Oligogenic Causes of Human Differences of Sex Development: Facing the Challenge of Genetic Complexity

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Keywords

Differences of sex development · Genetics of sex determination · Genetics of sex differentiation · Steroidogenic factor 1 (nuclear receptor subfamily 5 group A member 1) · Androgen receptor · Mitogen-activated protein kinase kinase kinase 1 · Hydroxy-Delta-5-Steroid Dehydrogenase 3 Beta- And Steroid Delta-Isomerase 2 · Zinc finger protein, friend of GATA family member 2 and GATA Binding Protein 4 · 17b-Hydroxysteroid dehydrogenase type 3; Mastermind-like domain-containing protein 1 · Oligogenic disorders · Human genetics

Abstract

Background: Deviations of intrauterine sex determination and differentiation and postnatal sex development can result in a very heterogeneous group of differences of sex development (DSD) with a broad spectrum of phenotypes. Variants in genes involved in sexual development cause different types of DSD, but predicting the phenotype from an individual's genotype and vice versa remains challenging.

Summary: Next Generation Sequencing (NGS) studies suggested that oligogenic inheritance contributes to the broad manifestation of DSD phenotypes. This review will focus on possible oligogenic inheritance in DSD identified by NGS

studies with a special emphasis on *NR5A1* variants as an example of oligogenic origin associated with a broad range of DSD phenotypes. We thoroughly searched the literature for evidence regarding oligogenic inheritance in DSD diagnosis with NGS technology and describe the challenges to interpret contribution of these genes to DSD phenotypic variability and pathogenicity. **Key Messages:** Variants in common DSD genes like androgen receptor (*AR*), mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*), Hydroxy-Delta-5-Steroid Dehydrogenase 3 Beta- And Steroid Delta-Isomerase 2 (*HSD3B2*), GATA Binding Protein 4 (*GATA4*), zinc finger protein friend of GATA family member 2 (*ZFPM2*), 17b-hydroxysteroid dehydrogenase type 3 (*HSD17B3*), mastermind-like domain-containing protein 1 (*MAMLD1*), and nuclear receptor subfamily 5 group A member 1 (*NR5A1*) have been detected in combination with additional variants in related genes in DSD patients with a broad range of phenotypes, implying a role of oligogenic inheritance in DSD, while still awaiting proof. Use of NGS approach for genetic diagnosis of DSD patients can reveal more complex genetic traits supporting the concept of oligogenic cause of DSD. However, assessing the pathomechanistic contribution of multiple gene variants on a DSD phenotype remains an unsolved conundrum.

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Introduction

Differences of sex development (DSD) comprise a heterogeneous group of congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical [1]. Typical sex development depends on a complex collaboration of numerous genes in a spatial and temporal correct fashion. Variants in involved genes may cause deviations in human sex development and thus lead to clinical manifestations with broad phenotypes summarized under the umbrella term DSD [2, 3]. The mechanisms causing the broad phenotypes in many forms of DSD are still unclear. In clinical practice, genetic diagnosis and clinical management for these patients remains challenging.

Due to the broad spectrum of conditions comprised in the group of DSD, underlying genetic etiologies cover the whole field of genetics and epigenetics. However, with current testing methods, single-nucleotide variants, deletions, and whole chromosome aneuploidies have been mostly described and the underlying genetic cause can be identified in less than half of the tested DSD individuals [4, 5]. DSD patients carrying variants in the same gene or even the same gene variant in a single family (e.g., nuclear receptor subfamily 5 group A member 1 [*NR5A1*]) may manifest with different phenotypes [6, 7]. Conversely, the same DSD phenotype may be produced by variants in >1 gene, for instance, severe undervirilization in patients with 46,XY DSD harboring variants in the androgen receptor (*AR*), the mastermind-like domain-containing protein 1 (*MAMLD1*), the steroidogenic factor 1 (*SF-1*)/*NR5A1*, the 17 β -hydroxysteroid dehydrogenase type 3 (*HSD17B3*), and the Steroid 5 α -Reductase 2 (*SRD5A2*) genes [7–12].

