Broad Phenotypes of Disorders/Differences of Sex Development in \textit{MAMLD1} Patients Through Oligogenic Disease

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Disorders/differences of sex development (DSD) are the result of a discordance between chromosomal, gonadal, and genital sex. DSD may be due to mutations in any of the genes involved in sex determination and development in general, as well as gonadal and/or genital development specifically. \textit{MAMLD1} is one of the recognized DSD genes. However, its role is controversial as some \textit{MAMLD1} variants are present in normal individuals, several \textit{MAMLD1} mutations have wild-type activity in functional studies, and the \textit{Mamld1}\textsuperscript{-knockout} male mouse presents with normal genitalia and reproduction. We previously tested nine \textit{MAMLD1} variants detected in nine 46,XY DSD patients with broad phenotypes for their functional activity, but none of the mutants, except truncated L210X, had diminished transcriptional activity on known target promoters \textit{CYP17A1} and \textit{HES3}. In addition, protein expression of \textit{MAMLD1} variants was similar to wild-type, except for the truncated L210X. We hypothesized that \textit{MAMLD1} variants may not be sufficient to explain the phenotype in 46,XY DSD individuals, and that further genetic studies should be performed to search for additional hits explaining the broad phenotypes. We therefore performed whole exome sequencing (WES) in seven of these 46,XY patients with DSD and in one 46,XX patient with ovarian insufficiency, who all carried \textit{MAMLD1} variants. WES data were filtered by an algorithm including disease-tailored lists of \textit{MAMLD1}-related and DSD-related genes. Fifty-five potentially deleterious variants in 41 genes were identified; 16/55 variants were reported in genes in association with hypospadias, 8/55 with cryptorchidism, 5/55 with micropenis, and 13/55 were described in relation with female sex development. Patients carried 1-16 variants in 1-16 genes together with their \textit{MAMLD1} variation. Network analysis of the identified genes revealed that 23 genes presented gene/protein interactions with \textit{MAMLD1}. Thus, our study shows that the broad phenotypes of individual DSD might involve multiple genetic variations contributing towards the complex network of sexual development.

Keywords: whole exome sequencing, \textit{MAMLD1}, disorders/differences of sex development, hypospadias, phenotype variability, oligogenic disorder
INTRODUCTION

Disorders/differences of sex development (DSD) occur when there is a discordance between chromosomal, gonadal, and genital sex (Ostrer, 2014). DSD may be due to mutations in any of the genes involved in sex determination and development in general, as well as gonadal and/or genital development specifically (Ostrer, 2014).

MAML1 (Xq28, OMIM 300120) is one of the recognized DSD-related genes (Fukami et al., 2006; Baxter et al., 2015). Variations in MAML1 sequence have been described mainly in 46,XY DSD individuals, mostly associated with hypospadias (Fukami et al., 2006; Kalfa et al., 2008; Chen et al., 2010; Kalfa et al., 2012; Metwalley and Farghaly, 2012; Camats et al., 2015; Igarashi et al., 2015; Eggers et al., 2016), but also with other DSD phenotypes, including micropenis (Chen et al., 2010; Kalfa et al., 2012; Camats et al., 2015; Liu et al., 2017), and/or cryptorchidism (Fukami et al., 2006; Kalfa et al., 2008; Kalfa et al., 2012; Camats et al., 2015), 46,XY with female external genitalia (Fukami et al., 2006; Camats et al., 2015) and 46,XY with complete gonadal dysgenesis (Ruiz-Arana et al., 2015). Furthermore, one homozygous MAML1 variant was also reported in a 46,XX patient with gonadal dysgenesis, primary amenorrhea, bilateral streak gonads and clitoromegaly (Brandao et al., 2011).

However, the role of MAML1 in sex development is controversial for several reasons: a) some MAML1 variants are present in the normal population (Fukami et al., 2006; Chen et al., 2010; Gaspari et al., 2011; Kalfa et al., 2011); b) the same MAML1 variant may be present in patients with different phenotypes (Camats et al., 2015); c) MAML1 variants are not present in all DSD individuals of the same family (Fukami et al., 2006); d) several MAML1 mutations present wild-type activity in functional studies (Camats et al., 2015); and e) the Mamld1-knockout male mouse presents with normal genitalia and reproduction (Miyado et al., 2012; Miyado et al., 2017).

MAML1 is expressed in human fetal and adult testis and in human ovaries (Fukami et al., 2006; O’Shaughnessy et al., 2007; Camats et al., 2015), and seems to be involved in sex development in fetal life and in adult reproductive function. Yet its exact role is not clear. It is expressed in mice gonadal cells during start of androgen biosynthesis up to male external genitalia formation and is therefore thought to be involved in the expression of Leydig-cell genes (Miyado et al., 2012), as well as supporting testosterone production in critical periods of male development (Fukami et al., 2008; Nakamura et al., 2011). In contrast, Mamld1-KO mice present normal external genitalia (but small testes and reduced seminiferous tubule size and proliferating germ cells) and reproduce similarly to wild-type mice (Miyado et al., 2012; Miyado et al., 2017). These findings challenge the role of MAML1 in sex development.

In a previous study, we tested functional activity of nine MAML1 variants detected in nine 46,XY DSD patients with broad phenotypes (Camats et al., 2015). None of the MAML1 mutants, except truncated L210X, had diminished transcriptional activity on known target promoters CYP17A1 and HE3. In addition, protein expression of MAML1 variants was similar to wild-type, except for the truncated L210X. We therefore hypothesized that MAML1 variants may not be sufficient to explain the phenotype in 46,XY DSD carriers, and that further genetic studies should be performed to search for additional hits explaining the broad variability.

In the past decade, High throughput sequencing (HTS) has changed the genetic approach in research and diagnostics. Whole-exome sequencing (WES) has led to the discovery of many new genes and has given insight into complex traits. Oligogenic inheritance is currently discovered for several disorders by HTS. In the field of sex development, digenic inheritance has recently been suggested in a 46,XY DSD patient with gonadal dysgenesis (NR5A1 and MAP3K1 variants) (Mazen et al., 2016); in a family with 46,XY DSD males (NR5A1 variants) and 46,XX POF females (NR5A1 and TBX2) (Werner et al., 2017); as well as in a DSD patient with ambiguous genitalia, micropenis, and inguinal testes (SEMA3A and AKR1C4) (Fan et al., 2017). Similarly, we found oligogenic origin of disease in heterozygous NR5A1 46,XY DSD patients by performing WES (Camats et al., 2018). In addition, in patients with hypospadias, an oligogenic origin was suggested by two other NGS studies (Kon et al., 2015; Eggers et al., 2016).

