Disorders of sex development: a genetic study of patients in a multidisciplinary clinic

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Abstract

Sex development is a process under genetic control directing both the bi-potential gonads to become either a testis or an ovary, and the consequent differentiation of internal ducts and external genitalia. This complex series of events can be altered by a large number of genetic and non-genetic factors. Disorders of sex development (DSD) are all the medical conditions characterized by an atypical chromosomal, gonadal, or phenotypical sex. Incomplete knowledge of the genetic mechanisms involved in sex development results in a low probability of determining the molecular definition of the genetic defect in many of the patients. In this study, we describe the clinical, cytogenetic, and molecular study of 88 cases with DSD, including 29 patients with 46,XY and disorders in androgen synthesis or action, 18 with 46,XX and disorders in androgen excess, 17 with 46,XY and disorders of gonadal (testicular) development, 11 classified as 46,XX other, eight with 46,XX and disorders of gonadal (ovarian) development, and five with sex chromosome anomalies. In total, we found a genetic variant in 56 out of 88 of them, leading to the clinical classification of every patient, and we outline the different steps required for a coherent genetic testing approach. In conclusion, our results highlight the fact that each category of DSD is related to a large number of different DNA alterations, thus requiring multiple genetic studies to achieve a precise etiological diagnosis for each patient.

Key Words
- disorders of sex development
- DSD
- disorders of ovarian development
- disorders of testicular development

Introduction

Sex development is a multistep process under genetic control, implying a delicate network of molecular events that direct both the bi-potential gonads to become either a testis or an ovary (sex determination), and the consequent divergent differentiation of internal ducts and external genitalia (sex differentiation). Correct dimorphic sex
determination and differentiation achievement can be
interrupted by a large number of genetic and non-genetic
factors altering any of the molecular signals that specify
sex-specific development of sex organs or endocrine
function. The term disorders of sex development (DSD)
embraces all the medical conditions characterized by an
atypical chromosomal, gonadal, or phenotypical sex (1).
Thus, a wide number of pathologies are included under the
same DSD definition; they show different frequencies and
their severity ranges from genital anomalies that do not
impair sexual definition or functionality, such as hypos-
padias, to conditions characterized by sexual ambiguity or
discordance between chromosomal and internal or exter-
nal sex anatomy. In 2006, Hughes et al. (1) proposed the
latest recommended classification of DSD, based on the sex
chromosomal findings and on the step of gonadal
development or phenotypic differentiation in which the
alteration had occurred. Current understanding of the
genetic control of sex development is still incomplete,
resulting a low probability of determining the molecular
definition of the causal defect in many of the patients with
DSD. Anyhow, proper and thorough clinical evaluation
and laboratory investigations are the necessary procedures
for obtaining the most accurate diagnostic definition. This
can more efficiently be achieved through a multidisciplin-
ary assessment of patients performed by different dedicated
specialists with long-standing experience and both pedi-
atric and adult practice. In this study, we describe the
results of studies carried out on 88 patients with DSD
evaluated and followed in the outpatient ‘Centre for
diagnosis, care and treatment of DSD’ at San Camillo
Forlanini Hospital, Sapienza University of Rome. In total,
we found a genetic alteration in 56 out of 88 cases, leading
to the correct clinical classification of every patient. Each
category of DSD was found to be related to a large number of
different DNA alterations, thus requiring multiple genetic
studies to possibly achieve a precise etiological diagnosis in
every patient.