Several studies tried to characterize phenotype-genotype correlations to ameliorate the diagnosis of DSD. But singlecandidate gene testing via Sanger sequencing, the “traditional” approach, is tedious as phenotypes of DSD frequently overlap and several genes needed to be screened one by one [13]. In recent years, Next Generation Sequencing (NGS) became available for routine genetic diagnosis of DSD, first in the form of gene panels and then as whole exome sequencing (WES) [11]. Research platforms even use whole genome sequencing (WGS) nowadays. While the candidate approach informs only about variants of suspected genes, NGS provides a large number of gene variants of unknown biological relevance. Subsequent sophisticated filtering is essential to identify disease-causing gene variants. Recently, NGS has shown that several DSD patients harbored >1 pathogenic or likely

pathogenic (LP) variant in genes associated with sex development. Several variants of unknown significance (VUS) in known DSD genes were also identified in oligogenic combinations [8, 11, 12, 14–19]. Thus in some patients, DSD may be caused by oligogenic variants working in concert as the single variant cannot explain their phenotype.

This review summarizes the oligogenic hits identified so far by recent NGS technology in DSD individuals. We also discuss the challenges to assess the effect of a combination of gene variants involved in the oligogenic inheritance with regard to pathogenicity and phenotypic variability. Overall, we show that the genetics underlying DSD are more complex than we thought.

Methods

We reviewed the literature for evidence regarding oligogenic inheritance and genetic diagnosis of DSD. We searched articles from peer-reviewed medical journals indexed in PubMed using the search terms *DSD* and/or *diagnosis of DSD* combined with *DNA sequencing*, *Next Generation Sequencing* or *high throughput nucleotide sequencing*, *multifactorial inheritance*, *oligogenic inheritance*, *digenic inheritance* or *oligogenic disease*. We also screened reference lists of recent key publications for further articles on oligogenic inheritance using NGS in DSD-related disorders, and added two articles about hypospadias, where oligogenic origin was also suggested by NGS studies.

Oligogenic Hits and *AR* Variants in DSD Patients

Variants in the *AR* gene are the leading cause of 46,XY DSD with a varying degree of androgen insensitivity [20]. The *AR* gene is located on the X chromosome and encodes the AR. It mediates the actions of androgens, which are required for male sexual development and secondary characteristics [21]. Next generation sequencing-targeted panels for DSD identified *AR* variants in 46,XY and 46,XX DSD patients in combination with variants in other genes related to sex development (online suppl. Table 1; see www.karger.com/doi/10.1159/000519691 for all online suppl. material).

Eggers et al. [14] conducted a large international study of 278 patients with 46,XY DSD and 48 with 46,XX DSD to improve clinical genetic diagnosis. They used a targeted NGS gene panel comprising 64 known DSD genes and added 967 candidate genes [14] and identified thirteen 46,XY DSD patients with >1 variant. Five out of 13 classified as 46,XY DSD of unknown origin carried pathogenic/

LP *AR* variants and additional hits in *SRD5A2*, *HSD17B3* and *SOX9*, *ZFPM2*, *NR5A1* and *FGRF2*, or *WDR11* gene [14]. An NGS study from Poland investigated 37 DSD candidate genes and 21 congenital hypogonadotropic hypogonadism (CHH) genes in 35 46,XY DSD patients. Of those, five carried variants in the *AR* gene together with variants in genes involved in sex development (*SOX9*, *CBX2*, and *DMRT1*) or in androgen biosynthesis (*POR* or *DHCR7*) [10]. In NGS studies of 46,XY patients with hypospadias, three patients had pathogenic/LP *AR* variants together with other gene variants. One patient carried additional variants in the *HOXB6* gene and in the *MAMLD1* gene, another a variant in the *PROKR2* gene, and a third one a variant in the *TRIM17* gene [8, 9].

Oligogenic Hits and Mitogen-Activated Protein Kinase Kinase 1 Variants in DSD Patients

Variants in the mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*) have been associated with complete or partial gonadal dysgenesis (CGD or PGD) in 46,XY DSD. The MAPK signaling pathway is important during testes development [22, 23], and variants in *MAP3K1* affect downstream signaling targets such as *SOX9* [24]. The international NGS study of Eggers et al. [14] found eleven 46,XY DSD individuals with LP variants or VUS in the *MAP3K1* gene. Of those, three 46,XY DSD individuals with *MAP3K1* variants also carried variants in the *LHCGR* or *WT1* or *WDR11* genes [14]. The *WDR11* gene is a known regulator of sex development and it has been also associated with hypogonadotropic hypogonadism [25, 26] (online suppl. Table 1).

