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► To cite this version:

L. Mary, M. Fradin, L. Pasquier, C. Quélin, P. Loget, et al.. Role of chromosomal imbalances in the pathogenesis of DSD: A retrospective analysis of 115 prenatal samples. *European Journal of Medical Genetics*, 2023, 66 (6), pp.104748. 10.1016/j.ejmg.2023.104748 . hal-04066527

HAL Id: hal-04066527

<https://hal.science/hal-04066527>

Submitted on 16 May 2023

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Role of chromosomal imbalances in the pathogenesis of DSD: a retrospective analysis of 115 prenatal samples

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Abstract

Differences of sex development (DSDs) are a group of congenital conditions characterized by a discrepancy between chromosomal, gonadal, and genital sex development of an individual, with significant impact on medical, psychological and reproductive life. The genetic heterogeneity of DSDs complicates the diagnosis and almost half of the patients remains undiagnosed. In this context, chromosomal imbalances in syndromic DSD patients may help to identify new genes implicated in DSDs. In this study, we aimed at describing the burden of chromosomal imbalances including submicroscopic ones (copy number variants or CNVs) in a cohort of prenatal syndromic DSD patients, and review their role in DSDs. Our patients carried at least one pathogenic or likely pathogenic chromosomal imbalance/CNV or low-level mosaicism for aneuploidy. Almost half of the cases resulted

from an unbalanced chromosomal rearrangement. Chromosome 9p/q, 4p/q, 3q and 11q anomalies were more frequently observed. Review of the literature confirmed the causative role of CNVs in DSDs, either in disruption of known DSD-causing genes (*SOX9*, *NR0B1*, *NR5A1*, *AR*, *ATRX*,...) or as a tool to suspect new genes in DSDs (*HOXD* cluster, *ADCY2*, *EMX2*, *CAMK1D*,...). Recurrent CNVs of regulatory elements without coding sequence content (i.e. duplications/deletions upstream of *SOX3* or *SOX9*) confirm detection of CNVs as a mean to explore our non-coding genome. Thus, CNV detection remains a powerful tool to explore undiagnosed DSDs, either through routine techniques or through emerging technologies such as long-read whole genome sequencing or optical genome mapping.

Keywords: genital – chromosomal imbalance - CNV – DSD – CMA – prenatal

Introduction

Differences of sex development (DSDs) are congenital variations characterized by a discrepancy between chromosomal, gonadal, and genital sex development. Prevalence of DSDs differs greatly depending on the variation considered: from 1 in 20,000 births for 46,XY complete gonadal dysgenesis or 46,XX testicular DSD to 1 birth in 250 boys for hypospadias (Atlas et al., 2021). Identification of a DSD often lead to a lifelong multidisciplinary medical management, with significant impact on medical, psychological and reproductive life of the patients (Hughes et al., 2006). Moreover, delay in diagnosing DSDs may burden a patient's life prognosis, since some DSDs increase the risk of gonadal malignancies (Cools et al., 2018).

The genetic landscape underlying these differences depends on the type of DSD considered. Gonosomal aneuploidies account for 33% to almost 50% of all DSD patients (Globa et al., 2022; Mazen et al., 2021). Variations in gonadal development are frequently due to *SRY* translocation on one X chromosome (50-90%, (Mazen et al., 2021)). A large amount of the remaining DSDs are monogenic, with whole exome sequencing (WES) reaching a diagnosis efficiency of around 40-60% in these forms

of DSD (Globa et al., 2022; Mazen et al., 2021). In addition to point variations, copy number variants (CNVs) and large chromosomal imbalances are rarely identified in cohorts of isolated DSD patients (4%, (Mazen et al., 2021)), but can hit 30% when syndromic DSDs are also considered (Ledig et al., 2010). However, deciphering the sexual development complexity remains challenging. Pathogenic CNVs identified in syndromic DSD patients may help to (1) identify new genetic mechanisms in known DSD genes, (2) highlight new DSD genes, (3) help to assess the role of the non-coding genome in DSDs. In this study, we aimed at describing the CNV burden in DSD patients extracted from a cohort of 4657 prenatal samples addressed for a polymalformative syndrome, and review the role of pathogenic CNVs in DSDs.

Patients and Methods

Patients

We retrospectively analyzed clinical and genetic data from fetal consecutive samples addressed to our laboratory between 2010 and 2021. A total of 4657 cases with polymalformative syndromes diagnosed through prenatal ultrasound examination were considered. Genital malformations were diagnosed and classified according to the Chicago DSD consensus (Hughes et al., 2006). Fetuses or neonates presenting with DSD and carrying at least one pathogenic chromosomal imbalance were retained for inclusion in this study. CNVs' classification was established based on the recommendations of the French array-CGH (aCGH) network (AChro-Puce <https://acpa-achropuce.com/>). DSD linked to gonosomal aneuploidies, trisomy for chromosomes 13, 18 and 21, samples without clinical data, and patients carrying only CNVs considered benign were dismissed. Standardized autopsy procedures were performed, including skeletal X-rays, macroscopic examinations and histology analyses. All samples were obtained with informed consent according to the protocols approved by the local ethics committee (Rennes hospital).

Methods

Karyotype: RHG-banding karyotypes were performed on cultured amniocytes or on chorionic villi (for all the fetuses, except P6 and P9) and cultured lymphocytes obtained from peripheral blood (for newborn and parents) according to standard cytogenetic procedures.