Therefore, in this study, we performed WES in seven 46,XY patients with DSD (Camats et al., 2015) and one 46,XX patient with ovarian insufficiency, who all carried MAML1 variants. WES data were filtered by common tools and a disease-tailored algorithm including MAML1-related and DSD-related known and candidate genes. Additional hits in likely disease-causing genes were detected in all eight MAML1 carriers. Our results suggest that oligogenic origin of disease may contribute towards the broad phenotypes of human MAML1.

PATIENTS AND METHODS

Patients

The study was approved by the Ethics Committee of Hospital Universitari Vall d’Hebron (Barcelona, Spain) (CEIC: PR(IR)23/2016). Written informed consent was obtained from the patients for the publication of their cases. Eight DSD patients (seven 46,XY and one 46,XX) each carrying one MAML1 variant were analyzed using WES. Clinical and genetic characteristics of 46,XY patients were previously reported in detail in Camats et al., (2015) and are summarized in Table 1 together with the 46,XX patient.

DNA Extraction, WES and Bioinformatic Analysis

DNA was extracted from blood leukocytes using QiaCube (Qiagen, Hilden, Germany) or manually using a DNA isolation kit (Qiagen). WES was performed by CNAG (Centre Nacional d’Analisi Genòmica, Barcelona, Spain). Libraries were prepared with a SureSelect Human All Exon V5 capture kit (Agilent, Santa Clara, CA, USA) and sequenced with a HiSeq™ 2000 sequencing system (v3, 2x100, Illumina, San Diego, CA, USA). Putative candidate variants were confirmed by Sanger sequencing.
| Patient | Karyotype assigned sex | Phenotype and origin | Gonadal function (age) | Adrenal function (age) | MAMLD1 variant | Variants after filtering by gene list (A) | Candidate variants (B) | Candidate genes (C) |
|---------|-----------------------|----------------------|------------------------|------------------------|----------------|---------------------------------------------|------------------------|---------------------|
| 1       | 46,XY Female          | Penoscrotal hypospadias. Small penis. | Normal hCG test. | Normal Synacthen test. | p.V505A NM_005491:c.1514T>C | rs17405666 | 547 | 9 | 7 |
| 2       | 46,XY Male            | Penoscrotal hypospadias. Small penis. | Normal baseline T (3m). | Normal hCG test (3m). | p.A503E NM_005491:c.1508C>A | 492 | 1 | 1 |
| 3       | 46,XY Female          | Penoscrotal hypospadias. Small penis. | Normal baseline (12m). | NA | p.S730S NM_005491:c.2190G>A | 570 | 2 | 2 |
| 4       | 46,XY Female          | Penoscrotal hypospadias. Testes 2 ml. | Normal hCG test (2y). | Normal baseline (2y). | p.H347Q NM_005491:c.1041C>A | rs62641609 | 633 | 4 | 4 |
| 5       | 46,XY Male            | Penoscrotal hypospadias. | Normal hCG test. | Normal baseline (2.5y). | p.H347Q NM_005491:c.1041C>A | rs62641609 | 929 | 6 | 6 |
| 6       | 46,XY Male            | Penoscrotal hypospadias. | Normal prepubertal baseline T (15 m). | Normal AMH. | p.L724V NM_005491:c.2170C>G | 710 | 16 | 16 |
| 7       | 46,XY Male            | Hypospadias. | Normal baseline (70y). | Normal baseline | p.Q501Q502 NM_005491:c.1503_1504dupCAGCAG | 429 | 5 | 5 |
| 8       | 46,XX Female          | Female external genitalia. Small ovaries and uterus, with fallopian tubes. | High gonadotropins and low/normal estradiol (27y). | Normal (27y). | NM_005491:c.*126C>MIT | 574 | 14 | 13 |

All patients presented one hemizygous/heterozygous variant in MAMLD1. In parentheses: patients in Camats et al. (2015); NA, not analyzed; d, day(s); m, month(s); y, year(s). (A) Filtered by DSD-related and MAMLD1-related gene list. (B) Number of candidate variants per patient: related to sex development, DSD phenotypes, and/or in MAMLD1-related genes, and with MAF ≤ 0.01. (C) Number of candidate genes per patient: genes containing at least one candidate variant per patient.
The genomic datasets were annotated (alignment with human genome hg19/GRCh37) and filtered with the functional annotation of genetic variants from HTS data (ANNOVAR; http://annovar.openbioinformatics.org/) (Wang et al., 2010), visualized and explored in Integrative Genomics Viewer (IGV, Broad Institute, Cambridge, MA, USA; https://www.broadinstitute.org/igv/ (Robinson et al., 2011). Frequencies of variants of relevant candidate genes were obtained from the Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org/about/) (Lek et al., 2016) and the Collaborative Spanish Variant Server (CSVS; CIBERER BIER, Valencia, Spain; http://csvs babelomics.org/; August 2018) (Dopazo et al., 2016). gnomAD includes gene variants from exome and genome sequencing data: 123,136 exomes and 15,496 genomes from unrelated individuals (from population and disease-specific studies). CSVS database includes (among others) exomes from a population of 267 healthy unrelated subjects.

WES data were filtered by a disease-tailored list of MAML1-related and DSD-related known and candidate genes (n = 606) similar to the algorithm previously set up for Camats et al., (2018). We generated a project-specific filter for DSD-related and MAML1-related genes by searching in published literature and databases. DSD-related genes are part of our DSD-gene database and tools (Camats et al., 2018), which have been currently updated. The DSD-related gene list included genes with reported (potentially) deleterious variants in patients with 46,XY and 46,XX DSD, genes with reported (potentially) disease-causing variants in syndromic patients with involvement of sex development, those “related” to DSD conditions in KO/mutant animal models (mice and rats), and also overexpressed, upregulated or downregulated genes in rodent embryonic gonadal cells (Camats et al., 2018). For the search for functional human partners of MAML1 and for possible interactions within interesting genes, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://string-db.org/) (Jensen et al., 2009) and the Biological General Repository for Interaction Datasets (BioGRID, thebiogrid.org) (Stark, 2006) were used.

