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Ovotesticular Difference of Sex Development: Genetic Background, Histological Features, and Clinical Management

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Ovotestis · Difference of sex development · Sexual development · Gonadal dysgenesis

Abstract
Background: Ovotesticular disorder/difference of sex development (DSD) refers to the co-presence of testicular and ovarian tissue in one individual. Childhood management is challenging as there are many uncertainties regarding etiology, gonadal function, and gender outcome. ***Summary:*** Ovotesticular DSD should mainly be considered in 46,XX children with atypical genitalia and normal adrenal steroid profiles. Various underlying genetic mechanisms have been described. Histological assessment of ovotestes requires expert revision and has many pitfalls. Neonatal sex assignment is essential, but as gender outcome is unpredictable, this should be regarded as provisional until a stable gender identity has developed. Therefore, it is crucial not to perform any irreversible medical or surgical procedure in affected individuals until adolescents can give their full informed consent. Gonadal function mostly allows for spontaneous pubertal development; however, fertility is compromised, especially in boys. Specific long-term outcome data for ovotesticular DSD are lacking but can be extrapolated from studies in other DSD populations. ***Key Messages:*** Management of ovotesticular DSD has changed in recent years, prioritizing the child's future right for autonomy and self-determination. The benefits and pitfalls of this new approach have not been documented yet and require intensive monitoring on an international scale.

Introduction

Gonadal differentiation is the embryonic process during which the bipotential gonad differentiates as either testes or ovaries. Typically, these developmental pathways are mutually exclusive and are driven by a complex interplay of antagonistic genes. Unscheduled variations in the spatiotemporal expression or dosage of these genes can lead to simultaneous activation of both pathways and hence ovotesticular development [1]. Ovotesticular disorder/difference of sex development (DSD), previously called true hermaphroditism, refers to the situation where functional ovarian and testicular tissue co-exist in one individual, either as ovotestes or as asymmetrically developed gonads [2]. The karyotype is almost always 46,XX, or rarely 46,XX/46,XY (chimerism) or 46,XX/47,XXY [3]. Several pedigrees have been described in which some 46,XX individuals have ovotesticular DSD, some have

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Table 1. Overview of genes currently involved in *SRY*-negative ovotesticular DSD

Gene	Inheritance	Mechanism	Phenotype	Allelic disorders	Reference
SOX9	AD	Duplications of enhancer region	XX (ovo)testicular DSD	Campomelic dysplasia, isolated Pierre Robin sequence, isolated XY DSD	[12]
RSPO1	AR	LoF mutations	XX (ovo)testicular DSD, palmoplantar hyperkeratosis, predisposition to squamous cell carcinoma, hearing impairment, or ophthalmologic features	/	[4]
SOX3	XL	GoF structural variants	XX (ovo)testicular DSD	Intellectual disability, hypopituitarism	[13]
SOX10	AD	22q13.1 duplication	Syndromic XX (ovo)testicular DSD	PCWH spectrum	[14]
NR5A1	AD	Missense mutations	XX (ovo)testicular DSD	POI, 46,XY DSD, male infertility	[5, 15, 16]
WT1	AD	Mutations affecting the fourth zinc finger	XX (ovo)testicular DSD	WT1 disorder	[6]
NR2F2	AD	LoF mutations	XX (ovo)testicular DSD, CHD, BPES, CDH	Isolated CHD	[17]
WNT4	AR	LoF mutation	SERKAL syndrome	Mullerian duct regression and hyperandrogenism	[18]

DSD, difference of sex development; BPES, blepharophimosis, ptosis, epicanthus inversus syndrome.

testicular DSD, and others are asymptomatic carriers [4–6]. Thus, the condition represents a spectrum of phenotypes and is characterized by variable penetrance. Overall, ovotesticular DSD is a rare form of DSD, with an estimated incidence of <1/20,000 individuals, representing <5% of all DSD cases, although it appears to be more common in some African countries such as Kenya or South Africa [7, 8]. The diagnosis is most often made in the first months of life, following investigations for the presence of atypical genitalia. Sex assignment may be very challenging and gender outcome largely unpredictable. If left untreated, the testicular and ovarian fractions both produce sex steroids at puberty, leading to phallic growth and breast development. Thus, the condition poses a number of diagnostic, clinical, and ethical problems, which will be reviewed below.