Oligogenic Hits and HSD3B2 Variants in DSD Patients

Variants in *HSD3B2* (coding for steroid enzyme 3 β -hydroxysteroid dehydrogenase type 2, 3 β HSD2II) cause a rare type of congenital adrenal hyperplasia (CAH). Activity of the enzyme 3 β HSD2II is essential for all steroid hormone biosynthesis in the adrenal cortex and the gonads [27]. In men, gene variants causing severe 3 β HSD2II deficiency lead to adrenal insufficiency and incomplete masculinization, manifesting with severe hypospadias, micropenis, bifid scrotum, and undescended testis [27–29]. In girls, 3 β HSD2II deficiency leads to adrenal insufficiency as well as a broad range of atypical genital phenotypes, including enlarged clitoris, incomplete labial fu-

sion, and genital hyperpigmentation at birth. Later in life, these women may develop signs of androgen excess such as premature pubarche, hirsutism, menstrual disorders, and infertility [30–32]. Distinct phenotypes may be explained by the dosage of enzymatic activity loss caused by biallelic variants in *HSD3B2*, but expression variability in patients with the same variants needs further explanation. Second hits have been recently identified in NGS studies in the *GNRHR* gene, known to cause CHH and in the *SRD5A2* gene necessary for dihydrotestosterone synthesis, in two patients with hypospadias [9, 14] (online suppl. Table 1).

Oligogenic Hits and GATA4 or Zinc Finger Protein, Friend of GATA Family Member 2 Variants in DSD Patients

Variants in genes *GATA4* (GATA Binding Protein 4) and zinc finger protein- friend of GATA family member 2 (*ZFPM2*) are mostly associated with congenital heart diseases [33]. But *GATA4* variants have also been reported in 46,XY DSD [34–36] and *ZFPM2* missense variants in patients with isolated 46,XY PGD, or CGD [37]. The transcription factor encoded by the *GATA4* gene is essential for early testis development. It interacts with several proteins including *ZFPM2*, *SF-1*, and *WT1* to regulate the expression of sex determining genes *SRY*, *SOX9*, and *AMH*, as well as genes involved in sex differentiation including *STAR*, *CYP17A1*, *CYP19A1*, *INHA*, and *HSD3B2* [38–42]. Five patients with hypospadias had LP variants in *ZFPM2* together with variants in *GATA4* or *MAP3K1* [14]. In addition, a variant in *GATA4* coexisted with a pathogenic variant in the *LHCGR* gene in one 46,XY DSD patient and a VUS in *LRP4* in another [18]. The *LRP4* protein is involved in the extracellular communication between epithelial and mesenchymal cells through Wnt and BMP signaling, both involved in sex development [43].

Oligogenic Hits and HSD17B3 Variants in DSD Patients

Variants in the *HSD17B3* gene cause 46,XY DSD due to a defect in androgen biosynthesis manifesting clinically as lack or low testosterone and dihydrotestosterone [44]. 46,XY newborns with autosomal recessive *HSD17B3* deficiency are born with severely undervirilized or ambiguous genitalia [45]. NGS studies of 35 46,XY children

with DSD have identified pathogenic variants in *HSD17B3* in combination with variants in *CYP17A1*, a gene essential for all steroid hormone biosynthesis [10]. The 46,XY DSD patient with *HSD3B2* and *CYP17A1* variants had a disorder of androgen synthesis. In a cohort of 80 46,XY and 46,XX DSD patients tested with a targeted NGS panel for DSD, a variant in *HSD17B3* in coinheritance with a variant in the *CYP11A1* gene was found in a patient manifesting with hypospadias and penoscrotal transposition [11]. In the same cohort of patients, two pathogenic variants in *HSD17B3* and one variant in the *MAMLD1* gene were found in two 46,XY patients with *HSD17B3* deficiency, respectively.