We used 30 pathogenic predictors to predict possible impact of amino acid substitutions on the structure, function and evolutionary conservation of corresponding human proteins and to predict impact on splicing. These in-silico predictors were accessed through ANNOVAR (Wang et al., 2010) annotation and run through Alamut Visual 2.11 (https://www.interactive-biosoftware.com/es/alamut-visual/). Functional exonic predictors were CADD (Combined Annotation Dependent Depletion of single-nucleotide and insertion/deletion variants, http://cadd.gs.washington.edu/) (Kircher et al., 2014), SIFT (Scale-invariant feature transform; http://sift.jcvi.org/), PolyPhen-2 (Polymorphism Phenotyping v2: HumDiv, HumVar; http://genetics.bwh.harvard.edu/pph2/index.shtml), Provean (http://provean.jcvi.org), MutationAssessor (http://mutationassessor.org/r3/), Mutation Taster (http://www.mutationtaster.org/), LRT, FATHEMM, Fathom-MKL, PROVEAN, VEST3 (Variant Effect Scoring Tool), MetaSVM, MetaLR, MCAP, DANN and fitCons. Exonic predictors on evolutionary conservation were: GERP++, phyloP (vertebrate and mammalian), phastCons (vertebrate and mammalian) and SiPhy. Splicing predictors were: splicing predictors from dbscSNV ADA and RF and SPIDEX splicing predictor (DPSI), and those splicing predictors from Alamut Visual software were: SSF, MaxEnt, NNSPLICE, GeneSplicer and Ex-Skip.

The following bioinformatics software tools were used for the interpretation and classification of variants: InterVar (http://wintervar.wglab.org/), clinical interpretation of genetic variants by the ACMG/AMP 2015 guideline, VarSome (The Human Genomics Search Engine, https://varsome.com/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and Alamut Visual 2.11 (https://www.interactive-biosoftware.com/es/alamut-visual/). We searched for reported (potentially) disease-causing variants with the Human Gene Mutation Database (HGMD® Professional 2018.2, http://www.biobase-international.com/product/hgmd; Biobase) and dbSNP (http://www.ncbi.nlm.nih.gov/snp/). We used STRING for the search for interactions within genes carriers of interesting variants (DSD-related and/or MAML1-related). Data from STRING are extracted from known interactions (curated databases, experimentally determined interactions), predicted interactions (gene neighborhood, gene fusions, gene co-occurrence) and other inferred evidences such as text mining, co-expression and protein homology. We used Pubmed (https://www.ncbi.nlm.nih.gov/pubmed/) and OMIM (https://www.omim.org) to build our DSD gene list and for further data analysis. The datasets generated for this study are publicly available in dbSNP (Sherry, 2001): https://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewBatch.cgi?sbid=1063030.

**Variant Analysis Per Patient**

After annotation, variant analysis was performed by the following steps. A) Each patient’s WES data were first filtered by our MAML1- and DSD-related known and candidate genes. B) We kept variants with MAF (minor allele frequency) ≤0.015 or not detected in gnomAD, and variants with the following predicted type, consequences and locations: splicing (intronic or exonic), exonic, intergenic, regulatory. C) We confirmed the correct annotation and location of variants by checking their alignment data in IGV (alignment with human genome hg19/GRCh37) (data not shown). D) We excluded variants that were considered non-relevant for our study: E.g. 1) variants found in more than two patients, 2) variants in repeat regions, 3) variants in genes or gene regions with high variability, 4) variants with low coverage and/or low quality, 5) variants with non-similar allelic depths. E) We revised variants with the annotated pathogenic predictors: functional exonic, evolutionary-conservation and splicing predictors (ANNOVAR and Alamut Visual software), as previously described. F) We run InterVar and VarSome to classify the variants, searched for reported (potentially) human disease-causing variants with the HGMD, and revised evidences of relationship with DSD, sex development and clinical phenotype of each patient with literature and database search. G) We used STRING to find out interactions among genes carriers of interesting variants (DSD-related and/or MAML1-related) (Figures 1 and 2). H) We checked MAF in a healthy cohort of Spanish population (CSVS: 267 unrelated healthy controls). I) We rejected variants with MAF ≥ 0.01 (gnomAD, CSVS,
August 2018), thus less plausible to be a DSD-causing variant. Importantly, synonymous variants were not rejected because it has been shown that they may affect splicing.

RESULTS

WES performed in eight unrelated subjects (seven 46,XY and one 46,XX) harboring hemizygous/heterozygous MAMLD1 variants revealed several candidate gene variants that potentially contribute to each patient’s phenotype. A detailed summary of patients’ characteristics and number of variants and genes is shown in Table 1. A list of identified candidate variants and corresponding information from literature is given in Table 2.

We identified a total of 55 potentially deleterious/candidate heterozygous/hemizygous variants in 41 genes in the eight hemizygous/heterozygous MAMLD1 patients (Tables 1 and 2). In the seven 46,XY patients 1–16 variants were found in a total of 1–16 genes, while the 46,XX MAMLD1 patient revealed 14 additional variants in 13 genes. (Tables 1 and 2).