Chromosomal microarray analysis (CMA): Oligonucleotide aCGH was performed using the Agilent Human Genome CGH microarray 44K, 60K, 105K or 180K (depending on the year the sample was received), with a genome-wide median probe spacing 43 kb, 41 kb, 22 kb and 13 kb respectively (Agilent Technologies, Santa Clara, CA, USA). The experiments were performed according to the manufacturer's protocol. Patients' gDNAs were obtained from chorionic villi, amniotic fluid, peripheral blood or frozen tissues using Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) before 2016 and a Prepito instrument (PerkinElmer Inc., Turku, Finland) after this year. A graphic overview and analysis of the data were obtained using Agilent softwares. The probes were mapped using the GRCh37 genome version.

FISH: FISH analyses using in-house BAC probes were used to confirm chromosomal imbalances and to study their inheritance, when parental samples were available (probes available on demand).

Classification of the variants was based on data found in the literature and international CNV databases (DECIPHER, DGV, gnomAD), and on inheritance and gene content of the CNV, in accordance with recommendations by the French aCGH network (AChro-Puce).

Results

Genital malformations were present in approximately 2.5% (115) of the 4657 patients. Of note, in our cohort, 3 patients without genital anomalies carried pathogenic CNV encompassing genes previously reported in DSD (*SOX9*, *SOX3*, *GATA4*, not detailed in this work). An inclusion chart is presented in Figure 1a. Global description of the CNVs observed in the reported patients is summarized in Figure 1b.

Fourteen patients were retained for inclusion in this work. A termination of pregnancy (TOP) was decided in 13/14 cases due to severe malformations, and one pregnancy ended with a living birth.

Autopsy procedures were performed on 10 fetuses and 3 others benefited only from full external examination. Pediatricians evaluated the newborn patient shortly after birth and at 3 months of age. Nine patients had a XY chromosomal sex (P2 to P9, P12) and 5 patients a XX chromosomal sex (P1, P10, P11, P13 and P14). Chromosome 9 anomalies accounted for 28% of the CNVs found in our patients (4/14). Chromosomal imbalances were also observed for chromosomes 3, 4 and 11, in 14% of the patients each (2/14). All patients presented with a syndromic DSD. Complete phenotypes of the patients (including non-genital/gonadal malformations) are summarized in Table 1. For 46,XY DSD patients, the clinical findings were mainly micropenis, isolated (P3, P6 to P8) or associated with hypospadias (P4, P5, P12). Cryptorchidism and isolated hypospadias was observed in one case each (P2, P9). None of them presented any Müllerian remnants. 46,XX DSD presented with more variable phenotypes, ranging from isolated hypoplastic ovaries (P14) to almost complete sex reversal with ovotestis (P1). The 3 other 46,XX DSD patients presented with Müllerian malformations (P11 and P13) or agenesis (P10). Familial segregation of the anomalies are reported in Table 1 when obtained. Patients are presented according to the size of their imbalance(s) (larger or smaller than 5Mb, sSMC (small supernumerary chromosome marker)) and then by patient's numerical order.

Imbalances >5Mb

P1 presented a XX DSD with a urethral opening under the genital tubercle, fused labioscrotal swellings and absent vaginal introitus nor Müllerian remnants. Histologic examination of the gonads revealed a left gonad being an ovotestis: a fibrous gonad with a region of testicular-like architecture with albuginea and seminiferous tubes containing Leydig cells, near an ovary-like region with isolated germ cells in an undifferentiated stroma (Figure 2a). The architecture of the right gonad was closer to a testis with fibrous modifications (Figure 2b). This case presented with mosaicism for complete trisomy 9. The mosaicism was determined to be around 30% through CMA on amniotic fluid. A karyotype was performed on an umbilical cord sample, highlighting a 14% mosaicism, confirmed by FISH analyses on cultured fibroblasts using a chromosome 9 centromeric probe associated with a *CDKN2A* probe.

Karyotype on placental sample found a complete homogeneous trisomy 9, leading to the diagnosis of fetoplacental mosaicism. Genitourinary variations are observed in almost half cases of complete trisomy 9, including XX sex reversal (Stoll et al., 1993). XX sex reversal was also reported in trisomy 9 mosaic syndrome, despite the fact that cryptorchidism in XY individuals is the most frequent DSD observed ((Li et al., 2021; Solomon et al., 2007). P1 also presented with a *de novo* 567kb-sized 13q13.3 deletion, disrupting 5 genes, including a read-through transcript containing *SOHLH2* (OMIM*616066). This late gene is suspected to play a role in oogenesis and is considered as a candidate gene for premature ovarian failure (POF), but DSD were not reported in animal models (Choi et al., 2008). The other 4 genes were not related to gonadal or genital development. Finally, such deletions were reported neither in the literature nor in general populations' databases, and we considered this CNV as a variant of unknown significance (VOUS), without significant role in the patient's genital phenotype. Three patients (P2, P3 and P4) with a XY DSD had partial chromosome 9 trisomy. It is of note that P2 was the only living birth in our cohort. These chromosomal rearrangements were linked to the presence of a derivative chromosome from a balanced *de novo* (P4) or maternal (P3) reciprocal translocation. No paternal karyotype was performed for P2 and the mother had a normal karyotype. Except for P2, a second pathogenic CNV was present in these patients. P2 was a XY syndromic DSD patient diagnosed shortly after birth with bilateral cryptorchidism. He presented with an almost complete 9p trisomy (9p24.3p11.2, 45.2Mb) due to a chromosome 15 derivative from a reciprocal (9;15) translocation. P3 was a syndromic XY DSD patient with micropenis. He showed a partial proximal 9p trisomy (9p24.3q21.13, 77.7Mb) and a 14q32.2q32.33 trisomy (3.3Mb) due to a supernumerary chromosome 9 derivative from a balanced maternal reciprocal translocation (9;14). P4 was a syndromic XY DSD patient with micropenis and hypospadias. He had a terminal 9q trisomy (9q33.3q34.3, 14.9Mb) and a 5.8Mb-sized 11q24.3q25 deletion linked to a chromosome 11 derivative of a *de novo* reciprocal translocation (9;11). Hypoplastic genitalia are reported in XY patients bearing pure 9p trisomy or partial distal 9q trisomy (Sato et al., 2020). Numerous genes are implicated in the imbalances diagnosed in patients P1 to P4, but two genes are known to be implicated in gonadal