Subjects and methods

Patients

A cohort of 88 individuals, aged from 1 day to 41 years
affected by non-syndromic DSD, were fully evaluated at the
DSD Centre of San Camillo-Forlanini Hospital, Rome
(Italy), by an experienced multidisciplinary team including
a pediatric surgeon, a pediatric endocrinologist, a clinical
psychologist, and a clinical geneticist. Patients were
identified on the basis of ambiguous genitalia or discor-
dance among chromosomal, gonadal and/or phenotypic
sex, or apparently minor genital abnormalities (Table 1,
modified from Hughes et al. (1)). Patients in whom the
presence of additional anomalies, such as dysmorphic
features and skeletal or visceral abnormalities, was detected
were excluded from the cohort with the exception of the
three syndromic patients (cases 6, 27, and 32) included in
the study. For each patient, hormonal, imaging, and

Table 1  Classification of the studied patients with DSD (based on Hughes et al. (1))

<table>
<thead>
<tr>
<th>Sex chromosome DSDs</th>
<th>45,X (Turner’s syndrome and variants)</th>
</tr>
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<tbody>
<tr>
<td>DSD 46,XX disorders of gonadal (ovarian) development</td>
<td>47,XXY (Klinefelter syndrome and variants)</td>
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<tr>
<td>DSD 46,XX androgen excess</td>
<td>45,XX/46,XY (mixed gonadal dysgenesis and ovotesticular DSD)</td>
</tr>
<tr>
<td>DSD 46,XX disorders in androgen synthesis or action</td>
<td>46,XX/46,XY (chimeric and ovotesticular DSD)</td>
</tr>
<tr>
<td>MURCS, Mullerian Renal Cervicothoracic Somite association; MRKH, Mayer Rokitansky Küster Hauser syndrome.</td>
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</table>

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genetic investigations were carried out. Individuals showing 45,X, 47,XXX, and 47,XXY karyotypes, as well as 46,XX patients with gonadal dysgenesis and 46,XY patients with minor genital anomalies that did not give rise to any doubts about sex assignment, were excluded from the present report. In contrast, 46,XX female patients with developmental anomalies of the Müllerian duct were included in this cohort. In all cases, a stepwise clinical diagnostic approach for evaluation was conducted and psychological support was constantly provided to both patients and their families. The genetic analyses included the study of AR, AMH, CYP11B1, CYP21A2, DMRT1, NR0B1, NR5A1, RSPO1, SHOX, SOX9, SRD5A2, SRY, and WNT4 genes chosen on the basis of the data reported in the literature (2, 3, 4, 5). The genes analyzed for each patient were selected according to the DSD category. All enrolled individuals gave informed consent for DNA analyses, approved by local ethics committees in accordance with the guidelines of Italian Society of Human Genetics (SIGU).

Karyotyping
Metaphase spreads were obtained from blood lymphocytes using standard procedures. Chromosome analysis was performed using standard G-bands by trypsin using Giemsa (GTG)-banding techniques on cultured lymphocytes.

Testing the presence or absence of SRY gene
Recognition of the SRY sequence was carried out on genomic DNA through polymerase chain reaction (PCR) amplification with specific SRY and control (ZP3) gene primers as described by Cui et al. (6).

Fluorescent in situ hybridization
SRY translocations in 46,XX male patients and DMRT1 deletions were investigated by fluorescent in situ hybridization (FISH) using two specific probes selected from a public database (http://genome.ucsc.edu), respectively, for SRY and DMRT1 genes.

Direct sequencing
The search for DNA point mutations in SRY, DHH, NR5A1, SRD5A2, AR, AMH, CYP21A2, CYP11B1, RSPO1, and WNT4 genes was carried out by PCR followed by direct sequencing. SRD5A2, CYP21A2, AMH, and CYP11B1 were analyzed as described previously (7, 8, 9). Primer sequences and annealing temperatures employed for amplification of SRY, DHH, NR5A1, AR, RSPO1, and WNT4 coding sequences are listed in Supplementary Table 1, see section on supplementary data given at the end of this article. Primers were designed in order to also detect possible splicing defects. Sanger sequencing was carried out using a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) on a 3500xl Genetic Analyzer (Thermo Fisher Scientific). Forward and reverse sequences were analyzed and compared with each gene’s mRNA reference sequence: SRY (NM_003140.1), DHH (NM_010144), NR5A1 (NM_004959), SRD5A2 (NM_000348.3), AR (NM_010144.2), AMH (NM_00479), CYP11B1 (NM_000497), CYP21A2 (NM_000500), RSPO1 (NM_001038633.2), and WNT4 (NM_030761). All novel missense and splicing mutations were searched for in 200 control chromosomes from unaffected subjects.