Patient 1 harbored nine variants in seven genes: CYP1A1, EVC, GRID1, NOTCH1, RET, RIPK4 and ZBTB16, all of them associated to gonadal/genital anomalies (Table 2). Patient 2 carried one variant in RECQL4, associated with syndromic hypospadias (Table 2). Patient 3 presented two variants in two genes: GLI2 (associated to gonadal/genital anomalies) and RECQL4 (associated to syndromic hypospadias) (Table 2). Patient 4 had four variants in four genes: CDH23, COL9A3, MAML1 and NOTCH1; all, except MAML1, have been proposed to be associated with gonadal development (Table 2). In patient 5, six variants in six genes were found: BNC2, FGF10, HSD3B2, IRX5, MAML2 and NOTCH2; all, except MAML2, have also been associated with hypospadias or gonadal development (Table 2). Patient 6 carried 16 variants in 16 genes: ATF3, BNC2, CYP1A1, EYA1, FLNA, FRAS1, GLI3, HOXA13, IRX5, IRX6, MAML1, NRP1, MAML3, PROP1, PTPN11 and WDR11 (Table 2). Thirteen of these genes are associated with risk of hypospadias and/or syndromes that include abnormal gonadal/genital development, whereas MAML1 is unrelated, MAML3 has been proposed to be associated with female gonadal development and PROP1 has only been associated with anterior pituitary insufficiency/hypogonadotropic hypogonadism. In addition, six of these genes have previously been described in patients with aortic diseases and cardiopathies (Table 2). Patient 7 presented five variants in five genes: EVC, MAML3, NOTCH2, PPARGC1B and WDR11; four of them associated with hypospadias or male gonadal development and one, MAML3, with female gonadal development (Table 2). Finally, patient 8 harbored 14 variants in 13 genes: CUL4B, DAPK1, EMX2, FREM2, IGFBP2, MAML2, MAML3, MYO7A, NOTCH1, PIK3R3, TGFBI, WNT9A and WNT9B.
FIGURE 2 | Interaction networks of DSD- and MAMLD1-related genes identified per single MAMLD1 individual. (A) to (F) correspond to the interaction networks per patient. For the search for functional human partner, the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, http://string-db.org/) was used. Nodes represent proteins. Filled nodes show proteins with known or predicted 3D structure. Empty nodes depict proteins with unknown 3D structure. Candidate genes are underlined. Known interactions correspond to curated databases (turquoise lines) and experimentally determined interactions (pink lines). Predicted interactions correspond to gene neighborhood (green lines), gene fusions (red lines) and gene co-occurrence (blue lines). Other interactions correspond to text mining (yellow lines), co-expression (black lines) and protein homology (violet lines). Genes with no interactions are on the right side of each network.
| Patient | Gene | Chromosome: Coordinates | Type/ consequence | HGVSc,HGVSsp | dbSNP ID | gnomAD: MAF | CSVS: MAF | Predictors | InterVar | ClinVar | VarSome | ACMG | HGMD: variant (7) | Gene characteristics: evidences for genotype-phenotype correlation (8) |
|---------|------|--------------------------|-------------------|--------------|-----------|-----------|-----------|-------------|----------|---------|--------|------|----------------|--------------------------------------------------|
| 1       | CYP1A1 | 15:75013644 snv/misssense | NM_000499.5:c.1162C>G:p.(His388Asp) | – | ND | ND | 24.9 | 13 | 6 | 0 | 5 | VUS | ND | VUS: PM2, PP3 | No Association to hypospadias (van der Zanden et al., 2012) |
| 1       | EVC   | 4:5798754 snv/misssense | NM_153717.2:c.1892C>T:p.(Thr631Met) | rs139481521 | 0.0003 | ND | 23.5 | 5 | 3 | 0 | 3 | VUS | VUS | VUS: BP4 | No Syndromic (Ellis-van Creveld syndrome) micropenis (D’Asdia et al., 2013; Ibarra-Ramirez et al., 2017), gonadal development? (Beverdam and Koopman, 2006; Jameson et al., 2012) |
| 1       | EVC   | 4:5785368 snv/synonymous | NM_153717.2:c.1653G>A:p.(Pro551=) | rs151293705 | 0.0003 | ND | 2.382 | NA | NA | 1 | 3 | Likely benign | other | VUS | VUS: BP7 | No Syndromic (Ellis-van Creveld syndrome) micropenis (D’Asdia et al., 2013; Ibarra-Ramirez et al., 2017), gonadal development? (Beverdam and Koopman, 2006; Jameson et al., 2012) |
| 1       | GRID1 | 10:87966142 snv/missense | NM_017551.2:c.499A>G:p.(Met167Val) | rs956188880 | 0.00004 | ND | 10.04 | 1 | 5 | 0 | NA | VUS | ND | VUS | VUS: BP7 | No Candidate to hypospadias (van der Zanden et al., 2012) |
| 1       | NOTCH1 | 9:139399213 snv/synonymous | NM_017617.5:c.4930C>T:p.(Leu1644=) | rs568700183 | 0.0003 | ND | 0.018 | NA | NA | 0 | 4 | Likely benign | Likely benign | VUS | S6, B7 | No Related to SHH and FGF10 (Chrinaps and Rey, 2014) |
| 1       | RET   | 10:43609955 snv/missense | NM_020975.5:c.1907C>T:p.(Thr636Met) | rs1035958105 | 0.00001 | ND | 23.0 | 6 | 6 | 0 | NA | VUS | VUS | VUS | Likely pathogenic: PM1, PM2, PP2, PP3 | DM; thyroid carcinoma Syndromic (CAKUT syndrome) cryptorchidism (Chatterjee et al., 2012), gonadal development? (Jameson et al., 2012; Li et al., 2014) |
| 1       | RIPK4  | 21:43161468 snv/misssense | NM_020639.2:c.1885G>A:p.(Asp629Asn) | rs190663994 | 0.00004 | ND | 7.899 | 4 | 4 | 4 | NA | VUS | ND | VUS: PM2 BP1 | No Syndrome (Popliteal pterygium syndrome) genital hypoplasia (Mitchel et al., 2012), micropenis, hypoplastic scrotum, inguinal hernia (Kaly et al., 2012), gonadal development? (Beverdam and Koopman, 2006; Jameson et al., 2012) |
| 1       | RIPK4  | 21:43176830 snv/misssense | NM_020639.2:c.3290G>T:p.(Ser110Leu) | rs200823657 | 0.00005 | ND | 22.7 | 3 | 5 | 0 | NA | VUS | ND | VUS: PM2 BP1 | No Syndrome (Popliteal pterygium syndrome) genital hypoplasia (Mitchel et al., 2012), micropenis, hypoplastic scrotum, inguinal hernia (Kaly et al., 2012), gonadal development? (Beverdam and Koopman, 2006; Jameson et al., 2012) |
| 1       | ZBTB16 | 11:114027160 snv/intronic | NM_008006.5:c.1366+4G>C | – | ND | ND | 13.81 | NA | NA | 1 | 4 | ND | ND | ND | VUS: PM2 BP1 | No Syndrome (skeletal 11q23) cryptorchidism and micropenis (Fischer et al., 2008) |

