figures 2 and 3), a molecular genetic analysis enables in many cases definition of the underlying defect. Monogenic, digenic, or even oligogenic inheritance of GnRH deficiency has been observed explaining the variable phenotypic spectrum [17, 28, 36]. Alterations in two or more distinct genes in one patient may induce a more severe phenotype than a single-gene mutation and lead to the overlap of two or more clinical syndromes. Rare genetic variants may further contribute to the susceptibility of individuals to functional changes in GnRH secretion such as hypothalamic amenorrhea, a common multifactorial disease [74]. Genetic counselling is offered in case of genetic diagnosis to first-degree family members. However, approximately 60–70% of cases with Kallmann's syndrome and 50% of patients with normosmic IHH are of unknown origin [60]. These patients and families should be encouraged to participate in ongoing research projects including DNA biobanking. In any case, early diagnosis of GnRH deficiency during childhood represents the requisite for induction of puberty in due time.

8. Hormonal Treatment of IHH

The hormonal induction of puberty in a hypogonadal adolescent aims to mimic normal pubertal development. Hormone replacement in adolescents is usually initiated with low dose of sex steroids and augmented over 3 to 5 years until

mature status is reached. In girls, estradiol orally is preferred, starting with one-sixths of the adult dose daily, increasing every 6 months by 1/6 and adding gestagens from the second year on day 1 to 12 of each month [75–77]. In boys, testosterone replacement is initiated most frequently with testosterone enanthate 50 mg per month intramuscularly, with increasing dose every 6 months until 250 mg is given every 3 weeks in the third year. While testosterone treatment effectively induces virilisation including penile growth, pubic and male hair and beard growth, change of voice, libido, and pubertal growth spurt, testicular volume remains small, lacking spermatogenesis. LH stimulates intratesticular testosterone secretion by Leydig cells inhibiting Anti-Müller's hormone production of the Sertoli cells, FSH induces testis growth via proliferation of seminiferous tubules, and both stimulate Inhibin B secretion by the Sertoli cells and sperm maturation. Therefore, induction of puberty using gonadotropins or pulsatile GnRH seems a more physiologic approach in the adolescent with hypogonadotropic hypogonadism and has been successfully used [78–82]. To further assess the benefit of GnRH or gonadotropin treatment for pubertal induction, prospective randomised trials are needed.

During adulthood testosterone replacement may be continued by daily transdermal application of testosterone gel or injection of the long-acting testosterone undecanoate intramuscularly every 3 months. Fertility treatment usually requires gonadotropin treatment with hCG and FSH or may alternatively, initiated by pulsatile GnRH treatment [80–82]. GnRH given every 90 minutes by a subcutaneous placed pump is the most physiologic therapy of GnRH deficiency, except in case of GnRH resistance, but is associated with higher costs and technical support. In rare cases of leptin deficiency, specific leptin treatment has been effective for treatment of hypogonadism [53, 54]. In general, long-term replacement of sex steroids is required not only for sexual and reproductive function but also for bone health and metabolic (glucose and fat) integrity in patients with hypogonadotropic hypogonadism.

9. Conclusion

The discovery of new genetic causes of hypogonadotropic hypogonadism gave new insights into the regulation of puberty and reproduction in humans. With the identification of genetic variants in GnRH-deficient patients, it became clear that monogenic, digenic, and oligogenic traits of inheritance may explain the variable phenotypic spectrum. In more than 50% of patients with IHH, the underlying defect is still unknown, demonstrating the need for further research activity in this field. The precise diagnosis facilitates appropriate treatment and counselling in affected patients. Established treatment procedures for hormonal induction of puberty might be reconsidered, since pulsatile GnRH and gonadotropin treatment are effective and more physiologic alternatives. To investigate the benefit of different therapeutic options on quality of life and fertility, prospective randomised controlled trials with long-term followup have to be conducted. For these future research directions, national and international scientific networking will be advantageous.

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Research Article

Clinical and Laboratorial Features That May Differentiate 46,XY DSD due to Partial Androgen Insensitivity and 5α-Reductase Type 2 Deficiency

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The aim of this study was to search for clinical and laboratorial data in 46,XY patients with ambiguous genitalia (AG) and normal testosterone (T) synthesis that could help to distinguish partial androgen insensitivity syndrome (PAIS) from 5α-reductase type 2 deficiency (5α-RD2) and from cases without molecular defects in the *AR* and *SRD5A2* genes. Fifty-eight patients (51 families) were included. Age at first evaluation, weight and height at birth, consanguinity, familial recurrence, severity of AG, penile length, LH, FSH, T, dihydrotestosterone (DHT), Δ4-androstenedione (Δ4), and T/DHT and T/Δ4 ratios were evaluated. The *AR* and *SRD5A2* genes were sequenced in all cases. There were 9 cases (7 families) of 5α-RD2, 10 cases (5 families) of PAIS, and 39 patients had normal molecular analysis of *SRD5A2* and *AR* genes. Age at first evaluation, birth weight and height, and T/DHT ratio were lower in the undetermined group, while penile length was higher in this group. Consanguinity was more frequent and severity of AG was higher in 5α-RD2 patients. Familial recurrence was more frequent in PAIS patients. Birth weight and height, consanguinity, familial recurrence, severity of AG, penile length, and T/DHT ratio may help the investigation of 46,XY patients with AG and normal T synthesis.