Genetic Background of Ovotesticular DSD

Recently, some highly informative mouse models have greatly contributed to our understanding of gonadal differentiation in general and of ovotesticular development in particular [9, 10]. It is unclear, however, to what extent these findings can be extrapolated to the human situation. The initiation of testis differentiation is triggered by the sex determining region on Y (SRY) gene by upregulation of *SRY*-box transcription factor 9 (SOX9) together with nuclear receptor subfamily 5 group A member 1 (NR5A1/SF1) [11]. Thus, paternal translocation of *SRY* in humans mostly leads to testicular but not ovotesticular DSD in a 46,XX individual. Gain-of-function changes of male sex-determining genes or its regulatory regions, for example, *SOX9*, or loss-of-function mutations of female sex-determining genes, for example, R-spondin 1 (*RSPO1*), have been implicated in syndromic and nonsyndromic ovotesticular DSD (Table 1) [4, 19]. Copy number variations disrupting the regulatory region of *SRY*-related genes such as *SOX3* and *SOX10* have also been described [3, 14, 20, 21]. It has been hypothesized that ectopic gonadal expression of these genes at a critical stage of development in these cases has triggered *SOX9* transcription and subsequent initiation of the male pathway [22].

NR5A1, apart from its crucial role in testis development, has also been implicated in the activation of early ovary-determining genes, possibly by upregulating nuclear receptor subfamily 0 group B member 1 (*NR0B1*), a repressor of *SOX9*, together with β-catenin, encoded by *CTNNB1* [5, 15]. Several families have been described in which a specific mutation in *NR5A1* c.274C>T (p.Arg92Trp) has led to ovotesticular or testicular DSD in some family members while others are asymptomatic carriers.

Although the exact mechanism remains to be elucidated, it has been hypothesized that the p.Arg92Trp protein specifically interferes with this NR5A1-mediated activation of ovarian development by an impaired interaction with β-catenin and by a loss of NR0B1-mediated suppression of *SOX9* [5, 15, 16]. Others illustrated no loss of *SOX9* repression and that the NR5A1 p.Arg92Trp as well as a novel p.Ala260Val mutants in itself did not reduce *NR0B1* promoter activity directly. The mutants did disrupt the β-catenin-mediated activation of this promoter and showed an increased repression of β-catenin-mediated WNT signaling resulting in reduced NR0B1 activity. It is suggested that WNT signaling is regulated by the NR5A1/β-catenin complex in a dose-dependent manner [23]. Additionally, a p.Arg92Gln mutation in *NR5A1* has been described in persons with and without ovotesticular DSD [24]. This variant did not show a reduced interaction with β-catenin [15].

Most recently, several cases of testicular and ovotesticular DSD caused by mutations affecting the fourth zinc finger of the WT1 transcription factor (*WT1*) gene have been reported [6, 25]. This resulted in the enhancement of male sex-determining genes and repression of *FOXL2* in a dominant negative manner. Mutated WT1 further showed interaction with β-catenin and upregulation of *SOX9* contrary to wild-type WT1 [6]. Mutations in some ovary-promoting genes may result in syndromic forms of (ovo)testicular DSD, including nuclear receptor subfamily 2 group F member 2 (NR2F2/COUPTFII), associated with cardiac defects and blepharophimosis, ptosis, epicanthus inversus syndrome [17]; *RSPO1*, associated with skin abnormalities (palmoplantar hyperkeratosis and increased risk of squamous cell carcinoma) [4]; and Wnt family member 4 (WNT4) which causes the SERKAL syndrome (renal, adrenal, and pulmonary dysgenesis) [18]. RSPO1 and WNT4 increase the expression of and stabilize β-catenin, and mutations in these genes may result in the escape of testis suppression through reduced β-catenin signaling [1].