development, namely *DMRT1* (9p24.3, OMIM*602424) and *NR5A1* (9q33.3, OMIM*612965). Both genes are sensitive to haploinsufficiency, but only *NR5A1* is triplosensitive. Duplications encompassing *DMRT* genes cluster were both reported in ClinVar and DECIPHER as likely benign or VOUS. However, *Dmrt1* copy number variants are suspected to play a role in sex determination in non-mammalian species ((Lambeth et al., 2014) and Discussion).

P6 presented with a syndromic XY DSD with micropenis, and the association of *de novo* terminal 4p duplication (4p16.3p15.2, 27Mb) and terminal 4q deletion (4q32.3q35.2, 25Mb). The duplication encompassed the Wolf-Hirschhorn Syndrome (WHS, OMIM#194190) critical region. The deletion involved at least 60 genes, including *NSD2* (OMIM*602952) and *NELFA* (OMIM*606026) that were linked to WHS core features, including DSD (Battaglia et al., 2015). The association of both 4p duplication and 4q deletion was reported in the literature in case of recombinant of chromosome 4 pericentric inversion (Maurin et al., 2009), with DSD mainly observed in XY patients (hypospadias, hypoplastic scrotum).

P7 had a syndromic XY DSD with micropenis. He carried an association of a 2.2Mb-sized distal 4q35.2 deletion containing 4 coding genes (*ZFP42* (OMIM*674572), *TRIML1*, *TRIML2* and *FRG1* (OMIM*601278)) and a 46.2Mb-sized distal 7q31.1q36.3 duplication encompassing more than 200 coding genes. Reports of 7q terminal duplications may include DSD (micropenis and cryptorchidism), especially duplications encompassing the 7q31 region (Bosfield et al., 2021). We suggest *FEZF1* (OMIM*613301) and *RBM28* (OMIM*612074) as possibly implicated in the DSD phenotypes reported in these patients. The genes contained in the 4q35.2 deletion are not considered as sensitive to haploinsufficiency and similar deletions were reported as VOUS or benign in ClinVar and DECIPHER databases. We thus considered the 7q duplication alone as responsible from the patient's phenotype.

P8 presented with a syndromic XY DSD with micropenis. Cytogenetic studies revealed homogeneous trisomy for the whole chromosome 3 long arm, due to a chromosome 14 derivative of a maternal reciprocal translocation (3;14). Partial or complete 3q trisomy are rare and reported in severe polymalformative phenotypes. Most XY patients present with micropenis, cryptorchidism and

hypospadias (Dworschak et al., 2017). *TBL1XR1* (OMIM*608628) and *SOX2* (OMIM*184429) are suggested as the main genes implicated in DSDs in 3q terminal region ((Dworschak et al., 2017) and Discussion). P9 had a syndromic XY DSD with hypospadias. This fetus carried both a 8.3Mb-sized 3p26.3p26.1 deletion encompassing 15 coding genes (notably *ITPR1* (OMIM*147265)), and a 34.5Mb-sized 3q26.1q29 duplication containing more than 150 coding genes, suggesting a derivative chromosome from a pericentric inversion. Parents were not available for testing. Chromosome 3p deletion syndrome is responsible for a syndrome first characterized by neurodevelopmental disorders, cerebellar ataxia and variable facial gestalt (Fernandez et al., 2008). Cryptorchidism was only reported once, in a patient carrying an associated 4q32.2qter duplication (Petriczko et al., 2012). Thus, we suggest the 3q trisomy observed in P8 and P9 as more likely to play a role in their DSD phenotype.

P11 had a syndromic XX DSD with unicornuate uterus. CMA revealed a terminal *de novo* 9.8Mb-sized 11q24.2q25 deletion containing at least 40 coding genes, leading to the diagnosis of Jacobsen syndrome (OMIM#147791). P4 (described above) also presented with a 5.83Mb-sized 11q24.3q25 deletion encompassing 18 genes, leading to partial Jacobsen syndrome, in addition to partial trisomy 9q. Cryptorchidism and hypospadias are reported in Jacobsen syndrome (Grossfeld et al., 2004; Penny et al., 1995), however uterine malformations were not reported to date, and no particular gene was linked to the occurrence of a genital phenotype. Common deleted genes in both P4 and P11 included, among others, *BARX2* (OMIM*604823), *NTM* (OMIM*607938) and *OPCML* (OMIM*600632), these two last genes predicted to be sensitive to haploinsufficiency. *BARX2* seemed the most likely to be linked to DSD, as discussed below, since *NTM* and *OPCML* functions seemed distant from genital development (neuronal migration and tumor suppression respectively).

sSMC

P12 had a syndromic XY DSD with a curved penis and a hypospadias. He carried a supernumerary isochromosome 12p, leading to homogeneous tetrasomy 12p (Pallister-Killian syndrome, (MIM#601803)). DSDs are frequently reported in both XX and XY patients presenting with Pallister-Killian syndrome (Izumi and Krantz, 2014; Wilkens et al., 2012). DSD phenotypes was not linked to a

particular gene on chromosome 12 short arm. However, we suggested *CHD4* (OMIM*603277) as a candidate gene for DSD in Pallister-Killian syndrome, as discussed below.