1. Introduction

The disorders of sex development (DSD) with sex ambiguity and 46,XY karyotype can be classified in three main groups: (1) disorders of gonadal development (ovotesticular DSD and partial gonadal dysgenesis), (2) disorders of testosterone synthesis (testosterone biosynthesis defects like steroidogenic acute regulatory protein (STAR) deficiency, side-chain cleavage (CYP11A1) deficiency, 3β-hydroxysteroid dehydrogenase type II (HSD3B2) deficiency, 17α-hydroxylase/17,20-lyase (CYP17A1) deficiency, 17β-hydroxysteroid dehydrogenase III (HSD17B3) deficiency, P450 oxidoreductase (POR) defect; cytochrome b5 (CYB5) defect, and defects in luteinizing hormone action (LHCGR defect)), and (3) disorders

of testosterone action [partial (PAIS) androgen insensitivity syndromes] or metabolization (5α-reductase type 2 deficiency) [1].

The main diagnosis for patients with ambiguous genitalia and 46,XY karyotype (46,XY DSD) with normal testosterone secretion and normal Müllerian duct regression is PAIS or 5α-reductase type 2 deficiency. Before puberty, the phenotypes of 46,XY DSD due to androgen insensitivity syndromes or 5α-reductase type 2 deficiency are, in general, indistinguishable, particularly when there is no parental consanguinity (5α-reductase type 2 deficiency is an autosomal recessive disorder) or family history consistent with X-linked inheritance (androgen insensitivity syndromes) [2–5].

Despite the multiple genetic causes of 46,XY DSD, around 30–40% of cases remain without diagnosis [6]. Currently, there is a frequent, nongenetic variant of 46,XY DSD characterized by reduced prenatal growth and lack of clear evidence for any associated malformation or steroidogenic defect. Additionally, other studies in undetermined 46,XY DSD report that around 30% of cases are associated with low birth weight, indicating that adverse events in early pregnancy are frequent causes of congenital nongenetic 46,XY DSD [7, 8].

For that reason, the aim of this study was to search for clinical and laboratorial features of 46,XY patients with ambiguous genitalia and normal testosterone synthesis that could help to distinguish PAIS from 5α-reductase type 2 deficiency and from cases without molecular defects in the *AR* and *SRD5A2* genes.

2. Methods

In the last 10 years (from January 2001 to December 2010), the Interdisciplinary Group for the Study of Sex Determination and Differentiation (GIEDDS) from the Clinical Hospital of the Faculty of Medical Sciences of State University of Campinas (UNICAMP), Brazil evaluated 58 patients (51 families) with ambiguous genitalia having a 46,XY karyotype and normal testosterone secretion after hCG stimulation. This study was performed according to the Helsinki declaration and was approved by the Ethical Research Committee of Faculty of Medical Sciences of UNICAMP. Informed consent was obtained from all participants and from parents of participants under 18 years of age.

All patients included in this study were born at term. They underwent laparoscopy during genitoplasty, orchidopexy, and/or gonadectomy, and no Müllerian structures were found.

Data were obtained regarding age at first evaluation, weight and length at birth, history of parental consanguinity, family history of ambiguous genitalia or infertility, severity of the ambiguous genitalia (according to Sinnecker et al. classification [9] and Ahmed et al. external masculinization score (EMS) [10]), penile length (in z score according to Gabrich et al. [11]), levels of LH, FSH, total testosterone (T), dihydrotestosterone (DHT), T/DHT ratio, Δ4-androstenedione, and T/Δ4 ratio. LH, FSH, and Δ4-androstenedione were evaluated by chemiluminescence immunoassay, T and DHT by radioimmunoassay. T was evaluated at basal levels in all patients, and in all prepubertal patients, a stimulation test was carried out by giving 1,500 IU of hCG by intramuscular injections for three consecutive days on an outpatient basis. Venous samples were taken before the test and approximately 24 hours after the third hCG injection and T was considered normal if above than 1,5 ng/mL [11]. In patients with complete puberty (Tanner 4 or 5), a hCG-stimulation test was carried out only if T was below 9 ng/mL.

Molecular analyses of *SRD5A2* and *AR* genes were performed for all patients. The eight exons of *AR* gene and the five exons of *SRD5A2* were amplified from genomic DNA using the polymerase chain reaction (PCR) followed by sequencing the fragments with Big Dye Terminator Cycle Sequencing Kit V3.1 Ready Reaction (ABI PRISM/PE Biosystems). The sequences were compared with the normal sequence of each gene (ENSEMBL: ENSG00000049319 and ENSG00000169083) using CLC Sequence Viewer v.6.2 (free software).

According to clinical and molecular data, the cases were classified in three groups of diagnosis: (1) 5α-reductase type 2 deficiency, (2) PAIS, and (3) undetermined (without molecular defects in either *AR* or *SRD5A2* gene).

Data were processed in the SPSS program for Windows, version 16.0, and descriptive analyses for continuous variables were made by calculating range, means and standard deviation. The data were compared among the three groups using Chi-square test or Fisher's exact test for categorical variables and Kruskal-Wallis test for continuous variables. For all analyses, a significance level of $P < 0.05$ was adopted.

3. Results

Data from all 58 patients are shown in Tables 1, 2, and 3. Nine patients (7 families) showed homozygous or compound heterozygous mutations in *SRD5A2* gene (Table 1), confirming the diagnosis of 5α-reductase type 2 deficiency in these patients. Only c.278delG mutation (patient 4: Table 1) was not yet described in the literature. Ten patients (5 families) showed hemizygous mutation in *AR* gene, confirming the diagnosis of PAIS (Table 2). The remaining 39 cases showed normal molecular analysis of *SRD5A2* and *AR* genes (Table 3).