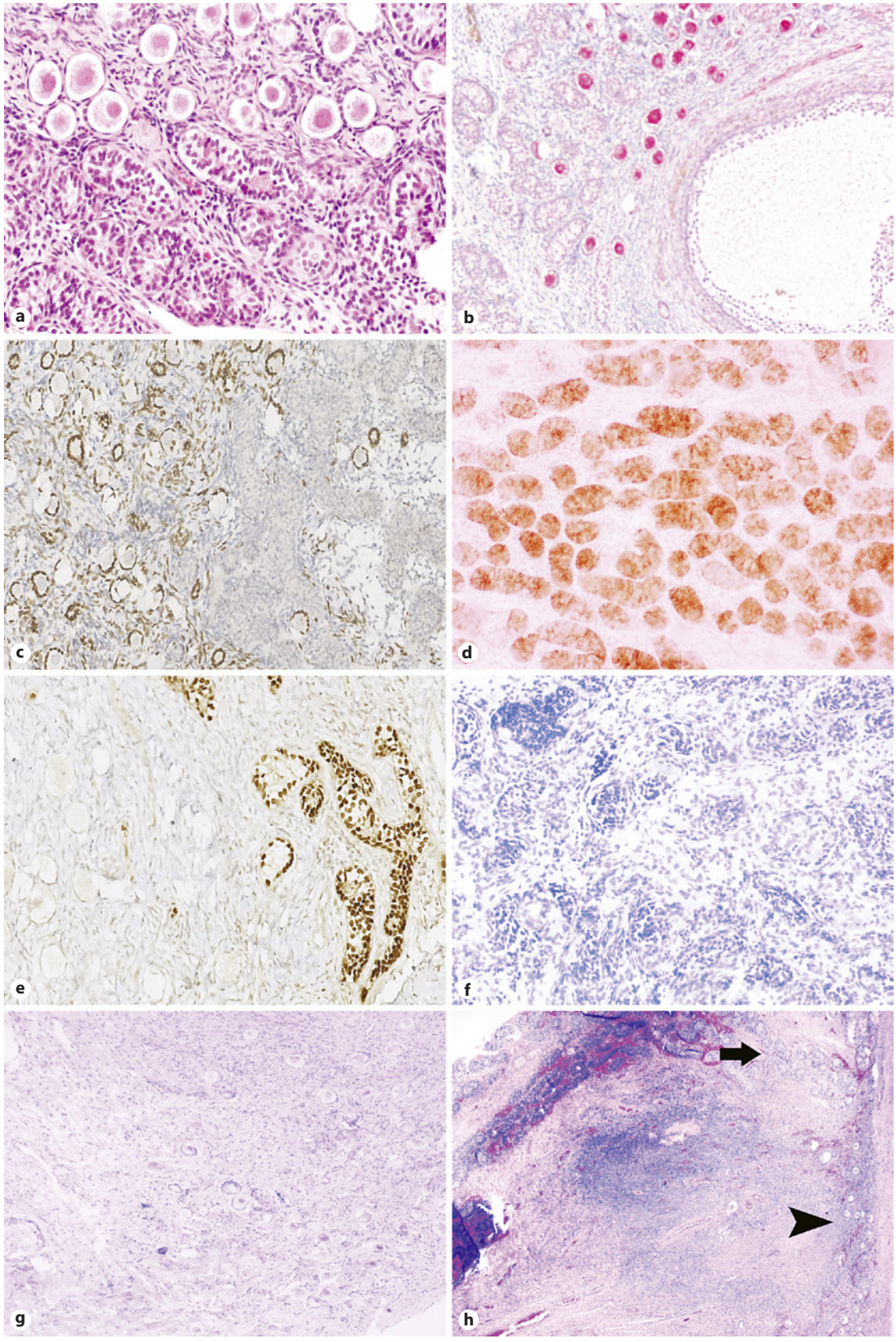
However, the molecular etiology for most ovotesticular DSD cases remains unknown. Mosaicism for mutations in sex-determining genes in individuals with an asymmetric reproductive system may be a possible underdiagnosed mechanism [26]. Furthermore, many variants in noncoding, regulatory elements of gonadal target genes as well as many structural variants (deletions, duplications, insertions, inversions, and other rearrangements) cannot be detected by currently used exome-based technologies or chromosomal microarray. The use of whole genome sequencing and long-read sequencing, when more readily available, may further uncover the missing heritability of ovotesticular DSD and DSD in general. Last, epimutations may explain an unresolved portion of ovotesticular DSD, illustrated by how hypermethylation of *SRY* may lead to XY DSD [27].