(Continued)
| Patient | Gene | Chromosome: Coordinates | Type/ consequence | HGVS_c/HGVS_p | dbsNP ID | gnomAD MAF | CSVS/MAF | Predictors | Interpretation/classification (6) | Evidence |
|---------|------|--------------------------|------------------|--------------|-----------|-------------|-----------|------------|----------------------------------|----------|
| 2 | REQL4 | 8:145738828 snv/misssense | NM_004260.3:c.2237C>T;p.(Ala746Val) | rs201883228 | 0.0002 | ND | 26.8 | 6 | 5 | NA | NA | VUS | VUS | VUS: BP1 | No | Syndromic (Rothmund-Thomson syndrome) hypospadias, bilateral inguinal hernia (Holmayer et al., 2009) |
| 3 | OGL2 | 2:121747688 snv/misssense | NM_005370.4:c.4198G>T;p.(Gly1400Cys) | rs143914758 | 0.0001 | ND | 23.4 | 7 | 2 | NA | NA | Likely benign | ND | VUS | VUS: - | No | Increased risk of hypospadias (Carmichael et al., 2013), male gonadal development (Jameson et al., 2012), masculinization of male external genitalia (Ray et al., 2000) |
| 4 | DCDH3 | 10:73539034 snv/homosense | NM_002124.5:c.7210C>T;p.(Thr2407*) | rs77363178 | ND | 0.002 | 35 | 5 | 6 | 1 | 3 | Likely pathogenic | ND | – | Pathogenic: PVS1, PM2, PP3 | No | Gonadal development? (Jameson et al., 2012; Li et al., 2015) |
| 5 | COLIA3 | 20:61448856 snv/misssense | NM_001853.4:c.116C>G;p.(Pro39Arg) | rs1029862816 | 0.00001 | ND | 22.7 | 12 | 5 | 0 | NA | VUS | ND | – | VUS: PP3 | No | Male gonadal development (Nat et al., 2005; Boerdam and Koopman, 2006; Jameson et al., 2012) |
| 6 | MAN1L1 | 5:17919386 snv/synonymous | NM_014757.4:c.1374C>T;p.(Arg458=) | rs81748799 | 0.003 | 0.004 | 0.089 | NA | NA | 0 | 1 | Likely benign | ND | – | VUS: BP7 | No | No (MAN1L1-related) |
| 7 | NOTCH1 | 9:13401832 p.| NM_017617.5:c.3597C>T;p.(Asp458=) | rs150863507 | 0.00009 | ND | 11.7 | 0 | NA | 0 | 3 | Likely pathogenic | Likely benign | Likely benign | Likely benign | Likely benign | BP6, BP7 | No | Related to SHH and FGF10 (Gorlin Syndrome) (Baxter, 2014) |
| 8 | BNC2 | 9:16436324 snv/misssense | NM_017637.5:c.1869C>T;p.(Arg623His) | rs114506656 | 0.022 | 0.002 | 5.290 | 8 | 6 | NA | NA | VUS | VUS | VUS: BP3 | No | Hypospadias (Bhoj et al., 2011; van der Zanden et al., 2012; Baxter et al., 2015; Kon et al., 2014), gonadal development? (Jameson et al., 2015) |
| 9 | PFGF10 | 5:44388817 snv/polyaami misssense | NG_011446.1:c.-33G>A | rs17233910 | 0.005 | 0.002 | 19.99 | NA | NA | NA | NA | ND | ND | – | VUS: - | No | Increased risk hypospadias in human (van der Zanden et al., 2012; Carmichael et al., 2013; Bhoj et al., 2011; Kellermayer et al., 2014), development of the glans penis (Rey et al., 2013) |
| 10 | HS2BS2 | 1:119664831 snv/misssense | NM_001983.3:c.707T>C;p.(Leu236Ser) | rs35887327 | 0.003788 | ND | 8.277 | 3 | 3 | NA | NA | Likely benign | VUS | VUS | Likely pathogenic: PM1, PP2, PP3, BP4 | DMT1, HSD11B2 deficiency | No | Hypospadias (Codner et al., 2004; Kon et al., 2015; Eggers et al., 2010), sex development (Baxter and Vian, 2013; Baxter et al., 2015), hormone synthesis (McCaughan et al., 2003) |
| 11 | JKS | 16:54967040 snv/misssense | NM_005853.6:c.707C>T;p.(Pro236Leu) | rs11549090 | 0.0098 | ND | 11.97 | 3 | 4 | NA | NA | Benign | ND | – | VUS: BP4 | No | Association to hypospadias (Catter and Rey, 2011; Grimsson and Rey, 2014), female gonadal development? (Nat et al., 2005) |