Table 4 shows the frequency of parental consanguinity, familial recurrence, and severity of ambiguous genitalia in the three groups. The frequency of parental consanguinity was significantly higher in patients with 5α-reductase type 2 deficiency ($\chi^2_{(2)} = 19.86$, $P = 0.00005$), whereas familial recurrence was significantly more frequent in the groups of PAIS ($\chi^2_{(2)} = 8.14$, $P = 0.02$). The severity of ambiguous genitalia according to Sinnecker et al. [9] classification ($\chi^2_{(2)} = 15.49$, $P = 0.0004$) and according to Ahmed et al. [10] score ($\chi^2_{(2)} = 20.89$, $P = 0.00003$) was significantly higher in the group of 5α-reductase type 2 deficiency and PAIS in relation to undetermined cases. Analyzing only the patients with 5α-reductase type 2 deficiency and PAIS, the Sinnecker et al. [9] classification was significantly higher in PAIS ($\chi^2_{(2)} = 6.41$, $P = 0.04$), while the Ahmed et al. [10] score did not show significant differences between these two groups of patients

Table 1: Data from 9 patients (7 families) of 5α-reductase type 2 deficiency.

Case	Age 1 (yr)	Birth weight (g)	Birth length (cm)	Penile (z)	Genital[1]	Genital[2]	T (ng/mL)	T/DHT	Mut1	Mut2
1[c]	0.06	3220	50	−4.6	4	3.0	2.8	28	p.G183S	p.G183S
2[c]	18.2	2900	48	−4.2	3	2.5	9.0	45	c.418delT	c.418delT
3[c]	14.6	2800	47	−4.1	3	2.0	2.8	28	p.R246W	p.R246W
4	5.4	2700	48	−4.1	2	9.0	2.2	73	c.278delG	c.278delG
5*[1]	3.0	2810	47	−4.0	3	1.0	1.8	60	p.Q126R	p.G158R
6*[1]	0.05	3500	50	−4.0	3	3.0	2.2	44	p.Q126R	p.G158R
7[c,r]	16.7	2600	47	−4.2	3	2.5	4.9	82	p.G196S	p.G196S
8*[2c,r]	17.3	2900	49	−3.9	3	5.5	13.6	68	p.Q126R	p.Q126R
9*[2c,r]	11.0	2600	48	−3.1	3	4.0	1.9	63	p.Q126R	p.Q126R

Age1: age at first evaluation, T: total testosterone, Mut1: mutation 1, Mut2: mutation 2, +: present, −: absent, *: related, c: presence of consanguinity, r: presence of familial recurrence, Genital[1]: external genitalia according to Sinnecker et al. [9], Genital[2]: external genitalia according to Ahmed et al. [10].

Table 2: Data from 10 patients (5 families) of PAIS.

Case	Age1 (yr)	Birth weight (g)	Birth length (cm)	Penile (z)	Genital[1]	Genital[2]	T (ng/mL)	T/DHT	Mutation
1*[1c]	28.8	2900	48	−8.7	4	1.0	10.0	33	p.L830F
2*[1c]	18.8	3330	48	−8.4	4	2.0	15.0	50	p.L830F
3*[1c]	3.0	2800	47	−6.2	4	2.0	3.2	32	p.L830F
4*[1c]	1.6	2650	46	−5.2	4	2.0	1.8	60	p.L830F
5*[1c]	0.2	3180	50	−6.2	4	5.5	2.0	67	p.L830F
6*[2]	0.8	2630	46	−2.7	2	5.5	1.9	63	p.A596T
7*[2]	0.2	2900	46	−7.5	2	2.0	1.7	34	p.A596T
8	1.3	3950	49	−3.1	2	6.0	2.9	72	p.A896V
9	19.2	3400	51	−7.5	3	1.0	9.6	46	p.R855H
10[c]	6.1	3150	49	−4.1	3	3.0	2.3	57	p.M742V

Age1: age at first evaluation, T: total testosterone, +: present, −: absent, *: related, c: presence of consanguinity, r: presence of familial recurrence, Genital[1]: external genitalia according to Sinnecker et al. [9], Genital[2]: external genitalia according to Ahmed et al. [10].

(Fisher = 1.00). However, these classifications showed high negative correlation ($r = -0.675$, $P = 0.0001$).

Table 5 shows range, mean and standard deviation of age at first evaluation, weight and height at birth, penile length (in z score), levels of T, Δ4-androstenedione, and T/Δ4 and T/DHT ratios in the three groups. The levels of LH and FSH were not compared because only a few patients were in pubertal age in each group of diagnosis (5α-reductase type 2 deficiency: 4 patients, PAIS: 1 patient, and undetermined: 2 patients). The age at first evaluation was significantly lower in idiopathic cases ($P = 0.02$). Weight ($P = 0.002$) and length ($P = 0.02$) at birth and T/DHT ratio ($P = 0.0001$) were significantly lower in undetermined cases, and penile length was significantly higher in this group ($P = 0.0001$). The T ($P = 0.07$) and Δ4-androstendione ($P = 0.12$) levels and T/Δ4 ratio ($P = 0.32$) did not differ among the three groups. All patients showed normal Δ4-androstendione levels for age and pubertal stage and T/Δ4 ratio above 0.8.

4. Discussion

Clinical and laboratorial investigation must include careful and precise anatomical and hormonal studies (both basal and after stimulation) prior to gender assignment in 46,XY patients with undermasculinization (46,XY DSD), which can be difficult in most cases [1, 8, 12]. The phenotypes of 46,XY DSD due to 5α-reductase type 2 deficiency, PAIS, and disorders in testosterone synthesis may be indistinguishable in newborns [1–6, 8, 12]. The differential diagnosis of PAIS and 5α-reductase type 2 deficiency should be established as soon as possible because individuals with PAIS are usually recommended to be raised as females, whereas those with 5α-reductase type 2 deficiency as males, when the diagnosis is made early in childhood [1, 6, 8, 12–14].