Histological Features and Pitfalls of Ovotesticular DSD

The histological hallmark of an ovotestis is the combined presence of ovarian and testicular differentiation in a single gonad [28]. The testicular and ovarian fractions are mostly organized in an end-to-end fashion, and sometimes separated by a transitional zone, or as an ovarian cap surrounding testis tubules, although more mixed patterns have been observed in the South African population [29–31], possibly representing different etiologies. FOXL2 and SOX9 immunopositive staining confirms granulosa and Sertoli cell differentiation, respectively [28, 32]. In the testicular component, prepubertal Sertoli cells also stain positive for podoplanin (unpublished observations), in line with previous findings [33]. Testis tubules rarely contain germ cells, and if present, they tend to disappear before puberty. Spermatogenesis is never observed in 46,XX individuals as this requires a Y chromosome. The tubules often undergo hyalinization and sclerosis in adults. Primordial and growing follicles populate the ovarian portion of an ovotestis, but absolute numbers may vary. These follicles may undergo ovulatory changes after puberty [29] (Fig. 1a–e).

In contrast to many other forms of DSD, the risk for malignant germ cell tumors (GCTs) in ovotesticular DSD is not or only marginally elevated, with only 9 cases reported to date [34]. Observational data, based on a meta-analysis of historical series, have suggested a risk of 2.6% [35]. This is likely an overestimation as in more recent series, complemented by a thorough review of 17 in-house available gonadal biopsy and gonadectomy samples from 7 patients, no cases of germ cell neoplasia in situ (GCNIS) or invasive GCT were found in a total of 120 patients ([29, 30, 36] and unpublished data). Indeed, GCT development in DSD has been associated with the presence of the Y chromosome, more specifically the testis-specific protein-Y encoded chromosome, and with incomplete testicular differentiation ("testicularization") of the gonad [37, 38], 2 risk factors that are absent in most ovotesticular DSD cases.

Nonetheless, histopathological assessment of ovotestes in the clinical setting has several pitfalls. Dysgenetic gonads are sometimes erroneously classified as ovotestes as they often present as ovarian-type stroma, sometimes



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with scattered germ cells and immature sex cords (Fig. 2). Such germ cells are typically not surrounded by a granulosa cell layer and are pre-meiotic, aberrantly expressing pluripotency markers like OCT3/4. Thus, they should not be misinterpreted as "immature follicles" in a young child. Sex cords consist of pre-Sertoli/granulosa cells that are blocked in their maturation and incapable of supporting the maturational process of neighboring germ cells [28, 35, 39]. Overall, such a pattern, which is mostly observed in 46,XY and 45,X/46,XY complete and partial gonadal dysgenesis, should be referred to as "undifferentiated gonadal tissue" and has a high risk for malignant degeneration, thus requiring prophylactic gonadectomy [38, 40]. A second problem lies in the inherent heterogeneity of ovotestes, which is typically not captured by a small gonadal biopsy, often obtained in the neonatal period for diagnostic reasons (Fig. 1f–h). Subsequently, the missed presence of an ovarian or testicular fraction in preserved gonads will lead to unexpected breast development or virilization ,respectively, in puberty, and, if not addressed early, may cause undesired and irreversible physical traits such as early epiphyseal closure and short stature in boys or deepened voice in girls.