Oligogenic Hits and *MAMLD1* Variants in DSD Patients

Variants in *MAMLD1* have been associated with a broad range of DSD phenotypes including mainly hypospadias, but also micropenis and/or cryptorchidism, 46,XY DSD with female external genitalia, 46,XY CGD, and 46,XX gonadal dysgenesis [14, 46–56]. *MAMLD1* is involved in fetal sex development and in adult reproductive function, but its exact role is unclear [48, 52]. A number of *MAMLD1* variants are present in the normal population, while the same *MAMLD1* variants are present in DSD with various phenotypes, questioning their relevance or at least indicating that these variants may not fully explain a DSD phenotype [48, 49, 52, 57]. An NGS study of 80 DSD patients (both 46,XY and 46,XX) found an LP *MAMLD1* variant together with two variants in the *AMH* gene and one variant in the *CBX2* gene in a patient with hypospadias [11]. We performed WES in eight patients with previously identified *MAMLD1* variants [12]; seven had 46,XY DSD and one 46,XX patient had primary ovarian insufficiency. Through a filtering algorithm including disease-tailored lists of *MAMLD1*-related and DSD-related genes, we found in additional 1–16 genes, 1–16 potentially deleterious variants per patient in a total of 41 genes. Seventeen of the total 55 variants were in genes previously associated with hypospadias (e.g., *ATF3*, *BNC2*, *CYP1A1*, *EMX2*, *EYA1*, *FLNA*, *GLI3*, *GRID1*, *GLI2*, *BNC2*, *FGF10*, *HOXA13*, *HSD3B2*, *IRX5*, *IRX6*, *PPARGC1B*, and *WDR11*), 8/55 with cryptorchidism (*BNC2*, *FLNA*, *RET*, *RECQL4*, *NRP1*, *PTPN11*, *RIPK4*, and *ZBTB16*), 5/55 with micropenis (*ZBTB16*, *BNC2*, *EVC*, *FGF10*, and *RIPK4*), and 13/55 with female sex development [12]. In a similar WES study in ten 46,XY DSD patients with *MAMLD1* variants, 43 potentially deleteri-

ous candidate variants of 18 genes (including *RET*, *CDH23*, *MYO7A*, *NOTCH2*, *MAML1*, *MAML2*, *CYP1A1*, *WNT9B*, *GLI2*, *GLI3*, *MAML3*, *WNT9A*, *FRAS1*, *PIK3R3*, *FREM2*, *PTPN11*, *EVC*, and *FLNA*) were identified [19]. The authors concluded that *MAMLD1*-related DSD may follow a complex oligogenic mode of inheritance.

Oligogenic Hits and *NR5A1* Variants in DSD Patients

Variants in *SF-1/NR5A1* are among the most frequently identified causes for DSD including phenotypes of 46,XY or 46,XX sex with CGD or PGD, ovotesticular DSD, 46,XX testicular DSD, and hypospadias [6]. In addition, *NR5A1* variants have been associated with primary ovarian insufficiency in women [6, 58–61]. The *NR5A1* gene encodes SF-1, which is an essential transcription factor for early sex determination and differentiation, steroidogenesis, and reproductive development [62]. Although the crucial role of SF-1 in sex and adrenal development is well established, the broad clinical manifestation associated with human variants of *NR5A1* remains a conundrum. It has been hypothesized that SF-1 works in concert with other gene partners to guide human sex development.

Data of several recent NGS studies of individuals carrying heterozygous *NR5A1* variants are in line with a possible oligogenic origin of the broad range of phenotypes. One 46,XY DSD patient with hypospadias [61] and another 46,XY DSD patient with gonadal dysgenesis [63] had pathogenic variants in the *NR5A1* gene in combination with a variant in the *ZFPM2* gene. A pathogenic variant *NR5A1* in coinheritance with a pathogenic variant in the *TBX2* (the DNA-binding domain of T-box factor 2) gene was found in another patient with 46,XY CGD [64], while an LP variant in *NR5A1* was found together with another variant in *MAP3K1* in a 46,XY patient with gonadal dysgenesis [15]. Further pathogenic/LP variants in *NR5A1* in combination with additional genetic hits were found in eight out of 70 46,XY DSD patients with phenotypes including moderate to severe undervirilization, micropenis, hypospadias, cryptorchidism, bifid scrotum, gonadal dysplasia, and female typical external genitalia, vulvar dysplasia, and primary amenorrhea. In these DSD patients, the additional gene variants were identified in *BMP2*, *SRY* and *FGF10*, *AR* and *MYH6*, *SOX3*, *WT1* and *HSD17B3*, *CST9* or *CST9*, and *EGF* [17] (online suppl. Table 1). Similar NGS studies revealed a combination of *NR5A1* variants with variants in *ATRX* and *AMH* genes in one 46,XY DSD patient