A correct and early diagnosis is very important because as a result of pre- and/or postnatal brain exposure to androgens, almost 70% of individuals with 5α-reductase type 2 deficiency and 46,XY karyotype raised as girls develop a male gender identity and change the gender behavior in adolescence or early adulthood [2, 4, 15–18]. The degree of external genital masculinization at birth does not seem to be related to gender role changes [18].

At puberty, the differential diagnosis of PAIS and 5α-reductase type 2 deficiency can be easier due to the presence of gynecomastia, little genital virilization, and body hair in patients with PAIS, whereas in patients with 5α-reductase type 2 deficiency, there is genital virilization, although not always with adequate penile growth, absence of gynecomastia, and absent or hypoplastic prostate [2–5]. Also at puberty, serum levels of LH and T are abnormally elevated in patients with PAIS [10]. However, in prepubertal patients with PAIS, serum concentrations of T and LH are generally normal and

Table 3: Data from 39 patients without molecular defects in *SRD5A2* and *AR* genes.

Case	Age1	Birth weight (g)	Birth length (cm)	Penile (z)	Genital[1]	Genital[2]	T (ng/mL)	T/DHT
1	0.6	2800	47	−4.0	3	5.5	3.7	18
2[r]	0.08	2650	46	−2.7	2	6.0	3.5	12
3	0.04	2740	47	−4.0	2	6.0	1.9	95
4	0.4	2410	46	−2.7	2	5.0	1.7	8
5	2.8	2700	48	−1.2	2	10.0	1.7	8
6[r]	4.1	2800	47	−1.1	2	10.0	2.1	7
7	0.6	1650	41	−3.4	2	6.5	2.1	10
8	0.8	2580	46	−3.0	2	8.0	2.7	13
9	13.8	2900	47	−6.5	3	5.0	1.7	1
10[r]	20.7	2700	46	−5.6	2	6.0	16.0	5
11	0.8	3000	47	−3.4	3	6.0	1.5	3
12	2.4	2500	49	−3.1	3	1.0	1.9	4
13[r]	2.3	2010	44	−3.7	2	5.0	1.8	6
14[r]	0.8	2900	47	−3.0	2	6.0	3.4	11
15	8.1	2800	48	−2.0	2	10.0	1.8	30
16[r]	0.3	2650	47	−2.7	2	5.5	3.6	18
17	10.2	2110	43	−3.1	2	8.0	1.8	18
18	3.8	1430	39	−4.0	2	5.5	2.0	22
19[r]	0.5	2500	45	−2.7	2	6.0	1.9	9
20	0.04	2810	46	−2.1	3	6.0	2.4	6
21	0.1	2160	43	−2.7	2	6.0	2.3	8
22	0.2	2600	47	−3.4	2	5.0	3.4	8
23[r]	0.1	3000	48	−4.2	2	5.0	2.0	10
24[r]	0.5	2700	48	−2.4	2	9.0	6.6	17
25	0.07	1740	42	−3.1	2	5.0	1.8	9
26[c]	0.4	1700	41	−2.1	2	9.0	2.7	9
27	1.9	2330	42	−2.9	2	6.0	3.4	17
28	0.02	2900	49	−2.7	2	6.0	1.8	9
29	0.2	1540	40	−4.5	2	6.0	1.7	8
30	0.06	2190	42	−3.4	3	3.0	2.6	5
31	0.7	1430	40	−3.4	2	6.0	1.6	8
32	0.2	2250	43	−2.4	3	5.5	1.6	55
33[r]	0.3	2990	49	−2.4	2	6.0	1.6	16
34	10.9	2800	47	−6.6	3	1.0	1.7	8
35	2.6	2700	49	−3.1	2	6.0	4.0	13
36	0.1	2800	50	−2.1	2	8.5	1.9	18
37[r]	3.7	2450	49	−2.7	2	4.0	2.5	12
38[c,r]	0.02	2900	48	−2.7	3	6.0	2.2	4
39[r]	2.7	3400	51	−5.9	2	1.0	1.9	12

Age1: age at first evaluation, +: present, −: absent, c: presence of consanguinity, r: presence of familial recurrence, T: total testosterone, Genital[1]: external genitalia according to Sinnecker et al. [9], Genital[2]: external genitalia according to Ahmed et al. [10].

do not help to establish the diagnoses [13]. In addition, T/DHT is usually elevated in prepubertal patients with 5α-reductase type 2 deficiency [4]. Surprisingly, in this sample, the T/DHT ratio did not allow differentiation between patients with PAIS and 5α-reductase type 2 deficiency, but it was important to differentiate these two groups from undetermined cases. Probably, this result was due to the sensitivity of laboratory methods. Unfortunately, we could not confirmed these results using more specific methods like liquid chromatography linked with tanden mass spectrometry (LC-MS/MS) or immunoassays after organic solvent extraction [19]. Δ4-androstenedione and T/Δ4 ratios were normal in all patients evaluated. According to George et al. [20], the diagnosis of 17β-hydroxysteroid dehydrogenase III deficiency in infants younger than 6 months can be excluded with a basal T/Δ4 ratio above 0.8, with a sensitivity of 100% and in prepubertal children with the same value of T/Δ4 ratio after hCG stimulation test, with a sensitivity of 90%.