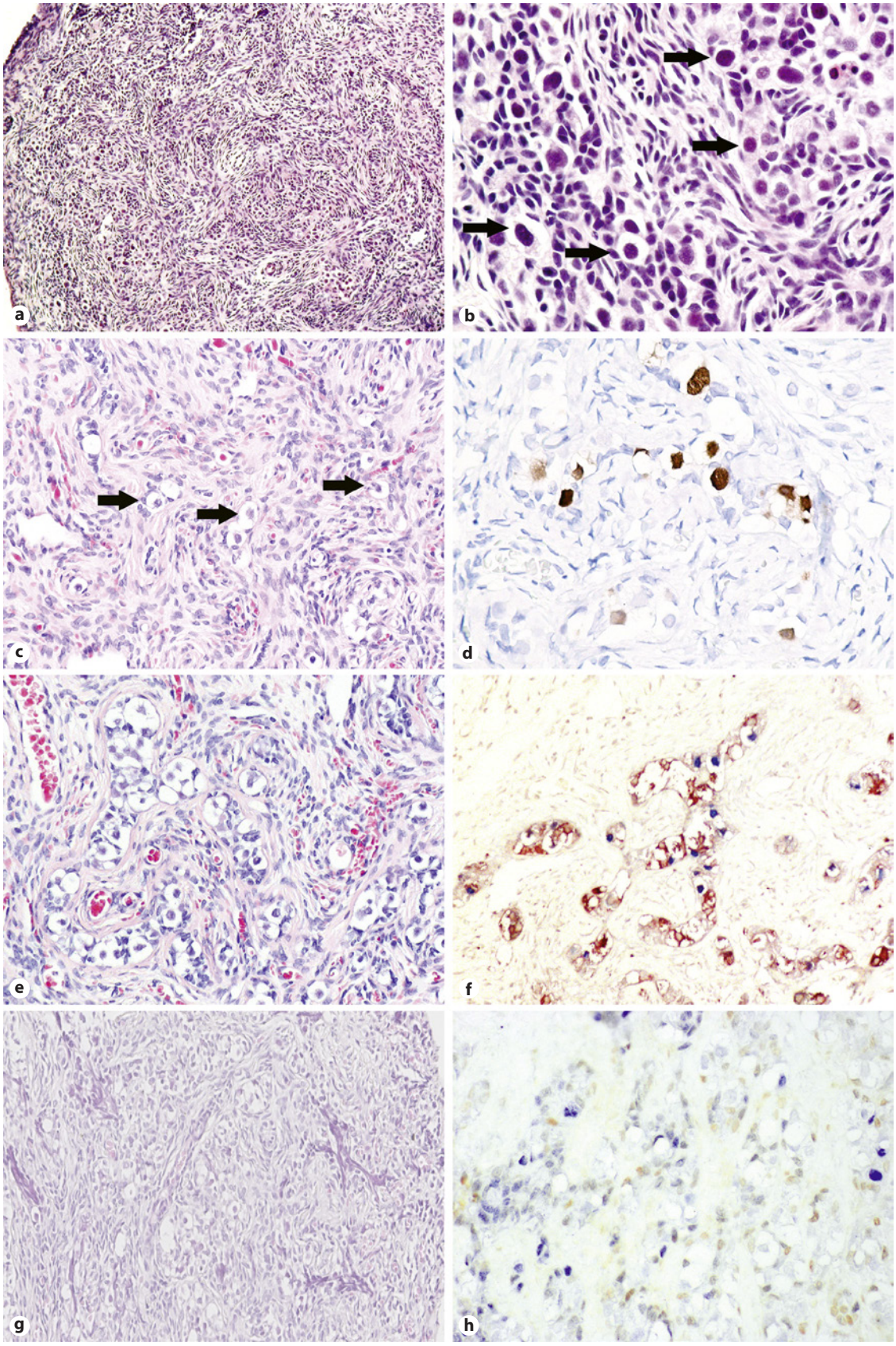
Management of Ovotesticular DSD

The general approach toward a child who has a DSD has been reviewed elsewhere. For parents of a newborn with atypical genitalia, an efficient and targeted diagnostic plan, extensive information, and adequate psychosocial support are the cornerstones of neonatal management [2, 41, 42]. The vast majority of virilized 46,XX neonates will have congenital adrenal hyperplasia, but the presence of 1 or 2 labioscrotal or inguinal gonads, a hemiuterus on ultrasound, or a normal adrenal steroid profile should raise suspicion of ovotesticular DSD [43]. Serum AMH is the most useful biochemical parameter to confirm the presence of testicular tissue in the neonatal period and throughout childhood, as testicular AMH levels are about 50-fold higher than and show no overlap with ovarian levels. In children with ovotesticular DSD, AMH is usually in between typical male and female ranges, depending on the amount of testicular tissue. If needed, a repeat measurement after 1–2 months can provide more certainty because AMH levels tend to transiently decline in the first week after birth. Serial AMH detection can be used to assess completeness of testicular tissue removal in girls with ovotesticular DSD (see below), but as serum AMH declines with rising intratesticular testosterone concentrations, its clinical value in DSD is limited to the prepubertal period [44].

In the child with ovotesticular DSD, normal serum gonadotropin levels suggest functional gonadal (testicular and/or ovarian) tissue. As for other forms of DSD, unstimulated testosterone levels are only helpful during mini-puberty and from puberty onward; in the first 2–3 weeks of life and in childhood, an hCG test is required to adequately measure Leydig cell function [45]. Insulin-like factor 3 has recently been proposed as a new Leydig cell marker that shows less diurnal variation; however, like testosterone, it is low in prepubertal children [46]. Stimulation of follicle development by intramuscular FSH to demonstrate the presence of an ovarian fraction has been proposed [47], but this test is not recommended in children for safety reasons.