P14 had a syndromic XX DSD with normal-appearing female external and internal genitalia, and histologically normal hypoplastic ovaries, one of them showing a small area of undifferentiated tissue without signs of dysgenesis. She presented with a sSMC derived from chromosome 22, resulting in a partial trisomy 22 (22q11.1q11.21, 5Mb) in a mosaic state. This marker contained the cat-eye syndrome (CES, OMIM#115470) region and the LCR-A to LCR-D 22q11.21 region. Genital anomalies are rare in CES: two XX patients are reported with hypoplastic female external genitalia or Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome (AlSubaihin et al., 2018). We suggest *CECR2* (OMIM*607576) may be a candidate in explaining the DSD observed in some CES (discussed below).

CNVs <5Mb

P5 had a syndromic XY DSD with micropenis and hypospadias. A 3.8 Mb-sized 4p16.3 deletion and a 6.7 Mb-sized 7p22.3p22.1 duplication due to a paternal reciprocal translocation (4;7) was detected. The 4p deletion contains the critical region for WHS and includes 45 coding genes, notably *NSD2* and *NELFA* (see above, (Battaglia et al., 2015)). The 7p22 duplication contained 54 coding genes, most of them not being considered as triplosensitive. The CNV did not seem to disrupt the frontier of a topologically associated domain (TAD) containing a DSD gene. However, smaller 7p22.1 duplications are reported in the literature, notably in 46,XY patients presenting with syndromic cryptorchidism, the authors suggesting *ACTB* (OMIM*102630) as the main candidate gene ((Ronzoni et al., 2017) and Discussion).

P10 presented with a syndromic XX DSD linked to a 2.14Mb-sized de novo 6q27 terminal deletion. Her DSD was characterized by uterine and fallopian tubes agenesis, associated with a complex cardiopathy and cerebral anomalies. The deletion encompassed 10 coding genes, including *ERMARD* (OMIM*615532) and *DLL1* (OMIM*606582). This patient was previously reported in (Lesieur-Sebellin et al., 2022). Terminal deletion 6q are known to be responsible from a recognizable syndrome mainly defined by cerebral anomalies, epilepsy and neurodevelopmental impairment, with *DLL1* as the main

candidate gene (Lesieur-Sebellin et al., 2022). However, one 46,XY patient with 6q terminal deletion was reported as also having genital hypoplasia (Striano et al., 2006). *DLL1* is a receptor for *NOTCH* signaling pathway, which is known to be implicated in genital and gonadal development, albeit not related to DSD in humans to date (see Discussion).

P13 had a syndromic XX DSD with normal-appearing female genitalia and gonads but a bicornuate, bicervix uterus and a vaginal septum. CMA found a 1.7Mb-sized *de novo* 19q13.42q13.43 deletion. Similar deletions were only reported in ClinVar without detailed phenotype assertion, but these CNV were considered likely pathogenic (nssv585173). The deletion contained 30 coding genes, including *UBE2S* (OMIM*610309), the only gene predicted to be impacted by haploinsufficiency, and located on a frontier between 2 TADs. These TADs did not seem to contain another DSD gene. This was the smallest CNV identified in the cohort.

Discussion

Our study focused on the role of chromosomal imbalances/CNVs in DSD. We confirmed the role of some imbalances previously reported (Supplementary Table 1) and reported CNVs containing either known or suspect DSD-causative genes.

Role of CNVs in DSDs

Some chromosomal syndromes are known to include genital features (ex: 9p- syndrome). Apart from recurrent cytogenetic anomalies such as gonosomal aneuploidies and *SRY* translocations, pathogenic CNVs in DSDs account for 4 to 30% of all cases (Ledig et al., 2010; Mazen et al., 2021). Diagnosing CNVs responsible from DSDs can lead to (1) identification of new genes, through determining the smallest region of overlap (ex: (Scott and Amarillo, 2022)), (2) identification of promoters and other regulating non-coding regions (ex: *SOX9*, Supplementary Table 1), (3) identify CNV as a new mechanism in a gene previously reported as DSD-causative through point variations (ex: *WNT4*, Supplementary Table 1). This study confirmed the prominent role of first tier routine detection of chromosomal imbalances or

CNVs through cytogenetic techniques (karyotype and aCGH). Supplementary Table 1 sums up known DSD-causative CNVs and related genes.

Novel small CNVs

Isolated partial 19q13 deletions are rarely described in the literature nor in patients' databases ((Rose et al., 2011), DECIPHER 400969, 421866, ClinVar nssv585173). To our knowledge, these patients were not reported as having DSD, contrary to patient P13 (bicornuate bicervix uterus and vaginal septum). The region deleted in P13 contains genes (*UBE2S*, *NLRP2* and *NLRP5*) implicated in oocyte maturation in humans (Mu et al., 2019, p. 2) and early embryonic development in animal models (Wang et al., 2016), but none of them were previously reported in DSD. *UBE2S* is an enzyme implicated in the ubiquitin-proteasome protein degradation pathway. Some authors reported *UBE2S* as a dosage-sensitive gene responsible for regulating *Sox2* expression in mouse early embryos (Wang et al., 2016, p. 2). *SOX2* (SRY-related HMG-box gene 2) is a promising candidate for sex development, since DSDs are reported in patients of both sexes presenting with loss of function variants in this gene or a complete gene deletion (AbuAlsaoud et al., 2021). Data are currently insufficient to definitely link the 19q13.4 deletion to the occurrence of DSD, but the region appears as an interesting candidate. Further cases are needed to get more insight on the role of *UBE2S* in sexual development.