In the present study, among 51 families evaluated, 7 (13.7%) showed homozygous or compound heterozygous mutations in *SRD5A2* gene, confirming the diagnosis of 5α-reductase type 2 deficiency, 5 (9.8%) showed hemizygous

Table 4: Data from 7 families with 5α-reductase type 2 deficiency, 5 families with PAIS, and 39 isolated undetermined cases.

		5α-reductase type 2 deficiency	PAIS	Undetermined	P value*
Parental consanguinity	+	5	2	2	$P = 0.00005$
	−	2	3	37	
Familial recurrence	+	3	5	13	$P = 0.02$
	−	4	0	26	
Ambiguous genitalia Sinnecker et al. [9]	**2**	1	3	29	$P = 0.0004$
	3	7	2	10	
	4	1	5	0	
Ambiguous genitalia Ahmed et al. [10]	**<3.5**	6	7	4	$P = 0.00003$
	≥3.5	3	3	35	

* Chi-square test.

Table 5: Data from 9 patients with 5α-reductase type 2 deficiency, 10 patients with PAIS, and 39 isolated undetermined cases.

	5α-reductase type 2 deficiency	PAIS	Undetermined	P value*
Age at first consultation (yr)	9.6 ± 7.5(0.05–18.2)	8.0 ± 10.0(0.2–28.8)	2.5 ± 4.5(0,02–20,7)	$P = 0.02$
Birth weight (g)	2890 ± 290(2600–3500)	3100 ± 400(2630–3950)	2470 ± 460(1700–3000)	$P = 0.002$
Birth length (cm)	48 ± 1(47–50)	48 ± 2(46–51)	46 ± 3(39–50)	$P = 0.02$
Penile length (z)	−4.0 ± 0.4(−4.6–−3.1)	−6.0 ± 2.1(−8.7–−2.7)	−3.2 ± 1.2(−6.6–−1.1)	$P = 0.0001$
T (ng/mL)	4.6 ± 4.1(1.8–13.6)	4.5 ± 4.4(1.7–15.0)	2.6 ± 1.4(1.5–9.1)	$P = 0.07$
Δ4 (ng/mL)	1.4 ± 0.4(0.8–1.9)	1.1 ± 0.4(0.7–1.9)	1.2 ± 0.3(0.7–1.7)	$P = 0.12$
T/Δ4 ratio	3.0 ± 1.9(1.5–7.2)	3.6 ± 1.9(2.1–7.9)	2.7 ± 0.9(1.8–7.0)	$P = 0.32$
T/DHT ratio	54 ± 19(28–82)	51 ± 15(32–72)	14 ± 16(1–95)	$P = 0.0001$

* Kruskal-Wallis test.

mutation in *AR* gene, confirming the diagnosis of PAIS, and the remaining 39 (76.4%) cases showed normal molecular analysis of *SRD5A2* and *AR* genes. Based on the literature available, we expected more cases of PAIS [21, 22] and up to 30–40% of undetermined cases [6, 8]. In addition, probably more patients would be diagnosed with PAIS and mutations in the *AR* gene if the *AR* promoter region and 3′UTR were evaluated, and we must also remember that the *AR* gene in target tissues from patients with hypospadias is more methylated than in control children, resulting in a decreased expression of the *AR*. This epigenetic alteration of the *AR* gene might be involved in the pathogenesis of hypospadias [23].

As expected, the frequency of parental consanguinity was higher in patients with 5α-reductase type 2 deficiency, which is a male-limited autosomal recessive disorder (OMIM 264600), whereas the familial recurrence was higher in PAIS, an X-linked disorder (OMIM 300068).

Weight and length at birth were lower in undetermined cases, suggesting that metabolic and endocrine disorders as fetal malnutrition could play a role in poor external genitalia development. Morel et al. showed that, in comparison with PAIS, undetermined 46,XY DSD was characterized by a high incidence of prematurity and/or intrauterine growth retardation (30%) [8]. In the present study, 12/39 (30.7%) undetermined cases had birth weight lower than 2,500 g. De Andrade Machado Neto et al. [24] showed an association between prenatal growth retardation and 46,XY DSD which may be due to genetic factors not clarified yet or to environmental factors which act early in gestation. Scaramuzzo et al. recently demonstrated that in the first days of life, small-for-gestational-age male pre-term newborns have reduced testosterone levels compared with adequate-for-gestational-age preterm newborns, independently from the presence of abnormalities of the external genitalia [25]. Low testosterone levels were not observed in our patients with low birth weight and length probably due to the gestational age, as all patients included in this study were born at term. Nutrition is the major intrauterine environmental factor that alters expression of the fetal genome and may have lifelong consequences (fetal programming). Alterations in fetal nutrition and endocrine status may result in developmental adaptations that permanently change the structure, physiology, and metabolism of the offspring, predisposing individuals to metabolic, endocrinological, and cardiovascular diseases in adult life [26, 27]. Furthermore, it may be that sexual plasticity during development explains the vulnerability of organisms to androgen influences, such as environmental oestrogens or endocrine disruptors [28–31]. The severity of ambiguous genitalia was higher in 5α-reductase type 2 deficiency in comparison with PAIS and undetermined cases. The 5α-reductase type 2 deficiency has frequently a classical syndrome of pseudovaginal perineoscrotal hypospadias, characterized by a predominantly female phenotype at birth and significant virilization at puberty [9]. Recent reports have shown the clinical spectrum to be heterogeneous, ranging from the classic phenotype to males with hypospadias and even micropenis [32].