[11] and the *SRD5A2* gene in another patient with hypospadias [63]. In our WES study of four 46,XY DSD patients with heterozygous (likely) pathogenic *NR5A1* variants manifesting with variable phenotypes, we found 19 additional variants (e.g., in *AKR1C3*, *CACNG4*, *CHD7*, *DENND1A*, *DOCK8*, *FBLN2*, *friend of GATA 2/ZFPM2*, *FSHR*, *GDNF*, *GLI2*, *INHA*, *NAV1*, *NCOR1*, *POR*, *SMAD6*, *SOX30*, *SRA1*, and *ZDHHC11*) [7]. Each patient had variants in 1–7 additional genes, which qualified as VUS or potentially deleterious variants. Eight of these genes were known to cause DSD. *ZFPM2* variants were previously associated with 46,XY/CHH, *DENND1A*, *FSHR*, *GLI2*, *INHA*, *POR* variants with 46,XX DSD/CHH/primary ovarian insufficiency, and *CHD7* and *SRA1* variants with both. Seven observed variants in *AKR1C3*, *DOCK8*, *NCOR1*, *FBLN2*, *NAV1*, *SMAD6*, and *GDNF* were not previously associated with sex development or gonadal function. In another study using a NGS targeted DSD panel for diagnostic workup of seven 46,XY DSD patients, combinatory heterozygous variants in *NR5A1* and *STAR* (VUS), *AMH* (VUS), and *ZFPM2* (LP) were reported [16].

In the literature, NGS workup of DSD suggests additional oligogenic combinations of unique gene variants to explain DSD; these are included in online supplementary Table 1. We foresee that the number of such findings will increase markedly with the recommended implementation of WES in routine genetic diagnostic of DSD and the increased use of methods such as WGS in research settings [65]. The validation and interpretation of these data will remain challenging.

The Challenges of Genetic Testing and Validating Genetic Data in DSD

Genetic testing is recommended as part of the first-line workup for diagnosing DSD [65, 66]. A variety of genetic tests including candidate gene testing, chromosomal microarrays, WES, and WGS are in clinical use. Despite this variety, a molecular diagnosis is not achieved in more than half of the patients [5]. Depending on the clinical manifestation, geneticists will recommend different methods for the initial genetic workup. Isolated DSD might be first tested with WES (or WGS), while in syndromic and complex DSD phenotypes a karyotype, multiplex ligation-dependent probe amplification or an array-comparative genomic hybridization (aCGH) may be applied [65] to detect large deletions and copy number variations. In the DSD-Translational Research Network

(DSD-TRN), 14% of DSD patients were diagnosed using aCGH [66], although balanced rearrangements, inversions, or translocations can be missed by aCGH.

Candidate gene testing remains a useful first approach for cases where the phenotype points clearly to a specific gene or in familial cases with known DSD gene variants (e.g., CAH and *CYP21A2* variants) [67]. But with candidate gene testing, large DNA insertions or deletions might be missed and thus further molecular approaches needed [68].

In patients without a specific clinical phenotype, NGS methods are recommended. Previously, specific gene panels were most common, while today unbiased WES is the method of choice in most routine laboratories. Panels comprise a limited number of specific genes and therefore WES with targeted gene analysis has replaced them in most laboratories. Whole exome sequencing may also reveal novel unsuspected gene variants and allows reanalysis of data [65, 66, 68]. Whole genome sequencing expands the diagnostic possibilities for DSD even further. Whole genome sequencing detected about 3% of coding variants that were missed and revealed less false-positive single-nucleotide variants compared to WES (17% vs. 78%). Additionally, WGS detects non-coding variants [69]. As the costs for WGS are still higher than WES and its interpretation is more demanding, it remains limited in clinical use despite the higher diagnostic validity.

The use of NGS technology in the genetic workup of DSD patients leads to identification of numerous VUS requiring sophisticated filtering and validation for disease-causing impact. Although initial analysis of such data has been standardized over the past 20 years by the genetic societies [70], analysis of data resulting from rare disorders like DSD or from disorders with possible oligogenic origin remains challenging. For initial variant calling and pathogenicity evaluation, several bioinformatics software have been developed to predict the possible biological impact (e.g., reviewed in [71]). However, for rare diseases, where big datasets for comparison are lacking or where polygenic inheritance might exist, additional disease-tailored data filtering may be necessary [3, 65, 72]. Therefore, we and others have recently designed DSD-tailored algorithms to analyze and interpret NGS data [7, 12, 19]. In these algorithms, candidate variants resulting from standardized population-based analysis are in addition interrogated for specific involvement in normal developmental biology, DSD, and cross-checked with clinical information (phenotype). With the perspective of possible oligogenic origin of some DSDs, combinatory effects of variants in >1 gene may be considered for fur-