CYP21A2 large deletions/conversions analysis
Investigation for large deletions/conversions affecting the CYP21A2 gene was conducted as described by Lee et al. (10).

Multiplex ligation-dependent probe amplification (MLPA) analysis
Screening for single and multi-exonic deletions/duplications affecting SOX9, NR0B1, NR5A1, SRY, CYP21A2, and WNT4 genes was carried out using the MLPA SALSA P185-B2 Intersex (version 05; April 22, 2011) (MRC Holland, Amsterdam, The Netherlands), following the manufacturer’s instructions. Information on probe sequences can be freely obtained from the MRC Holland website (www.mlpa.com). Relative quantification of copy number mutations was carried out using the Coffalyser Software (MRC Holland). DNA samples showing such a reduction or increase in the MLPA peak area values were reanalyzed by the same MLPA procedure. Only the samples showing consistent results between the two experiments were considered positive for a copy number alteration.

Real-time PCR
Copy number changes in NR0B1 genes identified by MLPA analyses and single/multi-exonic deletions/duplications of the SHOX gene were investigated using SYBR Green-based experiments on an ABI7900 HT Fast Real Time PCR System (Thermo Fisher Scientific). Primers were designed using the Primer Express 3.0 Software (Thermo Fisher Scientific; Supplementary Table 2, see section on supplementary data given at the end of this article).
The reference gene telomerase reverse transcriptase (TERT) was simultaneously quantified in a separate tube for each specimen. For each primer pair, the reaction efficiency parameter (R2) was assessed by a standard-curve analysis as reported in Supplementary Table 2. Results for each sample were expressed as N-fold changes in copies of each test exon, and normalized to TERT relative to the copy number of the test exon in the calibrator DNA, according to the following equation: amount of target = \(2^{-\Delta\Delta C_t} \) (11).

## Results

### Classification of patients with DSD

Classical cytogenetic techniques were employed in order to categorize each patient into the correct DSD class according to the karyotype. Thirty-seven patients showed a 46,XX karyotype, while 46 patients had a 46,XY karyotype. Out of 88 patients, five (Table 2, cases 1 to 5) showed an aberrant karyotype with mosaicism involving numerical and/or structural abnormalities of sex chromosomes. Those patients were then classified as affected by sex chromosome DSD. SRY absence/presence test, successively performed on the seven males showing a 46,XX karyotype, identified the SRY gene in two patients (Table 2, cases 7 and 8). FISH analysis performed with a SRY-specific probe defined those patients’ karyotype as 46,XX.ish der(X)(X;Y)(p22.3;p11.3)(SRY+), indicative of whole SRY gene translocation to the X chromosome in both cases. In accordance with karyotyping and FISH analyses, our cohort was determined to be composed as shown in Fig. 1. On the basis of subsequent clinical investigations, out of 88 patients, 29 (33%) were classified as carriers of a 46,XY disorder in androgen synthesis or action, 18 (20%) as carriers of a 46,XX disorder with androgen excess, 17 (19%) as carriers of 46,XY disorders of gonadal (testicular) development, 11 (13%) as affected by 46,XX DSD other, 8 (9%) as carriers of 46,XX disorders of gonadal (ovarian) development, and 5 (6%) as carriers of sex chromosome DSD.