(Continued)
| Patient | Gene | Chromosome: Coordinates | Type/ consequence | HGVS: HGVS| dbsNP ID | gnomAD: MAF | CSVS: MAF | Predictors | Interpretation/classification (8) | Evidence |
|---------|------|--------------------------|------------------|----------|-----------|------------|-----------|------------|---------------------------------|----------|
|         |      |                          |                  |          |           |            |           |            | Exonic predictor: CADD (1) |         |
|         |      |                          |                  |          |           |            |           |            | Exonic predictors: Functional impact (2) |         |
|         |      |                          |                  |          |           |            |           |            | Exonic predictors: conservation (3) |         |
|         |      |                          |                  |          |           |            |           |            | Splicing predictor (4) |         |
|         |      |                          |                  |          |           |            |           |            | Splicing predictors (Alamut) (5) |         |
|         |      |                          |                  |          |           |            |           |            | InterVar | ClinVar | VarSome | ACMG | HGMD: variant (7) | Gene characteristics: evidences for genotype-phenotype correlation (8) |         |
| 5       | MAML2 | 11:95820473 snv/missense | NM_002427.3:c.722G>A:p. (Arg241Gln) | rs119584864 | 0.005 | ND | 32 | 6 | 6 | 0 | NA | VUS | ND | – | VUs: - | No | No (MAML2-related) |
| 5       | NOTCH2 | 1:120469147 snv/ non-synonymous | NM_004469.3:c.980A>G:p. (Asp327Glu) | rs61752484 | 0.0037 | 0.004 | 20.4 | 6 | 6 | 0 | NA | Likely benign | Benign | Benign | VUS: BP6 | No | Primary ovarian failure (Patil et al., 2017); male gonadal development? (Jameison et al., 2013) |
| 5       | ATP3 | 2:12786564 snv/missense | NM_001674.3:c.181G>T:p. (Ala61Ser) | – | ND | ND | ND | 7.926 | 3 | 5 | 1 | NA | Benign | ND | – | VUS: PM2, BP4 | No | Hypoplasia (Bhoj et al., 2011); van der Zanden et al., 2012; Baxter et al., 2015; Kon et al., 2015), male gonadal development? (Jameison et al., 2012) |
| 6       | ATG2 | 9:11643624 snv/missense | NM_017637.5:c.1868C>A:p. (Pro623His) | rs114596065 | 0.0022 | 0.0022 | 5.2 | 8 | 6 | NA | NA | Benign | Benign | Benign | VUS: BP6 | No | Hypospadias (Bhoj et al., 2011); van der Zanden et al., 2012; Baxter et al., 2015; Kon et al., 2015), male gonadal development? (Jameison et al., 2012) |
| 6       | CYP1A1 | 15:7501279 snv/missense | NM_001319216.2:c.1303C>A:p. (Arg435Ser) | rs41279188 | 0.0047 | 0.0047 | 33 | 11 | 5 | NA | NA | VUS | ND | Benign | VUS: PP3, BP6 | No | Association to hypospadias (van der Zanden et al., 2012) |
| 6       | EYA1 | 8:72211882 snv/ synonymous | NM_000553.5:c.630T>C:p. (Ser210=) | rs373102227 | 0.0008 | 0.0008 | 10.56 | NA | NA | 1 | 4 | Likely benign | VUS | VUS | VUS: PP3, BP7 | No | Associated to hypospadias (Grinspon and Rey, 2014); Hwang et al., 2014), male gonadal development? (Jameison et al., 2012) |
| 6       | FLNA | X:153596078 snv/ synonymous | NM_001456.3:c.651C>T:p. (Asp217=) | rs34644500 | 0.0032 | 0.0032 | 5.473 | NA | NA | 1 | 3 | Likely benign | Likely benign | Likely benign | BP4, BP6, BP7 | No | Hypospadias, cryptorchidism, diminished androgen receptor (Carrera-Garcia et al., 2017); female gonadal development? (Jameison et al., 2013) |
| 6       | FRA5 | 4:70334181 snv/missense | NM_025074.6:c.4367T>C:p. (Ile1456Thr) | rs606902495 | 0.0003 | 0.0003 | 24.6 | 12 | 5 | NA | NA | VUS | ND | – | VUS: PM2, PP3, BP1 | No | Syndromic (Fraser syndrome) abnormal genitourinary system (Rattner et al., 2016; Kornacki et al., 2017); female gonadal development? (Jameison et al., 2012) |
| 6       | GLI2 | 7:42066017 snv/intronic | NM_002148:15p.1029G>A | rs74670269 | 0.0002 | 0.0002 | 3 | NA | NA | 0 | 3 | NA | ND | – | VUS: BP4 | No | Increased risk of hypospadias (Carmichael et al., 2013), early genital primordia (Ray and Grinspon, 2011), female gonadal development? (Jameison et al., 2012) |
### TABLE 2 | Continued

| Patient | Gene | Chromosome: Coordinates | Type/ consequence | HGVS: HGVSnp | dbSNP ID | gnomAD MAF | CSVS: MAF | Predictors | Interpretation/classification (6) | Evidence |
|---------|------|--------------------------|------------------|-------------|----------|------------|----------|-----------|-------------------------------|----------|
| 6       | HOXA13 | 7:27230079 snv/missense | NM_003022.4:c.618C>G p. (Phe206Leu) | rs774386575 | 0.0002 | ND | 22.3 | 5 | 5 | NA | NA | VUS | ND | – | VUS: PM4: nonframeshift deletion | No | 
| 6       | IRX5  | 16:54655347 deletion/insertion | NM_005863.5:c.240_243delCTC.p.[Ser81del] | rs1057518726 | ND | ND | – | NA | NA | NA | NA | NA | VUS | VUS | VUS | – | VUS: PM4: nonframeshift deletion | No | 
| 6       | MAML1 | 5:17913168 snv/missense | NM_014757.4:c.1157G>T p. (Lys2121Asn) | rs144047610 | 0.0014 | ND | 5.599 | 3 | 4 | 0 | NA | VUS | ND | – | VUS: - | No | 
| 6       | MAML3 | 4:14081687 snv/synonym | NM_007857:5:c.903C>T. (Asp301=) | rs151091776 | 0.0151 | 0.003 | 22.9 | 9 | 5 | 0 | NA | VUS | ND | – | VUS: - | No | 
| 6       | ARPP1 | 10:33469272 snv/missense | NM_003737.5:c.2504G>A p. (Gly835Asp) | rs115966590 | 0.0002 | 0.003 | 2.445 | 5 | 0 | NA | VUS | ND | – | VUS: – | No | 
| 6       | PROPO1 | 5:17741299 deletion/frameshift | NM_002621.4:c.1505delA p. (Arg53Asp/+112) | rs77776683 | ND | ND | – | NA | NA | NA | NA | NA | Pathogenic | Likely | pathogenic | VUS: PV5; PP5 | DM; pharyngeal hypoplasia deficiency | No | 
| 6       | PTPR1 | 12:112666827 snv/upstream | NM_002834.4:c.-85G>A | – | ND | ND | 16.23 | NA | NA | NA | NA | ND | – | VUS: PM2, PP3 | No | 
| 6       | WDR11 | 10:122686121 snv/missense | NM_018177.11:c.36710C>A p. (Gly1191His) | rs149486212 | 0.0001 | 0.004 | 34 | 14 | 5 | NA | VUS | ND | – | VUS: PP2; PP3 | No | 
| 7       | EVF | 4:5600565 snv/missense | NM_153717.2:c.2240C>T p. (Aal747Val) | rs151091776 | 0.0002 | 0.002 | 18.37 | 5 | 4 | 0 | NA | VUS | ND | – | VUS: - | No | 
| 7       | MAML3 | 14:14081709 snv/missense | NM_014757.15:c.881A>G p. (Asn294Ser) | rs115966590 | 0.0029 | 0.012 | 13.26 | 7 | 6 | 0 | NA | VUS | ND | – | VUS: – | No | 
| 7       | MTFCD2 | 1:120458982 snv/missense | NM_024089.3:c.6863G>C p. (Asp2287Asn) | rs144047610 | 0.0004 | 0.002 | 20.4 | 9 | 5 | NA | NA | VUS | VUS | VUS | VUS: - | No | 