Fig. 1. Interaction network of oligogenic variants found in combination with genetic variants in *NR5A1* in DSD individuals. The network was constructed using the MCODE & GeneMANIA software. Identified genes *MYH6*, *ZDHHC11*, and *SRA1* are not depicted as no direct link in the network was found with GeneMANIA. Predicted interaction comprises coexpression (violet lines), common pathways (light blue lines), shared protein domains (light brown lines), co-localization (blue lines), and genetic interactions (green lines). *NR5A1*, nuclear receptor subfamily 5 group A member 1; DSD, differences of sex development; MCODE, Cytoscape Molecular COMplex Detection; AR, androgen receptor; *SRD5A2*, steroid 5 alpha-reductase 2; *ZFP2*, zinc finger protein, friend of GATA family member 2; *HSD17B3*, 17b-hydroxysteroid dehydrogenase type 3.

ther analyses; this includes bioinformatics pathway analysis of candidate genes as well as inheritance testing, for example, by trio analysis [3, 14, 17, 18, 64, 72, 73].

Especially in field of rare disease with complex and variable phenotypes, an aggregation of rare variants in associated genes may explain the broad clinical spectrum [74]. We have applied pathway analysis in selected 46,XY DSD patients harboring heterozygous *NR5A1* variants in combination with variants in other DSD-related genes [7]. For this review, we performed similar analyses for all gene variants so far reported in combination with *NR5A1* variants (online suppl. Table 1) to illustrate the possible network of genes modifying a DSD phenotype through genetic variations. Gene clusters associated with *NR5A1* are shown in Figure 1 and protein clusters shown in Figure 2. The genetic network shown in Figure 1 demon-

strates relationships between genes with respect to (a) shared common pathways, (b) coexpression or colocalization, (c) genetic interactions, as well as (d) functional protein interactions encoded by the genes (e.g., regulatory transactivation of different genes by the transcription factor SF-1). The protein network shown in Figure 2 displays physical interactions between proteins (e.g., interactions of the SF-1 transcription factor with other transcription factors that together may regulate the expression of targeted genes). Only proteins that are known or recognized candidates for sex determination and/or sex differentiation (male or female pathway) identified in individuals harboring *NR5A1* variants are shown in Figure 3. These pathway analyses (shown in Fig. 1–3) illustrate how sophisticated bioinformatics data analysis may clarify the interrelationship between candidate genes and provides hypotheses how combined gene variants may lead to a specific phenotype.

All the oligogenic findings associated with DSD comprised in this review are displayed in online supplementary Table 1, including the phenotype of each patient as well as zygosity, pathogenicity, and function of each variant identified. In total, 71 patients with possible oligogenic DSD were found. They had two or more variants in different genes associated or not with DSD conditions, sex development, or gonadal function. Reporting investigators suggested that their broad range of phenotypes may be due to oligogenic origin. Seventeen percent of identified variants (43/257) were classified as pathogenic according to American College of Medical Genetics standards, 14% (36/257) as LP, 10% as likely benign (26/257), only 2% (6/257) as benign variants, and more than half of the variants (57%, 146/257) as VUS. Although the pathogenicity of VUS remains uncertain, we cannot exclude them as additional oligogenic hits, especially in DSD patients whose phenotype-genotype correlation is still unclear. Investigations and functional validation together with segregation analysis are needed to determine the possible pathogenicity of these variants.

Functional studies for evidence of pathogenicity for each specific variant have been performed only in 11% (28/257) of total reported variants included in this review. It is a big challenge to investigate and proof oligogenic pathogenicity of two or more variants identified in (DSD) patients. In silico prediction, tools that explore disease-causing oligogenic variant combinations may help in this matter. For instance, programs ORVAL (Oligogenic Resource for Variant AnaLysis) and VarCoPP (Variant Combination Pathogenicity Predictor) have been recently used for the analysis of genetic