### DSD 46,XX androgen excess

Eighteen 46,XX female patients with suspected adrenogenital syndrome derived from 21-hydroxylase enzyme deficiency (Fig. 1) were analyzed for CYP21A2 gene mutations. One of these patients, in whom the hormonal profile was indicative of a rare form of the disease, was secondly analyzed for CYP11B1 point mutations. In total, we found CYP21A2 DNA alterations in 14 out of 18 (78%) patients (Table 2, cases 10–23). Among the seven different identified CYP21A2 mutations, the c.293-13C/A>G change was present in heterozygosity in six out of 14 patients and in the homozygous condition in patient 10 (Table 2). CYP21A2 large deletion/conversion was detected in heterozygosity in six out of 14 cases and in homozygosity in three out of 13 patients (Table 2). Out of 14 individuals, 8 (57%) were compound heterozygous for CYP21A2 mutations, while six out of 14 (43%) were homozygous (Table 2). Parental DNA for cases 16 and 22 (Table 2) was available for determination of the origin of the mutations. CYP21A2 del/conv was paternally derived in patient 16, while the mother harbored the c.293-13C/A>G mutation. Regarding patient 22, the c.920_921insT (p.L307insT) and the c.293-13C/A>G alterations were inherited, respectively, from the mother and the father. The result of the CYP11B1 test indicated that the single analyzed patient (Table 2, case 24) was homozygous for the p.G379V mutation. All the identified pathogenetic alterations have been reported previously (13, 14, 15, 16, 17, 18), except for CYP21A2 c.365T>C (p.L122P) that was found to be novel.

### DSD 46,XX disorders of ovarian development

Both the five 46,XX testicular (SRY-negative) patients and the single 46,XX ovotesticular patient with DSD (Fig. 1) were first analyzed by MLPA for the presence of copy number imbalances affecting SOX9, NROB1, NRS5A1, SRY, CYP21A2, and WNT4 genes. In a single individual with 46,XX testicular DSD (Table 2, case 9), we identified a heterozygous duplication encompassing SOX9 exon 1.
Table 2: Clinical and genetic features of DSD mutated patients. The mutations not marked by any symbol were identified in heterozygosity/hemizygosity

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Disorder of sex development</th>
<th>Patient</th>
<th>Genes</th>
<th>Mutations</th>
<th>References</th>
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</thead>
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<tr>
<td>mos 45,X(7)/47.X;i(idic(Y)(q12), + mar.idic(Y))(DYZ1 + ?)(17)/46,X;i(idic(Y)(q12)(76) mos 46,X(20)/45,XY(80) mos 45,X(70)/46,X;i(idic(Y)(qter-&gt;p11.3::p11.3-&gt;qter)(30) 45,X,(51)/46,X;i(idic(Y)(q11.23)(49) 45,X(23)/46,XY(77) 46,XX</td>
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<td>Patient</td>
<td>Genes</td>
<td>Mutations</td>
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<td>AR</td>
<td>c.2290_2291inv.TA (c.2290A&gt;G;2291T&gt;A), p.Y763I</td>
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<td>AR</td>
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<td>SRD5A2</td>
<td>c.332_333delTC (p.L111Hfs*24)</td>
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<td>56</td>
<td>AMH</td>
<td>c.367C&gt;T (p.R123W)</td>
<td>(50)</td>
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MRKH, Mayer-Rokitansky-Kuster-Hauser; del/conv, deletion/conversion.
§ Homozygous mutations. The mutations not marked by any symbol were identified in heterozygosity/hemizygosity.
hybridization analyses (data not shown), were analyzed for the presence of WNT4 point mutations and for SHOX copy number imbalances. None of the patients were found to carry a WNT4 alteration, while a duplication of SHOX exon 6 in the heterozygous state was found in two out of ten MTKH patients (Table 2, cases 25–26).