Large chromosomal imbalances involve genes known or suspected to intervene in sexual development

Partial or complete 3q trisomy are rare and reported in phenotypes close to Cornelia-de Lange syndrome, associated with sacral anomalies, micropenis, cryptorchidism and hypospadias (Dworschak et al., 2017). These phenotypes are in accordance to what was observed in our patients (P8 and P9, micropenis and hypospadias). The authors suggested *TBL1XR1* (3q26.32, OMIM*608628) responsible from Pierpont syndrome, as a candidate gene for DSD. *TBL1XR1* encodes a protein capable of interacting with histones and promoting the expression of nuclear receptor's target genes. *SOX2* (3q26.33, OMIM*184429) is also a promising candidate, as described above.

Partial 4p monosomy, especially 4p16.3 deletion, is responsible for Wolf-Hirschhorn syndrome (WHS), a contiguous genes syndrome. Genital anomalies in WHS include hypospadias with cryptorchidism in

XY patients, and clitoral variations, streak gonads and absence of Müllerian derivatives in XX individuals (Battaglia et al., 2015). A few patients with 4p duplications of various sizes are also reported in the literature: most of the clinical features observed are close to those of WHS syndrome (Maurin et al., 2009). These observations are coherent with the phenotype of P5 (4p deletion) and P6 (4p duplication), namely micropenis and/or hypospadias. Two genes, namely *NSD2* and *NELFA*, seem to contribute to the core symptoms of WHS (Battaglia et al., 2015). *NSD2* encodes a SET domain-containing transcriptional regulatory protein with histone methyltransferase activity. In mouse embryonic stem cells, *Whsc1* (*NSD2* homolog) governs cell differentiation through the expression of key transcription factors (Nimura et al., 2009). *NELFA* encodes a protein of the negative elongation factor complex (NELF), implicated in the regulation of transcription elongation of RNA polymerase. Studies in mice models suggests that Nelf is required in early embryogenesis, to maintain the pluripotency state of embryonic stem cells. Patients with haploinsufficiency of these genes, especially *NSD2*, can present with genital variations (Zanoni et al., 2021).

To the best of our knowledge, patients with 6q terminal deletion encompassing *DLL1* (as observed in P10, Table 1) did not present with DSD. *DLL1* is an effector of the *NOTCH* pathway and, in mouse models, *Notch* pathway disruption can lead to cryptorchidism and abnormal Sertoli cell function ((Kamińska et al., 2020; Yuan et al., 2006). Moreover, some authors performed gene profiling on vaginal samples from MRKH patients and highlighted downregulation of genes implicated in *NOTCH* pathway, including *DLL1*. Since P10 presented with a MRKH phenotype, with absent uterus and Fallopian tubes and normal ovaries, it suggests *DLL1* as a DSD candidate, especially in MRKH.

Partial 7p or 7q terminal duplications are often observed in cases of derivative chromosome from parental reciprocal translocations, as for P5 (presenting with micropenis and hypospadias and a 7p terminal duplication). Some authors suggested *ACTB* (7p22.1, OMIM*102630) as the critical gene responsible from most of the phenotype observed in 7p duplications (Ronzoni et al., 2017). *ACTB* encodes beta-actin, an essential protein of the cytoskeleton, implicated in various functions such as cell shaping, migration and gene expression regulation. *ACTB* is responsible for Baraitser-Winter

cerebrofrontofacial syndrome, which includes DSD in XY patients, through a gain-of-function mechanism. Large 7q duplications encompassing the 7q31 region (Bosfield et al., 2021) were observed in patients with syndromic micropenis and/or cryptorchidism (as observed in P7). This region contains *FEZF1* (7q31.32, OMIM*613301) and *RBM28* (7q32.1, OMIM*612074) two genes responsible for recessive hypogonadotropic hypogonadism, a condition that can lead to XY DSD and primary amenorrhea in XX patients, although these genes are not currently known to be dosage-sensitive.

Mosaicism for complete trisomy 9 is a rare condition, with only a few patients reported to date (Li et al., 2021). Clinical findings are quite similar to those observed for partial 9p trisomy (Akalin et al., 2014). Genitourinary variations are commonly observed in both XX and XY patients, albeit more frequent in XY cases (Li et al., 2021; Solomon et al., 2007; Stoll et al., 1993). This is in line with the phenotype observed in P1, P2, P3 and P4 (XX sex reversal, micropenis, hypospadias, cryptorchidism). Among the leading genes for DSDs on chromosome 9, *DMRT1* (9p24.3, OMIM*602424) plays a critical role in the maintenance of the male phenotype by repressing pro-ovarian genes. XY mice KO for *Dmrt1* fully develop as males but present with abnormal Sertoli cells differentiation (Raymond et al., 2000). In birds, *Dmrt1* is responsible for sex determination in a dose-dependent manner, two copies of the gene leading to a male fate whereas a single copy is not sufficient to repress female-determining genes (Lambeth et al., 2014). In humans, disruption of *DMRT1* leads to prenatal focal testicular dysgenesis (Baetens et al., 2019) and *DMRT1* deletions are associated with XY sex reversal (Zarkower and Murphy, 2021). *NR5A1* (9q33.3, OMIM*184757) encodes SF1 (Steroidogenic Factor-1), a member of the orphan nuclear receptor family of transcription factors involved in reproduction, steroidogenesis, adrenal and gonadal development in both males and females. This gene is responsible from various phenotypes in humans when disrupted, including adrenocortical insufficiency, 46,XY or XX sex reversal, male or female infertility notably premature ovarian insufficiency (POI) (Baetens et al., 2019; Jaillard et al., 2020, p. 1). Interestingly, overexpression of *Nr5a1* in female mice impairs their reproductive functions through hyperandrogenism, disruption of ovarian functions, premature infertility and metabolic disturbance (Rotgers et al., 2021). Although isolated *DMRT1* or *NR5A1* duplications are not reported

to date in DSD patients, we cannot rule out that overexpression of male-determining genes in trisomy 9 plays a role in the occurrence of such phenotypes.