Fig. 2. Interaction network for proteins encoded by additional gene variants identified in DSD individuals harboring genetic variants in *NR5A1*. The network was constructed using the MCODE & STRING software. Proteins ATRX, NCOR1, DENND1A, POR, NAV1, CACNG4, MYH6, CST9, SRA1, ZDHHC11, CHD7, DOCK8, FBLN2, SOX30, and SRY are not depicted as there are no predicted clusters to include them in the protein network based on STRING and MCODE software. Predicted interactions correspond to database evidence (light blue lines), experimental evidence (purple lines), and coexpression evidence (black lines). DSD, differences of sex development; MCODE, Cytoscape Molecular Complex Detection; ZFP2, zinc finger protein, friend of GATA family member 2; NR5A1, nuclear receptor sub-family 5 group A member 1; AR, androgen receptor; HSD17B3, 17 β -hydroxysteroid dehydrogenase type 3; MAP3K1, mitogen-activated protein kinase kinase 1.

findings in patients with possible oligogenic DSD to investigate the probability of a combined pathogenic effect [75].

Final proof of oligogenic causation of DSD remains largely unsolved in most cases. Familial cases and mode of inheritance may provide convincing evidence in some cases. Experimental models used for monogenic DSD are of limited use. While single gene knockout mice models have helped in elucidating important mechanisms of sex development, testing of >2 gene variants simultaneously is difficult, if not impossible. Common cell-based models for functional studies suffer from the same limitations. Whether patient-derived pluripotent stem cell and reprogrammed cell models may offer a novel opportunity to study complex oligogenic constellations remains to be verified. They will comprise the unique genetic background of the patient, and first studies for investigating genetic variants causing CAH were successful and promising [76].

Conclusion and Perspective

For people with a DSD, a clear diagnosis is important. It translates to improved disease understanding, more accurate prediction of prognosis, better therapeutic and preventive care, and more specific genetic counseling [77]. It also stimulates research for new therapeutic solu-

tions. To this day, about every second DSD individual who attempted to receive a specific diagnosis at the genetic level remains undiagnosed [65]. In most forms of DSD, phenotype-genotype association seems poor. Several reasons may explain these facts: first, DSD are a large group of different disease entities with rare disorders. Thus, nearly all studies suffer from small sample size. Second, most case reports lack standardized and comprehensive (longitudinal) description of the broad phenotype. Third, genetic workup and analysis use very heterogeneous methods and may not consider oligogenic inheritance. It is therefore difficult to compare data between study cohorts. Fourth, experimental functional tests often do not reflect the heterozygote or possibly oligogenic mode of inheritance found in humans. Thus, validation of genetic findings is challenging.

While some of these obstacles are difficult to overcome, others can be addressed by joining forces of DSD individuals, care givers, and researchers internationally. The scientific community of the recent COST action DSDnet has identified the need for a comprehensive, ideally worldwide DSD registry, which collects all rare DSD cases with longitudinal clinical data in a standardized fashion [65, 78, 79]. This registry exists as I-DSD since 2005 and has recently been updated to comprise longitudinal data. Similarly, genetic data should be collected in a standardized fashion in a DSD-specific database related to the I-DSD registry; but this task is still pending. The rapid evolution of NGS should

Fig. 3. Interaction network of proteins encoded by additional gene variants identified in DSD individuals harboring genetic variants in NR5A1. The network was constructed using the Cytoscape – STRING software. Network partners were grouped based on their contribution to sex determination and biology of the biopotential gonad (**a**) and sex differentiation to the female and male pathways (**b**). Predicted interactions correspond to database evidence (light

blue lines), experimental evidence (purple lines), and coexpression evidence (black lines). DSD, differences of sex development; NR5A1, nuclear receptor subfamily 5 group A member 1; ZFPM2, zinc finger protein- friend of GATA family member 2; AR, androgen receptor; SRD5A2, steroid 5 alpha-reductase 2; HSD17B3, 17b-hydroxysteroid dehydrogenase type 3; MAP3K1, mitogen-activated protein kinase kinase kinase 1.

result in validated routine genetic protocols that are at the front line of an integrated approach for a genetic diagnosis of DSD. It is our hope that through such common efforts, the open questions regarding oligogenic DSD and broad phenotypes observed in many forms of DSD may be solved. The complex cascade of sexual development can produce

a wide spectrum of phenotypes, when several genetic variations with variable severity occur within this cascade. However, to proof the impact of multiple single-gene variants occurring in this cascade is an obstacle that we have not yet overcome. Once we can proof and understand the

genetic key players of this network, we can use this knowledge for improving patient care.

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Conflict of Interest Statement

Christa E. Flück is Associate Editor of the journal. The other authors have no conflicts of interest to declare.

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