(Continued)
| Patient | Gene | Chromosome: Coordinates | Type/ consequence | HGVSc, HGVSsp | dbsNP ID | gnomAD: MAF | CSVS: MAF | Predictors | Exonic predictor: | Splicing predictors: | InterVar | ClinVar | VarSome | ACMG | HGMD: variant (7) | Evidence |
|---------|------|--------------------------|------------------|-------------|-----------|-------------|-----------|------------|----------------|-----------------|----------|---------|---------|------|-----------------|----------|
| 7       | PPARC18 | 5:149210653 snv/missense | NM_130263.3:c.2668G>A | rs150637009 0.0056 | ND 19.3 5 5 0 NA | VUS ND – VUS: BP4 | No | Candidate to hypospadias (van der Zanden et al., 2012) |
| 7       | WDR11 | 10:122637000 snv/missense | NM_018117.11:c.1520C>G | rs77569615 0.00004 | ND 24.1 14 6 1 NA | VUS ND – VUS: PP2, PP3 | No | Hypospadias (Eigers et al., 2016, Fan et al., 2017), small testes (Fan et al., 2017) |
| 8       | CUL4B | X:119708447 snv/missense | NM_003688.3:c.260G>A | rs149016283 0.0002 | ND 18.73 1 NA 1 NA | Likely benign Likely benign Likely benign BP4, BP5 | No | Abnormal genital system (Ketterer et al., 2014) |
| 8       | DAPK1 | 9:9321476 snv/missense | NM_004938.3:c.3490G>A | rs937952689 0.00007 | ND 24.3 8 5 NA NA | VUS ND – VUS: - | No | Female gonadal development? (Jameson et al., 2012) |
| 8       | JMK2 | 10:119051533 snv/missense | NM_004098.3:c.407-10C >T | – 0.00004 | ND 9.098 NA NA 1 4 | ND ND – VUS: BP4 | No | 46,XX DSD (Li et al., 2012), six determination (Bacon-Laube, 2010, Jacob and Lovell-Badge, 2011; Eigers and Sinclair, 2012), (female) gonadal development (Riley et al., 2009, Jameson et al., 2012; 46,XY DSD (Furd et al., 2014) |
| 8       | FREM2 | 13:39454885 insertion/ frameshift | NM_207361.5:c.3472delCpC:p.(Gln2107Phe) | – ND ND – NA NA NA NA | ND ND – Likely pathogenic PV51, PV52 | No | Syndromic (Fischer syndrome) abnormal genitalia (Stano et al., 2018), female gonadal development (Jameson et al., 2012) |
| 8       | ISFB3 | 2:217525993 snv/missense | NM_005097.3:c.685G>A | p.(Gln229Lys) | – ND ND 23.7 8 6 0 NA | VUS ND – VUS: PM2, PP2 | No | Candidate gene in ovary development (Clement et al., 2007), female gonadal development? (Jameson et al., 2012; Mungler et al., 2013) |
| 8       | MAML2 | 11:95826675 snv/missense | NM_003247.3:6.600G>A | p.(Arg207Thr) | rs191391876 0.0002 | ND 24.4 9 5 0 NA | VUS ND – VUS: - | No | No MAML2-related |
| 8       | MAML3 | 4:140811709 snv/missense | NM_018171.5:c.881A>G | p.(Asp280Asn) | rs115966590 0.0028 | 0.004 13.26 7 6 NA NA | ND ND – – | No | Female gonadal development? (Jameson et al., 2012) |
| 8       | MYO7A | 11:76883787 deletion/intronic | NM_002663.1:1798-7_1798-6del40 chrAT | – ND ND – NA NA NA NA | ND ND – – | No | Male gonadal development? (Jameson et al., 2012; Li et al., 2014) |
| 8       | MYO7A | 11:76883780 deletion/ intronic | NM_002663:1:1798-4_1801delinsGGCTGCT | – ND ND – NA NA NA NA | ND ND – – | No | Male gonadal development? (Jameson et al., 2012; Li et al., 2014) |
| 8       | AHDCH1 | 1:39405111 snv/missense | NM_017617.5:c.2734C>T | p.(Arg912Trp) | rs201620398 0.002 | ND 31 12 6 0 3 | Likely benign VUS VUS VUS: PP2, PP3 | No | Related to SHH and FGF10 (Gripon and Rey, 2014) |
| 8       | PKC6 | 1:4621570 snv/missense | NM_003623.3:c.838G>A | p.(Asp281Glu) | rs186728731 0.0001 | ND 25.5 6 6 0 2 | VUS ND – VUS: PP3 | No | Female gonadal development (Boverdam and Kooiman, 2006), (Jameson et al., 2012) |
Among them, only MAML2 has not been related to gonadal or genitourinary system development (Table 2).

The following genes showed variants in two patients: CYP1A1 in patients 1 and 6; EVC in patients 1 (2 variants) and 7; IRX5 in patients 5 and 6; MAML1 in patients 4 and 6; MAML2 in patients 5 and 8; NOTCH2 in patients 5 and 7; RECO14 in patients 2 and 3 and WDR11 in patients 6 and 7 (Table 2). In addition, 2 genes presented variants in 3 patients: MAML3 (patients 6, 7 and 8) and NOTCH1 (patients 1, 4 and 8). Furthermore, RIPK4 presented 2 variants in patient 1. Finally, BNC2 variant c.1868C>A:p. (Pro623His) (MAF = 0.002) was detected in 2 patients (patient 1 and 7) and MAML3 variant c.881A>G:p.(Asn294Ser) (MAF = 0.0028) in patients 7 and 8 (Table 2).

We performed interactome analysis for the identified DSD genes using bioinformatic tools for the analysis of possible gene-protein interactions. The network comprising all genes identified is shown in Figure 1. Overall, a connection was found for 27 of the 41 genes. MAML1 connects directly to MAML1/2/3. Via NOTCH1/2 8 genes are in connection with MAML1, namely WNT9A/9B, GL2/3, FGFl0, RET, PRO1 and NRP1. Some of these genes are also central nodes for further connections; e.g. GL13 for EVC, FGFl0, GL12, RIPK4 and EYA1; and RET for PIK3R3 with PTPN11, which also is connected with RIPK4. RIPK4 itself is a central node for ZBTB16, CUL4B, GL13 and PTPN11. NRP1 is connected to FLNA and EYA1 connects with FRAS1 and FREM2. In addition, 2 isolated gene couples have been revealed by our analysis: CYP1A1-HSD3B2 and MYO7A-CDH23. These observations give an idea of the complex interactions among genes related to sex development.