Regarding chromosome 11q anomalies, genital features of Jacobsen syndrome mainly concern XY patients. Cryptorchidism (as observed in P4) is observed in 36-60% of males (Mattina et al., 2009). Uterine anomalies, as seen in P11, were not reported to date. Among the genes reported in the 11q telomeric region and included in the overlapping deleted region in our patients, we suggested *BARX2* (OMIM*604823) as a candidate gene for DSD. *BARX2* is a homeobox transcription factor able to bind regulatory elements of genes encoding neural adhesion molecules. In animal models, *Barx2* is known to play a role in neural and craniofacial structures, and hair follicles (Olson et al., 2005). *BARX2* was found to interact with estrogen receptor-alpha (*ESR1*) to regulate its splicing and expression (Stevens and Meech, 2006). Variations in genes regulating *ESR1* mRNA degradation led to hypospadias (as observed in P4), sex reversal and/or gonadal dysgenesis in XY patients (Tsai et al., 2020). A recent study also suggested *ESR1* as a candidate gene for MRKH syndrome (Brucker et al., 2017), a DSD linked to abnormal regression of the Müllerian ducts, as partially observed in P11. We can thus postulate that the DSD phenotypes observed in patients P4 and P11 are partially linked to *BARX2* haploinsufficiency and *ESR1* dysregulation.

Supernumerary marker chromosomes are a cause of DSD

As observed in P12, cryptorchidism is the most frequent DSD feature in Pallister-Killian patients presenting with DSD. We proposed *CHD4* (12p13.31, OMIM*603277) as an interesting candidate, since it encodes a protein from the nucleosome remodeling and histone deacetylase (NURD) repressor complex, which is involved in epigenetic regulation of gene transcription, DNA repair, and cell cycle progression. A recent study in a mouse model demonstrated the role of *Chd4* in spermatogonia survival and normal XY gonad development (de Castro et al., 2022).

P14 presented with hypoplastic ovaries and a sSMC leading to mosaicism for proximal trisomy 22. Such anomalies are usually responsible for CES (Rosias et al., 2001). The sSMC observed in P14 region encompassed various genes suggested to play a role in gonadal development and maintenance: *CECR2*,

COMT (OMIM*116790) and *HIRA* (OMIM*600237), of which only *CECR2* is included in the CES critical region (Footz et al., 2001). In CES, genital anomalies are rare, mainly observed in XY patients. Male mice KD for *Cecr2* are infertile with testis histological defects (Norton et al., 2022). This can suggest that variations of *CECR2* expression may impair cellular maturation and growth in gonads. *COMT* or *HIRA*, are suggested to play a role in oocyte maturation and POI (Jaillard et al., 2018), and may contribute to the hypoplastic ovaries observed in P14, but such a hypothesis needs to be further explored.

Phenotype complexity of DSD

In our cohort, micropenis and Müllerian anomalies were the most common phenotypes observed in XY and XX patients, respectively. Micropenis is a frequent symptom resulting from androgen insensitivity or ineffective testicular testosterone secretion. Beyond testicular dysfunction or defects, any disruption of the gonadotropic axis may lead to micropenis. Genes implicated in cerebral development or in ubiquitous cellular functions such as cell migration or chromatin remodeling may also cause micropenis (discussed in (Gazdag et al., 2016)). Similar conclusions can be made for Müllerian anomalies, which also rely on disruption of genes implicated in ubiquitous pathways (i.e. Wnt- β -catenin, *HOX* genes... (Kyei-Barffour et al., 2021)). Thus, in front of large genomic imbalances in DSD patients, it may be difficult to differentiate genes directly implicated in sex determination from DSD as an indirect consequence of disruption of ubiquitous functions. We carefully reviewed published functional studies and/or animal models for the suggested genes presented in this study, but the size of the imbalances highlighted in most of our patients remains a limitation in such approach. The imbrication of environmental factors and this genetic complexity leads to difficulties in diagnosing the etiology of DSDs, and almost 50% of the patients may be stuck in a “diagnostic odyssey” (Globa et al., 2022; Mazen et al., 2021). Phenotypic variability and emerging proofs of oligogenism in DSDs (Camats et al., 2020) complicate the interpretation of variants. High-throughput sequencing techniques such as WES, and more recently, whole genome sequencing (WGS), may be of help in addressing both CNVs and point variations detection (Audi et al., 2018; Délot and Vilain, 2021) in patients with normal

conventional karyotype. Finally, “mild” DSD are also underreported in syndromic patients presenting with severe non-genital symptoms, such as neurodevelopmental impairment (Gazdagh et al., 2016). Description of recurrent CNVs associated with DSDs still provide new genes and new questions, as demonstrated with the patients reported here.

Conclusion

This study shed the light on the variable phenotypes and chromosomal imbalances/CNV encountered in DSDs and highlights the clinical impact of these genetic variations. Molecular genetic analysis is becoming the gold standard for DSDs diagnosis but cytogenetics studies may still be relevant as (1) most of DSDs are linked to gonosomal aneuploidies or structural chromosomal anomalies, including low-level mosaicism, (2) non-coding genome CNV also probably account for a non-negligible part of DSDs, (3) such routine techniques may provide a good diagnostic yield at a relatively low cost. Innovative technologies such as long-read WGS and Optical Genome Mapping, may combine the abilities to detect both structural, copy number and point variations, thus increasing the diagnosis yield of DSDs and ending the patient’s “odyssey”.

Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of interest

The authors declare no conflict of interest.

Data sharing and accessibility

All patients reported here were registered in the DECIPHER database. Accession numbers are available in Table 1.

CRedit Author Statement

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Sylvie Jaillard: Conceptualization, Supervision, Writing - Review & Editing.

Figure 1: Inclusion and cohort flowcharts

Flowchart (a) sums up the inclusion criteria. Flowchart (b) describes the genetic imbalances found in our cohort. The threshold of 5Mb was considered according to (Le Caignec and Redon, 2009). sSMC: small supernumerary marker chromosome, der from t: derivative chromosome from reciprocal translocation, der from pericentric inv.: derivative chromosome from pericentric inversion, chr.: chromosome.

Figure 2: Histological aspect of patient P1's gonad.

Pane A represents the right testis-appearing gonad (upper) and the left ovotestis-like gonad (lower) at x10 magnification. Black and white arrowheads show up the clearly separated testis-like and ovarian-like regions respectively. Black star represents an area of undifferentiated tissue. Pane B contains x20 magnifications of the areas highlighted by the dashed boxes on Pane A.

Patient (DECIPHER ID)	Karyotype	aCGH	Array type	CNV size (Mb)	Age at diagnosis	Outcome / clinical evaluation	External genitalia	Internal genitalia	Gonads	Prenatal US findings	Additional findings at exam
P1 (488429)	mos 47,XX,+9[9]/46,XX[18]	arr[GRCh37] 9p24.3q34.3(271257_141008915)x3 [0.30],13q13.3(36826472_37394317)x1	60K	Chr9	31 GW	TOP / autopsy	Urethral opening under the genital tubercle Fused labioscrotal swellings Absent vaginal introitus	No müllerian remnants	Right gonad: fibrous testis Left gonad: ovotestis	Gallbladder agenesis	Facial gestalt Micrognathism Partial pancreas agenesis Intestinal malrotation Thymic hypoplasia
P2 (488430)	46,XY,der(15)t(9;15)(p11.2;p11)	arr[GRCh37] 9p24.3p11.2(204193_45469390)x3	180K	45.26	36 GW	Living birth / pediatric exam	Bilateral cryptorchidis m	No müllerian remnants	NA	Cardiomegaly, atrial septal defect Left superior vena cava Absent right superior vena cava Thin corpus callosum, dilated ventricles	Clubfeet Strabismus Brachydactyly, 5th finger clinodactyly Axial hypertonia Dyspnea Duplication of left renal pelvis
P3 (488431)	47,XY,+der(9)t(9;14)(q21.13;q32.2)dm at	arr[GRCh37] 9p24.3q21.13(271257_78004446)x3 dmat,14q32.2q32.33(101003974_104258824)x3 dmat	60K	77.73 and 3.25	15 GW	TOP / pediatric exam	Micropenis	NA	NA	Increased nuchal translucency Bilateral pyelectasia Hypoplastic left heart	Bilateral labiopalatine cleft Facial gestalt Brachydactyly
P4 (488433)	46,XY,der(11)t(9;11)(q33.3;q24.3)dn	arr[GRCh37] 9q33.3q34.3(126027848_141008915)x3,11q24.3q25(129034189_134868407)x1	60K	14.98 and 5.83	25 GW	TOP / pediatric exam	Micropenis, hypospadias	NA	NA	Bilateral pyelectasia Pericardic effusion	Retrognathism Microstomia Facial gestalt Microcephaly Supernumerary rib pair and vertebra

P5 (488435)	46,XY	arr[GRCh37] 4p16.3(51 dpat		and 6.72		autopsy	hypospadias	No müllerian remnants	Normal gonads	Bilateral renal hypoplasia, renal pelvis dilatation IUGR	Arachnoid cyst, Blake pouch cyst, ventricular dilatation Intestinal malrotation, Meckel diverticle, pancreas tail agenesis Rib asymmetry
P6 (488436)	No conventional karyotype performed, XY on aCGH	arr[GRCh37] 4p16.3p15.2(51,413-27,238,111)x3 dn,4q32.3q35.2(165,361,405- 190,896,674)x1 dn	44K	27.18 and 25.53	22 GW	TOP / autopsy	Micropenis	No müllerian remnants	Normal gonads	NA	Persistent truncus arteriosus Hypoplastic left heart Palatine cleft Postaxial polydactyly R hand SUA Intestinal malrotation 1 missing rib pair
P7 (488439)	46,XY,der(4)t(4;7)(q34;q22.1)dpat	arr[GRCh37] 4q35.2(188706640_190896674)x1,7q31.1q36.3(112844269_159088636)x3 dpat	60K	2.19 and 46.24	27 GW	TOP / autopsy	Micropenis	No müllerian remnants	Normal gonads	Cerebral ventriculomegaly Ventricular septal defect	Facial gestalt Lanugo White hair Punctate chondrodysplasi a Missing rib pair Hypoplastic distal phalanges of the digits
P8 (488440)	46,XY,der(14)t(3;14)(q11.1;p11.2)dmata	arr[GRCh37] 3q11.1q29(93580736_197840339)x3 dmata	60K	104.2 5	18 GW	TOP / autopsy	Micropenis	No müllerian remnants	Normal gonads	Anasarca Thymus hypoplasia Ventricular septal defect Micromelia	Labiopalatine cleft Facial gestalt Camptodactyly Pes equinovarus Vertebral