**DSD 46,XY disorders of testicular development**

Seventeen 46,XY patients (Fig. 1) were studied for variations in the SRY, NR5A1, and DHH genes. We identified the heterozygous c.301C>G (p.L101V) SRY missense mutation in patient 28 (Table 2) manifesting complete gonadal dysgenesis. Three patients with partial gonadal dysgenesis were heterozygous carriers of three different NR5A1 mutations: c.691_699 dupCTACAGCTG (p.L231_L233dup) (19) (Table 2, patient 29), c.86C>T (p.T29M) (Table 2, patient 30), and c.872_874delTGG (p.V291del) (Table 2, patient 31). None of the DHH point mutations were found in the analyzed patients. Following SRY, NR5A1, and DHH screening, the 13 out of 17 negative cases were tested for copy number alterations in NR0B1, WNT4 and DMRT1 genes. Patient 27 (Table 2) showing complete gonadal dysgenesis carried a heterozygous whole NR0B1 gene duplication. We identified a heterozygous deletion encompassing the DMRT1 gene in patient 32 (Table 2) manifesting partial gonadal dysgenesis. Finally, we did not detect a WNT4 imbalance in any of the investigated patients.

**DSD 46,XY disorders in androgen synthesis or action**

Sequence analysis of AR and SRD5A2 genes was performed in 29 patients with suspected DSD 46,XY defects in the synthesis or action of androgens (Fig. 1). The AMH gene was sequenced in a single case with the clinical diagnosis of persistent Müllerian duct syndrome. We found a genetic alteration in 24 out of 29 (83%) patients (Fig. 2). Among them, 15 individuals (Table 2, cases 33–47) were found to carry a hemizygous mutation in the AR gene. Out of 13 identified different sequence changes, six were missense mutations, five were truncating mutations, and two were splicing mutations. The c.1886-2A>G splicing alteration was detected in three unrelated patients (Table 2, cases 42–44). Six identified AR alterations were first reported in this study (c.906delC (p.S302Rfs*19), c.1249delG (p.A417Rfs*61), c.1769-13T>G, c.1886-2A>G, c.2290_2291inv.TA (c.(2290A>T;2291T>A), p.Y763I), and Cervico-thoracic Somite dysplasia; MRKH, Mayer-Rokitansky-Kuster-Hauser; CGD, Complete Gonadal Dysgenesis; PGD, Partial Gonadal Dysgenesis.

**Figure 1**

Etiological classification of 88 patients with DSD. After karyotyping and SRY-specific FISH analyses, the patients were classified using the classes suggested by Hughes et al. (1). MURCS, Müllerian aplasia, Renal aplasia, and Cervico-thoracic Somite dysplasia; MRKH, Mayer-Rokitansky-Kuster-Hauser; CGD, Complete Gonadal Dysgenesis; PGD, Partial Gonadal Dysgenesis.

**Figure 2**

Point mutation analysis of AR, SRD5A2, and AMH genes in 29 patients with 46,XY DSD disorders in androgen synthesis or action. Out of 29 patients, 15 (52%) harbored a mutation in the AR gene, 8 (27%) in the SRD5A2 gene, and 1 (3%) in the AMH gene. Out of the 29 patients, 5 (17%) did not show any point mutations in the coding sequences of the analyzed genes.
and c.165_788del (p.L56_L263del)). Eight patients carried a total of 12 different alterations in the SRD5A2 gene in compound heterozygosity (Table 2, cases 48 and 50–55) or in the homozygous state (Table 2, case 49). Out of 12 mutations, eight were missense, while four were truncating. Three missense mutations recurred in more than one patient: c.513G>C (p.R171S) (Table 2, cases 51 and 52), c.586G>A (p.G196S) (Table 2, cases 50 and 51), and c.1036A>T (p.R246W) (Table 2, cases 50 and 55). The c.513G>C (p.R171S) and the c.763T>C (p.X255Qfs*28) were, respectively, paternally and maternally derived in patient 51. Two of the identified alterations (c.564C>A (p.C188X) and c.332_333delTC (p.L111Hfs*24)) were found to be novel. Finally, a single patient (Table 2, case 56) was a compound heterozygote for a missense (c.367C>T (p.R123W)) and a novel nonsense (c.564C>A (p.C188X)) mutation in the AMH gene.