The age at first evaluation was lower in undetermined cases, and this data can be associated with severity of ambiguous genitalia: 5α-reductase type 2 deficiency and PAIS groups have more cases with Sinnecker et al. [9], classification grades 3 and 4, and Ahmed et al. [10] score below 3.5 than undetermined cases and may be more frequently underdiagnosed. The penile length, that was higher in undetermined cases, reinforces this hypothesis.

In conclusion, birth weight and length, parental consanguinity, familial recurrence, severity of ambiguous genitalia, penile length, and T/DHT ratio may help the investigation of 46,XY patients with ambiguous genitalia and normal testosterone synthesis.

Disclosure

The authors have nothing to disclose.

Author's Contribution

N. N. Veiga-Junior, P. A. R. Medaets, R. J. Petroli, and F. L. Calais made an equal contribution to this paper.

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Review Article

Long-Term Followup of Adolescent and Young Adult Females with Hypergonadotropic Hypogonadism

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The condition characterized by elevated gonadotrophins (gonadotropins elevated into the menopausal range), low sex steroids, and menstrual disorders was previously termed Premature Ovarian Failure (POF). However, over the last two years an effort has been made by many authors to have the term Primary Ovarian Insufficiency (POI) exclusively applied. Irrespective of the term, the condition concerns adolescent and young adult women under 40 years who experience cessation of menstruation for more than 3 cycles (whereas these women in the past had a rhythmic menstrual cycle) or amenorrhea for 4–6 months against the background of a previously disturbed menstrual cycle. Determining the cause of POI is difficult, and it is even harder to deal with problems arising from the paucity of estrogen as well as to draw up the plan for long-term monitoring of these patients. This paper presents long-term therapeutic management strategies concerning emotional health, hormone replacement therapy, maintenance of bone health, family planning, other associated disorders as well as possible research options for the future.

1. Introduction

Several different terms have been used to describe what Fuller Albright in 1942 originally named "Primary Ovarian Insufficiency" (POI) [1, 2]. A search by Cooper et al. of PubMed [3] revealed that the following terms have been used to describe POI: gonadal dysgenesis, premature ovarian failure (POF), premature menopause, early menopause, hypergonadotropic hypogonadism, ovarian dysgenesis, primary ovarian failure, hypergonadotropic amenorrhea, primary ovarian insufficiency, climacterium praecox, or menopause praecox.

Investigation into POI is very complex, and in the overwhelming majority of cases (90%) a clear-cut cause is not found [4]. The most common causes are presented in Table 1, while rare cases where POI is the single manifestation of an multiorgan syndrome have also been reported [5]. POI occurs through two major mechanisms either follicle dysfunction or follicle depletion.

Diagnosis of POI is confirmed when in the investigation of menstrual disorders the following are observed: FSH levels greater than 30–40 mIU/mL (varying between different laboratories), in two measurements in a one-month period, and estradiol levels less than 50 pg/mL (which indicate hypoestrogenism). The most commonly observed menstrual disorder is secondary amenorrhea, while for younger patients primary amenorrhea is also observed [6]. Bleeding patterns may also include oligomenorrhea, polymenorrhea, or dysfunctional uterine bleeding usually prior to final cessation of menstruation. In the case of teenagers, definitive early diagnosis is difficult because these concomitant disorders are very frequent in puberty and less than 10% of these adolescents suffer ultimately from POI [7]. The reason we need to expedite diagnosis is the rapidity of establishment of osteopenia (up to 60% of adolescents at the time of diagnosis) or, rarely, osteoporosis caused by it [8].

As mentioned earlier, POI results from either rapid atresia of primordial oocytes or the impairment of existing follicles. Ultrasonographic identification of follicles >3 mm, the advent of menstruation, and the measurement of plasma oestrogens do not prove normal ovarian function [9]. Measurement of Anti-Mullerian hormone and inhibin B has been used to estimate remaining functional follicles. According to ACOG (American College of Obstetricians and

Table 1: Common and less common causes of POI.

Most common investigating causes of POI	Less common investigating causes of POI
Chromosomal abnormalities (gonadal dysgenesis with or without Turner syndrome)	Part of a multiple endocrinopathy (i) hypoparathyroidism, (ii) hypoadrenalism, (iii) & mucocutaneous candidiasis
Premutation of X-chromosome (FMR1 gene) Fragile X syndrome	Autoimmune diseases such as (i) autoimmune lymphocytic oophoritis, (ii) dry-eye syndrome, (iii) myasthenia gravis, (iv) rheumatoid arthritis, (v) systemic lupus erythematosus
Damage from chemotherapy or radiation therapy	Viral infections Galactosemia
Surgical oophorectomy (surgical extirpation)	Sarcoidosis

Gynecologists) the measurements of all the above are not necessary for the diagnosis of POI [10].

Therapeutic goals of POI are emotional health, hormone replacement therapy, maintenance of bone health, family planning, and concern about associated disorders [4]. In the event of coexistence of other endocrinologic issues, abnormalities of karyotype or iatrogenic causes of POI, there are several additional diagnostic procedures which must take place, but they are beyond the scope of this paper.

2. Emotional Health

An important problem in treating these patients is that it is difficult to announce the medical situation to both the teenage girl and her parents. The most common words young patients use to describe how they feel after receiving the diagnosis are "shocked," "confused," and "devastated" [11].The family feel themselves confronted by an immense problem that will affect their daughter's capacity for fertilization while also creating in them a feeling of "stigmatization" as well as an acute sense of anxiety and insecurity [12].