Laparoscopic evaluation of internal genital structures, including gonadal biopsies for histological assessment of gonadal differentiation, is sometimes needed to finalize the diagnostic process. However, as explained above, small biopsies will often not reflect the heterogeneity of gonadal tissue present and should not be regarded as conclusive regarding the absence of functional testicular or ovarian tissue if not present in the biopsy specimen [48]

Fig. 1. Typical histological features in ovotesticular DSD. **a** Ovotestis of unknown cause in a 46,XX child, 6 years old. Ovarian portion (top) with an abundance of ovarian follicles adjacent to the testicular portion (bottom), consisting of immature Sertoli-cell-only tubules, but without signs of testicular dysgenesis. No Leydig cells are found, in line with the patient's age. HE, ×200. **b** Same child as (**a**). DDX4 staining indicates primordial follicles in the ovarian fraction, the growing follicle stains negative. DDX4 staining is negative in the testicular fraction, indicating the absence of spermatogonia. DDX4 staining, ×100. **c** Ovotesticular DSD of unknown origin in a 46,XX child, 10 years old. FOXL2 staining demonstrates granulosa cell (brown) differentiation in the ovarian fraction (left side). No granulosa cells are found in the testicular part (right side). FOXL2 staining, ×200. **d** Same child as **c**. Podoplanin staining, indicating immature Sertoli cells, in accordance with the patient's age, and normal testicular architecture with Sertoli-cell-only tubules. Podoplanin staining, ×100. **e** Ovotesticular DSD in a 3-year-old 46,XX girl who has the c.274C > T (p.Arg92Trp) variant in *NR5A1*. SOX9 stains Sertoli cells in the testicular component (right). The ovarian fraction (left) does not contain SOX9-positive cells. SOX9 staining, ×200. **f** Right gonadal biopsy in a 3-year-old girl with 46,XX ovotesticular DSD. The biopsy sample contains testis tissue only. HE, ×200. **g** Left gonadal biopsy in the same child. The biopsy sample contains ovarian tissue only. HE, ×100. **h** Right gonadectomy in the same child at 10 years of age. The gonadal specimen consists of an ovotestis; the testicular fraction is indicated with an arrow, the ovarian fraction with an arrowhead. HE, ×40. DSD, difference of sex development.