In total, we found a genetic alteration in 56 out of 88 (64%) patients (Table 2). Figure 3 summarizes the genetic tests, and the respective results, performed on patients with 46,XX disorders of ovarian development, 46,XX androgen excess, 46,XX other, and disorders of testicular development, and 46,XY disorders in androgen synthesis or action. Figure 3

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of patients</th>
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<tbody>
<tr>
<td>DSD 46,XX testicular SRY +/-</td>
<td>2</td>
</tr>
<tr>
<td>MLPA intersex (SOX9)</td>
<td>1</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
</tr>
<tr>
<td>DSD 46,XX ovotesticular RSPO1</td>
<td></td>
</tr>
<tr>
<td>CYP21A2</td>
<td>1</td>
</tr>
<tr>
<td>CYP11B1</td>
<td>1</td>
</tr>
<tr>
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<td>DSD 46,XX androgen excess WNT4</td>
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<tr>
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<tr>
<td>DSD 46,XX other MLPA intersex (NROB1)</td>
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<tr>
<td>SRY</td>
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<tr>
<td>NRSA1, DHH</td>
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</tr>
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<tr>
<td>DSD 46,XY partial gonadal dysgenesis MLPA intersex + SRY, DHH</td>
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</tr>
<tr>
<td>NRSA1</td>
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<tr>
<td>DMRT1</td>
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<tr>
<td>DSD 46,XY ovotesticular MLPA intersex + SRY, NRSA1, DHH</td>
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<tr>
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<tr>
<td>DSS 46,XY disorders in androgen synthesis or action AR</td>
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<td>SRD5A2</td>
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<td>AMH</td>
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<tr>
<td>Negative</td>
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</table>

Figure 3

Genetic studies and molecular results for 83 patients with 46,XX and disorders of ovarian development, 46,XX and androgen excess, 46,XX other, 46,XY and disorders of testicular development, and 46,XY and disorders in androgen synthesis or action. DSD clinical classes and genetic tests performed are listed on the y-axis. For each molecular analysis, the number of positive (mutated) and negative (not mutated) patients is symbolized by gray bars. SRY +/-, SRY presence/absence test; MLPA Intersex: MLPA SALSA P185-B2 Intersex; RSPO1, CYP21A2, CYP11B1, WNT4, SHOX, SRY, NRSA1, DHH, DMRT1, and SRD5A2; molecular study of the listed genes.

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testicular development, and 46,XY disorders in androgen synthesis/action. The positions of all the identified point mutations along the coding sequences of the SRY, AMH, SRD5A2, NR5A1, CYP11B1, AR, RSPO1, and CYP21A2 genes. Exons are symbolized by gray boxes and introns (not in scale) by black lines. Functional protein domains are represented by black stripes above each gene diagram. HMG, High-Mobility Group; TGFB, transforming growth factor Beta; ZnF_C4, c4 zinc finger; HOLI, ligand-binding domain of hormone receptors; FU, furin-like repeats; TSP1, thrombospondin type 1 repeats. Protein domains were predicted using the SMART software (30).

Figure 4
Positions of the identified point mutations along the coding sequences of the SRY, AMH, SRD5A2, NR5A1, CYP11B1, AR, RSPO1, and CYP21A2 genes. Exons are symbolized by gray boxes and introns (not in scale) by black lines. Functional protein domains are represented by black stripes above each gene diagram. HMG, High-Mobility Group; TGFB, transforming growth factor Beta; ZnF_C4, c4 zinc finger; HOLI, ligand-binding domain of hormone receptors; FU, furin-like repeats; TSP1, thrombospondin type 1 repeats. Protein domains were predicted using the SMART software (30).