When parents have fully understood the problem of their teenage daughter, they can help her emotionally and further provide her with support at different times when the teenage experiences emotional swings. Parents must be aware that talking about POI with their daughter is an ongoing process. Responses should be short and clear. Dialogue must always be encouraged, and emotional feedback has to be obtained periodically. Should the patient's questions seem irrelevant, parents need to understand what the hidden message is. The patient's outlook should be encouraged to be positive, and her emotional state must be externalized. Parents must help their daughter develop a sense of herself and her purpose beyond biological motherhood. Since the patient may become secretive and withdrawn, both the adult adolescent patient and her parents need to seek out resources for support. Patients' parents should share their faith or sources of hope and understanding with the child [13]. All the above-mentioned procedures are long-lasting and have to form the basis for a peaceful environment in which such teenagers will grow up in.

3. Hormone Replacement Therapy (HRT)

Contrary to the case of menopausal women, teenagers need to treat POI via long-term therapy, which not infrequently may be necessary even before puberty. The combined hormone therapy (oestrogen + progestin) should not be administered before the completion of puberty (full development of secondary sexual characteristics). Previously there were no guidelines on dosage and styles of hormones. However, recently the ACOG published a Committee Opinion (Number 502) on Adolescent Health Care [10]. Suggested doses and available routes in the prepubertal patient are 25-microgram estradiol-17β transdermal patch (or 0.3 milligram conjugated estrogen orally or 0.2–0.5 milligram micronized estradiol orally) and 2.5–5 milligrams/day oral medroxyprogesterone acetate for 12–14 days every 30–60 days (or 100 milligrams/day oral micronized progesterone for 12–14 days every 30–60 days).

In the postpubertal patient treatment options can include combined hormone contraception or hormone replacement. Suggested doses and available routes in this group are 100-microgram estradiol-17β transdermal patch (or 0.625–1.25 milligrams/day oral conjugated estrogen) and 2 milligrams/day oral micronized progesterone with 10 milligrams oral medroxyprogesterone acetate daily for 12–14 days every 30–60 days (or 200 milligrams oral micronized progesterone daily for12–14 days every 30–60 days).

The dosage and route of administration of HRT is extremely complex because of the chronicity (many years of treatment) and because during that period of time many changes take place at both the physical and psychological level. There are rare cases where the estrogen needs are greater in younger women than in menopausal women because the first category is required to achieve peak bone mass, while the latter simply seeks maintenance of bone health [7]. The HRT for the period before completion of secondary sexual characteristics should be the smallest possible (estrogen alone) in order to achieve maximum height increase. Progestogen compound must be added when breakthrough bleeding occurs, in order to induce regular withdrawal bleeding.

In virtually all cases treatment should be individualized to the patient. It must be borne in mind that oral administration of any estrogen exposes the liver to higher concentrations than any other tissue. In contrast, a transdermal patch provides estrogen as 17β-estradiol allowing easy absorption, rapid metabolism, and low bioavailability. Several studies have been conducted comparing use of oral versus transdermal estrogens. Use of oral estrogens leads to suppression of insulin-like growth factor I (IGF-I) concentration, while transdermal estrogen did not have a negative effect on IGF-I [14]. In case-control studies

of postmenopausal women, transdermal estradiol has been associated with a lower risk of venous thromboembolism than has oral estrogen [15].

Despite the fact that the final decision for route of therapy remains between any individual patient and her clinician, based on current data, transdermal HRT should be the first choice for adolescent patients [16]. On the other hand, there are several regions around the world, such as the Mediterranean countries, where the warm and humid climate does not allow the use of patches. To date, however, few studies have actually examined whether HRT administration reduces the long-term risks of POF and no evidence-based guidelines for management of POF exist [17]. Despite the fact that HRT is still a controversial topic as regards menopausal women, there is no doubt among physicians that all women with POI must replace their missing steroid hormones (both estrogens and progesterone) for the time period until they reach the usual age of menopause [18]. All patients with POI who take HRT must be aware that they have a 5–10% possibility of conceiving [19] and for this reason they must use barrier contraceptive methods such as a condom or intrauterine devices. The HRT should be discontinued in the event of a positive pregnancy test and the woman must be advised by a fetal specialist as concerns her possible new "needs" of estrogen and progesterone, this time for the safer maintenance of the starting pregnancy. Although combined oral contraceptives (COCs) appear to be an acceptable method for adolescents' HRT, they provide more steroid hormone than is needed for physiologic replacement and are therefore not recommended as first-line management [4]. Alternative methods for HRT, such as percutaneous estradiol gel and transvaginal progesterone gel, have been introduced over the last few years and seem to be well tolerated, reducing the incidence of the psychologic side effects (emotional lability, sleep disorders, depression) of estrogen single therapy [20].

4. Family Planning and Sexual Function

In cases of POI, couples typically have the options of adoption or egg donation. As mentioned above, women with POI may experience sporadic resumption of ovarian function and spontaneous pregnancies can occur in 5–10% of those with idiopathic POI [19, 21]. Currently, there are no known markers that are associated with an increased rate of remission and there are no therapies that have been shown to restore ovarian function and fertility. Evidence suggests that pregnancies might occur if women with POI managed to suppress their high FSH concentrations, either with ethinyloestradiol or with gonadotrophin-releasing hormone analogues, and then followed ovulation induction protocols using low-dose gonadotrophins [22].