			Journal Pre-proof								segmentation anomalies Partial pancreatic agenesis
P9 (488441)	No conventional karyotype performed, XY on aCGH	arr[GRCh37] 3p26.3p26.1(93949_8411977)x1,3q26.1q29(162391365_197840339)x3	60K	8.31 and 35.44	15 GW	TOP / pediatric exam	Hypospadias	No müllerian remnants	NA	IUGR Hydronephrosis Ventricular septal defect Rhombencephalosynapsis Small corpus callosum Partial vermian agenesis	Imperforate anus Foot syndactyly
P10 (Lesieur-Sebellin et al., 2022)	46,XX	arr[GRCh37] 6q27(168845632_170892302)x1	44K	2.04	25 GW	TOP / autopsy	Normal-appearing female	Uterine and Fallopian tubes agenesis	Normal gonads	Congenitally corrected transposition of the great arteries Cerebral ventricular dilatation Small cerebellum	Facial gestalt Ventricular septal defect Partial vermian agenesis, cerebellar hypoplasia SUA Left kidney agenesis, pelvic right kidney Vertebral anomalies
P11 (488442)	46,XX	arr[GRCh37] 11q24.2q25(125,101,271-134,927,114)x1 dn,22q12.3(36,668,889-36,971,301)x3 mat	180K	9.82	14 GW	TOP / autopsy	Normal-appearing female	Unicornuate uterus	Normal gonads	Bilateral renal cystic dysplasia Left superior vena cava	Accessory spleen Intestinal malrotation
P12 (488443)	47,XY,+i(12)(p10)dn	arr[GRCh37] 12p13.33p11.1(163593_34398316)x4 dn	180K	34.23	13 GW	TOP / autopsy	Curved penis, hypospadias	No müllerian remnants	Normal gonads	Dandy-Walker anomaly Nasal bones agenesis Omphalocele Diaphragmatic hernia	Imperforate anus Truncus arteriosus Facial gestalt Hexadactyly of the hands
P13 (488444)	46,XX	arr[GRCh37] 19q13.42q13.43(55784730_57496219)x1 dn	44K	1.71	22 GW	TOP / autopsy	Normal-appearing female	Bicornuate, bicervical uterus	Normal gonads	Cystic hygroma Microcephaly Corpus callosum agenesis	Accessory spleen SUA

			Journal Pre-proof					Vaginal septum			
P14 (488445)	mos 47,XX,+der(22)(pter- >q11.21:)[5]/46,XX[25]	arr[GRCh37] 22q11.1q11.21(17096855_22055183)x2-3[0.50]	60K	5	27 GW	TOP / autopsy	Normal- appearing female	Normal- appearing female	Hypoplasti c ovaries	NIPD: non conclusive x2 IUGR	Delayed bone maturation Facial gestalt Mild skeletal anomalies

Table 1: Phenotype and genotype description of the patients reported in this study.

TOP: termination of pregnancy, exam: examination, NA: not assessed, NIPD: non-invasive prenatal diagnosis, IUGR: intrauterine growth retardation, SUA: single umbilical artery.

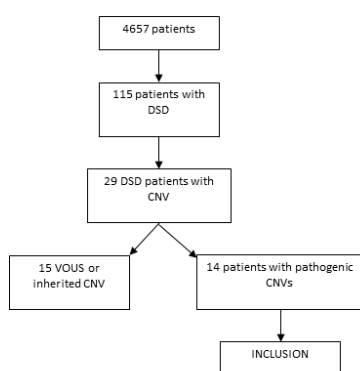
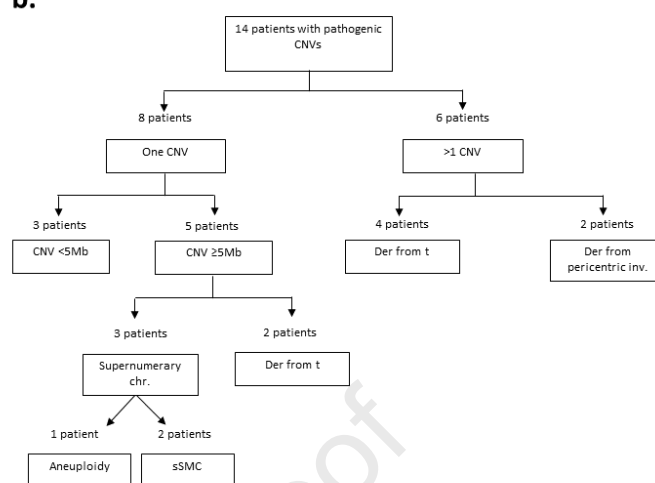
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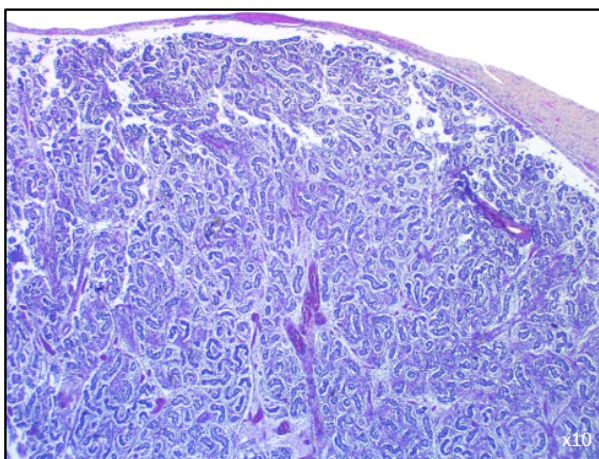
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