Discussion
DSDs are complex conditions related to a vast number of different causes. Establishing the specific etiology may be crucial for choosing the more adequate sex of assignment, for the clinical management of patients, and to permit the family to plan informed further pregnancies. However, molecular characterization cannot be reached in a consistent number of cases, due to the still limited knowledge of etiological determinants. In this report, we describe the clinical assessment and the cytotagnetic and molecular findings in a large cohort of patients with DSD. In our hands, it was possible to identify a genetic defect in 64% of them and to assign each of the examined patients to a specific category in accordance with the current DSD classification. Accordingly, a specific survey could be planned for each patient. This study excluded 45,X as well as 47,XXX and 47,XXY patients and individuals with sex chromosome mosaicism identified during prenatal diagnosis, but that did not display abnormalities of the genital tract after birth. Karyotype analysis performed during this study showed that five out of 88 (6%) patients harbored mosaic sex chromosome anomalies, confirming that standard cytogenetic analyses can detect frequent
genetic causes of DSD. Moreover, the initial classification based on clinical and cytogenetic findings was revealed to be an important starting point to carry out the further appropriate molecular testing, specific for each DSD subgroup. Out of 88 patients, 7 (8%) were classified as 46,XX testicular DSD and, between them, two out of seven carried a SRY translocation onto the pseudoautosomal region PAR1 of one X chromosome. This aberration is considered the major cause of testicular development in individuals with 46,XX testicular DSD (20, 21, 22). Conversely SRY translocation appeared to be involved only in less than a third of our cases. Thus, we assumed that other molecular determinants were responsible for the other 46,XX testicular DSD cases not carrying the SRY translocation. The involvement of the SOX9 gene has already been demonstrated in a number of 46,XX testicular patients with DSD (5, 23, 24). Particularly, Cox et al. (24) and Vetro et al. (5) reported a 178-kb duplication and a 96 kb triplication, respectively, 600kb and 500 kb upstream of SOX9, in 46,XX, SRY-negative male patients. These alterations were assumed to enhance the promoter activity leading to SOX9 overexpression. In this study, we demonstrated the presence of a SOX9 exon 1 duplication in a 46,XX testicular patient with DSD (Table 2, case 9) not harboring an SRY translocation. Although it is not possible to affirm with certainty that the duplication identified in our patient is causative of his DSD, data from the literature permit speculation about its role in the determination of the abnormal gonadal development (5, 23, 24). Owing to the lack of the patient’s DNA, it was not possible to investigate whether the rearrangement identified in case 9 extended upstream of SOX9, but we cannot exclude the involvement of its promoter. In addition, as patient 9 belongs to north African ethnic group and lives in Africa, DNA neither from other family members nor from healthy controls of his population was available for testing the origin and the possible recurrence of the rearrangement. Our series of SRY-negative 46,XX testicular patients with DSD were also investigated for RSPO1 gene alterations as this gene has already been described as recessively mutated in two familial cases with 46,XX testicular DSD (25). Those patients showed genital anomalies accompanied by additional features, in particular palmpoplantar hyperkeratosis. We did not find any RSPO1 point mutations in our 46,XX testicular DSD cases, implying that the RSPO1 gene may not be involved in 46,XX testicular DSD without palmpoplantar hyperkeratosis. Our series of patients included a single 46,XX ovotesticular DSD case showing palmpoplantar hyperkeratosis. This patient was born from consanguineous parents and harbored the c.286+1G>A (p.I32_I95del) RSPO1 mutation in homozygosity (12).

The 21-hydroxylase deficiency is considered the most frequent cause of DSD with genital ambiguity. Genetic analysis of CYP21A2 performed in patients with a definitive or presumptive clinical diagnosis of adrenogenital syndrome allowed the identification of the molecular defect in 14 out of 18 (78%) cases. Among the four negative patients, case 24 was afterward recognized to be affected by a very rare form of congenital adrenal hyperplasia related to 11-β-hydroxylase deficiency. This patient was born as the result of a consanguineous mating and presented ambiguous genitalia at birth. Her clinical and hormonal profile could be defined only after the first weeks of life, when the CYP21A2 study had already been started. She was found to carry the homozygous p.G379V alteration in the CYP11B1 gene. The remaining three out of 18 CYP21A2-negative patients, in whom adrenogenital syndrome was suspected, showed regression of clitoral hypertrophy throughout the late neonatal period. The evolution of their clinical presentation together with the molecular and hormonal findings led to definitive exclusion of the initial diagnostic hypothesis in these infants.