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and unpublished data. Genetic tests include urgent karyotyping, with exclusion of Y chromosomal material by fluorescent in situ hybridization or quantitative polymerase chain reaction, a next-generation sequencing-based gene panel, focusing on genes known to be involved in (ovotesticular) DSD and a high-resolution analysis of genomic imbalances by chromosomal microarray [49].

The current consensus favors that all children, after careful consideration of all diagnostic tests, have a social sex assigned at birth, for reasons including mental well-being of the child, integrity of the family, and avoiding stigma [43, 50]. However, neonatal sex assignment is exceptionally difficult in children who have ovotesticular DSD, as individual gender development, although often in line with the sex of rearing, is unpredictable. The decision is the result of a multidisciplinary team discussion, including the parents, and is based on careful analysis of all results. Factors that may influence this decision are, among others but not limited to, the degree of external virilization, the estimated amount of testicular or ovarian tissue present, uterine development, and parental preferences. The recent advent of technical possibilities for expert consultation on a European level within the European Reference Networks (ERN) offers important opportunities to discuss the most difficult cases with a wider team [51]. Although mental well-being was reported to be impaired in many, gender issues were not observed in a French long-term outcome study including 33 individuals with ovotesticular DSD of whom 12 were raised male and 21 female [29]. In contrast, a recent South African series observed gender dysphoria in several individuals with ovotesticular DSD who were all initially raised as females [36].

In view of the unpredictable gender outcome and in line with recent more general recommendations and with human rights principles, early and/or unconsented genital surgery, including (partial) gonadectomy, should be absolutely avoided in any child with ovotesticular DSD [41, 52]. GnRH analog-mediated blockage of mini-puberty has been attempted in 5 children with ovotesticular DSD to avoid clitoris growth. Although interesting, this treatment should be regarded as experimental in attendance of further data, which can best be collected in a standardized way through international collaboration given the rareness of the condition [30, 53].

With the advent of puberty, gonadectomy will be needed to avoid undesired hormone production; however, this should be postponed further when gender identity is not stable yet (see below). When there is a clear demarcation between the (presumed) ovarian and testicular fraction of an ovotestis, gonad-sparing partial gonadectomy can be attempted, with close postoperative monitoring of AMH or estradiol levels in confirmed females and males, respectively, in order to ascertain complete removal of the testicular or ovarian part, respectively. Conclusive intraoperative ultrasound assessment of gonadal differentiation has also been reported [54]; however, in many cases, complete removal of the ovotestis will be unavoidable. The importance of regular clinical follow-up should be emphasized in all adolescents with ovotesticular DSD who had partial gonadectomy, as accidentally remaining functional gonadal tissue can cause irreversible effects such as breast development and early closure of epiphyseal plates in boys or deepening of the voice and clitoral growth in girls. When gender identity is still uncertain, the temporary use of GnRH analogs for a maximum of 3–4 years is well established nowadays, analogous to management of young people who have gender dysphoria [55, 56].