Recent results of cryopreservation techniques of ovarian tissue have raised hopes of fertility preservation in women who are due to receive chemotherapy for cancer treatment. An alternative to cryopreservation of ovarian tissue is ovarian stimulation, provided that cancer treatment can be delayed [23]. Oocytes or embryos can be cryopreserved after stimulation. Although there may be future developments in fertility treatment, such as in vivo maturation of oocytes from stem cells or primordial follicles, in vitro fertilization using donor oocytes remains the mainstay of treatment for POI women who wish to become pregnant. However, according to several authors, these pregnancies are at increased risk for first trimester hemorrhage, gestational hypertension, and/or preeclampsia, the delivery of small for gestational age (SGA) neonates, and postpartum hemorrhage, although these findings are controversial [24].

Since patients with POI have estrogen deficiency, it is expected that there will be a degree of vaginal dryness. This leads to reduced arousal, less frequent sexual encounters, and increased pain [25]. The above-mentioned factors diminish the sense of sexual well-being of young women with POI and, in the case of adult patients who are embarking upon their first sexual experiences, can lead to complex psychosexual impairment [26]. Adequate dosage of estrogen replacement and occasionally androgen replacement, along with sexual counseling, may be helpful in the management of sexual dysfunction.

5. Maintaining Bone Health

Estrogens have important anticatabolic and anabolic effects on bone remodeling. Consequently, estrogen deficiency among patients with POI results in an imbalance between osteoclast and osteoblast activity and a progressive loss of bone mineral density (BMD). Popat et al. reported that women with POI have lower bone density compared to regularly menstruating women [27]. The same research team concluded that delay in diagnosis of POI contributes to reduced bone density by delaying proper therapy. There are no reports that suggest increased likelihood of fracture in patients with primary ovarian insufficiency.

Since HRT has not proven to be completely effective in prevention of osteoporosis, additional preventive measures such as calcium and vitamin D intake and weight-bearing exercises should be taken. Adult and young women with POI should engage in a variety of exercises, such as walking, jogging, and stair climbing, along with resistance exercises since these not only aid bone acquisition but also contribute to improvement of cardiovascular circulation. The North American Menopause Society guidelines for perimenopausal and postmenopausal women recommend an intake of 1200 mg of elemental calcium and at least 800 to 1000 IU of vitamin D3 per day [28].

There is uncertainty (i.e., no clear purpose) as regards the frequency of dual-energy X-ray absorptiometry (DEXA) scanning in adolescents with estrogen deficiency. In a recent Committee Opinion (no. 502), the American College of Obstetricians and Gynecologists refers to the proposal made by a number of experts for annual monitoring of bone density during early to midpuberty in order to document peak bone accrual, and then every 2 years through late adolescence. By contrast, other experts claimed that there is no need for an annual DEXA scan, as the implications of a low bone mineral density result in this population are unclear, given the low risk of fracture and the potential for long-term treatment of osteopenia or low bone mass. Up

to now, use of bisphosphonates is not recommended in the young population because of uncertain adverse effects and safety profiles, especially in cases of spontaneous pregnancy.

Crofton et al. recently published a study stating that the type and profile of hormone replacement are critical and can have considerable effects on the bone health of women with POI [29]. The author concluded that physiological sex steroid replacement (transdermal estradiol, 100 μg daily for week 1, 150 μg for weeks 2–4; vaginal progesterone, 200 mg twice daily for weeks 3-4) has better effects on bone mass acquisition and turnover compared with standard HRT (oral ethinyloestradiol 30 μg and 1.5 mg norethisterone daily for weeks 1–3, week 4 "pill-free"). Further research in this area is needed.

6. Cardiovascular Disease

In 1978, the Framingham Study established that there is an association between early menopause and increased mortality from cardiovascular disease, with an estimated 1.8 relative risk of mortality from ischemic heart disease in those with menopause under the age of 40 compared with those with menopause at 49–55 [30, 31]. Kalantaridou et al. published results demonstrating that young women with POF have significant vascular endothelial dysfunction. Early onset of endothelial dysfunction associated with sex steroid deficiency may contribute to the increased risk of cardiovascular disease and mortality in these women. Hormone therapy restores endothelial function within 6 months of treatment [32]. Estrogen deficiency has also been correlated with adverse effects on lipid profile (increased triglycerides, reduced high-density lipoprotein cholesterol)[33], reduced insulin sensitivity [34], and the metabolic syndrome [35], all recognized risk factors for the development of cardiovascular disease.

Adult and young women with POI should be counseled as to tobacco avoidance, daily exercise for obesity prevention, and an appropriate Mediterranean diet in order to achieve optimal cardiovascular health. Blood pressure screening, lipid profile measurements, and cardiac imaging should be performed and monitored by a specialist. HRT results in an increased carotid pulsatility index [36], decreased blood pressure, improved renal function, and lowered activation of the renin-angiotensin system in this age group. In a comparison drawn between physiological sex steroid replacement regimens (transdermal estradiol and vaginal progesterone) and standard (oral ethinylestradiol and norethisterone) therapy for 12 months, the first cited regimens showed better results in the above-mentioned cardiovascular parameters [37]. Because these therapies are long-lasting, the risk of thromboembolic events such as pulmonary embolism or deep vein thrombosis must be extensively and repeatedly examined.

7. Conclusions

Every prepubertal girl, teenager or young woman, who is diagnosed with primary ovarian insufficiency should undergo extensive search by a group of specialists in a referral center while, moreover, benefiting from special handling of her case that will comprise affection and understanding. The followup, which of necessity will be long term, needs to take place twice annually. The final decision for mode of therapy will be one taken mutually between the individual patient and her clinician. Health care providers who counsel these patients must possess comprehensive knowledge of female reproductive biology as well as special sensitivity to the emotional needs of these patients and their parents, especially at the time of diagnosis. Further investigation must also be carried out into the extent and severity of POI complications and due consideration given to the taking of all necessary precautionary measures.

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