Regarding 46,XX patients with DSD with abnormal development of the Müllerian structures, our results demonstrated the presence of a duplication involving SHOX exon 6 in two out of ten unrelated cases with MRKH syndrome, a condition of still mostly unclear etiology. Nevertheless, our targeted investigations permitted replication of the results obtained from the study by Gervasini et al. (4), which reported a SHOX intragenic duplication in five patients with abnormal development of the Müllerian ducts. Although the mechanism that may relate SHOX duplications and the development of Müllerian ducts has not been clarified, our data and those described by Gervasini et al. (4) indicate a possible functional role of SHOX in the MRKH syndrome. Based on the results obtained from the study by Philibert et al. (26) describing a WNT4 heterozygous mutation in four cases with MRKH syndrome and hyperandrogeism, we sequenced the WNT4 gene in 46,XX patients with DSD with abnormal development of the Müllerian structures, but did not find any DNA alteration. In accordance with results from other studies reporting the absence of WNT4 gene mutations in MRKH women (27, 28), it is possible that the involvement of this gene is restricted to cases with an atypical form of the syndrome.

Investigations of patients affected by 46,XY DSD with a defect in testicular development led to the molecular characterization of six out of 16 (37%) cases.
These outcomes are consistent with the still incomplete understanding of the molecular events that underlie testicular development, indicating the need to search for novel genes associated with gonadal dysgenesis in 46,XY patients. The NR5A1 gene, studied in 46,XY patients with a diagnosis of partial gonadal dysgenesis, was found to be mutated in heterozygosity in 3 out of 8 (37%) cases. These results consistent with those described in recent reports that identify mutations in NR5A1 as a major cause of 46,XY DSD with a defect in the testicular development. Among the three mutations identified in NR5A1, the genetic location of c.86C>T (p.T29M) seems to affect the binding of the protein to DNA, while p.L231_233dup and p.V291 lay in the domain regulating transcription after hormone binding. Interestingly, in the three NR5A1-mutated patients, no Müllerian structures seemed to be present.

The analysis of the DHH gene yielded negative results in all cases of 46,XY DSD with partial gonadal dysgenesis, even if DHH alterations have already been described as the possible cause of a consistent number of 46,XY DSD cases with a defect in the testicular development (29).

Concerning patients with 46,XY DSD with a defect in the synthesis or action of androgens, 24 out of 29 (83%) cases were characterized at a molecular level. Among the 12 different identified SRD5A2 genetic alterations, one maps in the transmembrane region, possibly affecting the protein localization, and 11 in the protein catalytic domain. Sequence analysis of the AR gene identified 13 different mutations, including nine alterations lying in the functional protein domains: three out of nine in the zinc finger domain responsible for the DNA binding, and six out of nine in the domain that regulates the transcription after hormone binding. The incomplete diagnostic sensitivity of the applied molecular studies in 46,XY patients with DSD with a defect in the synthesis or action of androgens might be the cause of the failure of diagnosis in those partial androgen insensitivity syndrome (PAIS) patients for whom negative results were obtained according to AR analysis. These patients may indeed harbor DNA alterations in non-canonically investigated AR regions (introns or regulatory sequences), or in different known or as yet unidentified genes.

Our results highlight that each category of DSD is related to a large number of different DNA alterations, thus requiring multiple genetic studies to possibly achieve a precise etiological diagnosis in every patient. Currently, as a consequence of the incomplete knowledge concerning the genetic factors involved in the differentiation of testes and ovaries, DSD associated with anomalies in gonadal development still often lacks a molecular diagnosis.

A multidisciplinary and specialized DSD center is the key for the correct clinical management of neonates in cases of ambiguous genitalia. Moreover, the introduction of new technologies for massive parallel sequencing is becoming helpful for the molecular characterization of patients with DSD by analyzing previously known genes as well as candidate genes.