Very few studies report on long-term fertility outcomes of ovotesticular DSD. XX males are sterile due to the absence of the Y chromosome, but also immature spermatogonia are generally absent from biopsies taken outside the neonatal period. Whether chimeric individuals can have

Fig. 2. Histological pitfalls and differential diagnoses of ovotestes. **a**, **b** Gonadal dysgenesis in a 6-month-old girl with 46,XY DSD of unknown origin. In the clinical setting, such a gonad is often described as ovarian-type stroma containing follicles, or in the context of an XY karyotype, as "ovotestes." However, germ cells (arrows) are not enclosed by a granulosa cell layer and thus should not be designated as follicles but lay isolated in between the stromal cells. The latter have not differentiated as mature Sertoli or granulosa cells. Thus, the gonad should be described as "undifferentiated gonadal tissue" and has a high risk for malignant degeneration, requiring prophylactic gonadectomy. **a** HE, ×100; **b** HE, ×400. **c** Undifferentiated gonadal tissue in a girl, 14 years old, who has a 45,X/46,XY karyotype. Isolated germ cells are indicated by an arrow. HE, ×200. **d** Same child. Primordial germ cells aberrantly express the pluripotency marker OCT3/4, indicating their malignant potential due to maturation block. OCT3/4 staining, 400. **e**, **f** Same child. Some areas of the dysgenetic gonad display sex cords, consisting of a single layer of germ cells lining up with immature Setoli/granulosa-like cells. The blue OCT3/4 staining indicates pluripotent germ cells with malignant potential; the red DDX4-positive cells represent more mature germ cells. **e** HE staining, ×200; **f** OCT3/4-DDX4 double staining, ×200. **g**, **h** Undifferentiated gonadal tissue consisting of ovarian-type stroma and sex cords. Some of the supportive cells are SOX9 positive, indicating Sertoli cell differentiation, while others stain positive for FOXL2, showing their granulosa cell differentiation. **g** HE, ×300; **h** SOX9-FOXL2 double staining, ×200. DSD, difference of sex development.

spermatogenesis is unknown. Limited data indicate that most XX boys with preserved testicular tissue progress through puberty at a normal age and pace; however, testosterone production may decline over time, with testes becoming atrophic and requiring hormone replacement therapy [29]. Cyclic menstruation seems common in girls with ovotesticular DSD who have ovarian tissue in place, and some uneventful pregnancies have been reported [29, 30]. It is currently unclear if their follicle pool is at risk of early decline and/or may be affected by long-term preservation of adjacent testicular tissue or long-term GnRH analog use, but in the absence of evidence, it seems prudent to offer monitoring of ovarian reserve to pubertal girls with ovotesticular DSD and discuss options for oocyte cryopreservation in young adult women.

Adult psychosexual outcome has not been studied specifically in individuals with ovotesticular DSD, and thus data can only be extrapolated from larger studies in a DSD population with mixed diagnoses. The DSD-Life study, performed in 14 European centers with data collected in 1,040 individuals with various DSD conditions, has been very informative in this respect. This study showed a specific profile of issues related to body image and self-esteem per specific diagnosis; however overall, openness about the condition and reduction of stress and anxiety were associated with better outcomes, underscoring the importance of individualized management, adequate psychosocial counseling, and peer support throughout life for each individual who has a DSD and their families [57].

Conclusion

Ovotesticular DSD is a very rare congenital condition that poses several clinical and ethical dilemmas and pitfalls that can only be addressed by multidisciplinary expertise and individualized holistic care. It illustrates like no other condition the challenges that are associated with the increased awareness for safeguarding the child's autonomy as a principal right. Although data on long-term outcome are lacking, its management has greatly evolved in recent years, with changing societal perspectives and based on studies in larger DSD populations. Great efforts should be put in optimizing psychological well-being and in decreasing taboo and promoting openness about the condition. Rigorous data collection and analysis on an international scale is the most effective way to understand as early as possible the beneficial and eventual negative effects of this new approach on the long-term outcomes of individuals who have this condition.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

H.S. took part in data collection, text writing, and revision and approval of the final manuscript; K.V.D.V. was involved in data collection, text writing, and revision and approval of the final manuscript; M.C. contributed to overall coordination, data collection, text writing, and revision and approval of the final manuscript.

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