The specific interactome of identified genes in patients 1 and 4 to 8 is shown in Figure 2. In patients 1, 4, 5, 7 and 8, MAML1 and MAML1-related genes (MAML1, MAML2 or MAML3) are directly related to NOTCH1/2 (Figures 2A–C, E, F). In patient 1, there are 2 networks: ZBTB16-RIPK4 and MAML1-NOTCH1-RET (Figure 2A). In patient 6, GL13, EYA1 and FRAS1 as well as FLNA and NRP1 seem directly related (Figure 2D). In patient 8, NOTCH1 plays a central role connecting to WNT9A, WNT9B and MAML1 network (Figure 2F).

**DISCUSSION**

Sex development is a very complex biological event which requires the concerted collaboration of a large network of genes in a spatial and temporal correct fashion. In the past, much has been learned about human sex development from monogenic DSD, but the broad spectrum of phenotypes in numerous DSD individuals remains a conundrum. Oligogenic disease has been proposed. In fact, multiple genetic hits, which might not be deleterious by themselves, have been found in several individuals with DSD (Kon et al., 2015; Eggers et al., 2016; Mazen et al., 2016; Werner et al., 2017; Camats et al., 2018). In a previous study of 46,XY DSD patients carrying MAML1 variants, we showed that none of the variants were functionally pathogenic except for a stop variant (Camats et al., 2015). In the present study, we searched for additional genetic hits in DSD patients harboring MAML1 mutations and manifesting with unexplained broad phenotypes. Using HTS and a custom-made
algorithm including DSD- and MAMLD1-related genes from literature and databases, we identified potentially deleterious genetic variants in additional genes in all MAMLD1 individuals. Thus, we believe that the broad phenotype of individuals carrying MAMLD1 variants is due to additional genetic hits.

In our study, we identified 55 additional heterozygous/hemizygous variants in 41 genes in seven 46,XY DSD hemizygous and one 46,XX DSD heterozygous MAMLD1 patients. Among the 41 genes, 16 have been previously reported in humans with hypospadias (ATF3, BNC2, CYP1A1, EMX2, EYA1, FLNA, GLI3, GRD1, GLI2, FGF10, HOXA13, HSD3B2, IRX5, IRX6, PPARC1B and WDR11) and 2 have been related to cryptorchidism (BNC2, FLNA, RET, RECQL4, NRPI, PTPN11, RIPK4 and ZBTB16), and 5 genes have been found in patients with micropenis (BNC2, EVC, FGFI0, RIPK4 and ZBTB16). Also, 15 genes have been described in other types of DSD (CUL4B, EMX2, FRAS1, FREM2, HSD3B2, NOTCH2 and NRPI) and/or or were reported in different syndromes (CYP1A1, EVC, FRAS1, HOXA13, PTPN11, RECQL4, RET, RIPK4 and ZBTB16) (Table 2). In addition, 27 genes had been previously described in the context of sex or gonadal development (ATF3, BNC2, CDH23, COL9A3, DAPK1, EMX2, EVC, EYA1, FLNA, FRAS1, FREM2, GLI2, GLI3, HOXA13, IGFBP2, IRX5, MAMLD1, MYO7A, NOTCH1, NOTCH2, NRPI, PIK3R3, RET, RIPK4, TGFBI, WNT9A and WNT9B) of sex or gonadal development (Table 2).

According to OMIM, almost all of our patients presented at least one variant in a gene with autosomal dominant inheritance (AD) (COL9A3, GLI2, FGFI0, FLNA, EYA1, GLI3, HOXA13, NOTCH1, NOTCH2, PTPN11, RET, TGFBI and WDR11), while other genes (CDH23, MYO7A and PPARC1B) may have both AD and autosomal recessive (AR) inheritance. FLNA and CUL4B are X-linked (XLR), while CYP1A1, FREM2, EVC, HSD3B2, IRX5, PROP1, RAS1, RECQL4, RIPK4 and ZBTB16 are known for AR inheritance. No information on inheritance is currently available for the remaining genes including ATF3, BNC2, GRD1, DAPK1, IRX6, IGFBP2, MAMLI1, MAML2, MAMLD3, NRPI, PIK3R3, WNT9A and WNT9B.

The seven MAMLD1 patients with 46,XY DSD presented phenotypes from female external genitalia (patient 4) to variable degrees of hypospadias, cryptorchidism and small penis (Table 1). Interestingly, patient 4 with female external genitalia had normal T secretion. Similarly, patient 5 carrying a heterozygous HSD3B2 variant, had normal levels of 17OH-pregnenolone, DHEA and DHEA-S (data not shown). Patient 6, who presented with a right aortic arch, was found to carry variants in five genes (BNC2, FLNA, MAML1, NRPI and PTPN11) that have been previously described in patients with heart and/or vascular anomalies (Tartaglia et al., 2002; Bhoj et al., 2011; Lee et al., 2014; Shaheen et al., 2015; Preuss et al., 2016; Chen et al., 2018). The 46,XX patient (patient 8), with primary amenorrhea, hypergonadotropic hypogonadism, normal female external genitalia and small uterus harbored gene variants involved in gonadal development and DSD (CUL4B, DAPK1, EMX2, FREM2, IGFBP2, MAMLI3, MYO7A, NOTCH1, PIK3R3, TGFBI, WNT9A and WNT9B; Table 2). Five of these genes (DAPK1, IGFBP2, MAML3, PIK3R3 and WNT9A) have so far only been related to female gonadal development (Table 2).

Overall, the genes detected in our eight studied patients with MAMLD1 variants have been previously reported in humans with hypospadias, cryptorchidism, micropenis, and other urogenital abnormalities; or they have been found involved in sexual and gonadal development. Also, some of them have been associated with specific syndromes in patients with genitourinary anomalies: CAKUT syndrome, Ellis–van Creveld syndrome, Fraser syndrome 1, Fraser syndrome 2, hand–foot–genital/Guttmacher syndrome, Noonan syndrome, Mayer–Rokitansky–Küster–Hauser syndrome, Popliteal pterygium syndrome and Rothmund–Thomson syndrome (see Table 2). However, none of the present patients presented a complete phenotype for any of these syndromes, maybe because none of the variants completely impairs gene expression and protein function, as inferred by the in silico analyses. Detailed information on these genes from current literature is given in Supplementary Materials (S1).

A search for an underlying network comprising variants in the identified genes related to MAMLD1 revealed a considerable number of genes which showed gene-gene, gene-protein or protein-protein interactions (Figures 1 and 2) suggesting that genetic variations in these genes may affect sex development. In addition, MAMLD3 was found in a network related to female gonadal development (Jameson et al., 2012). Accordingly, one variant in MAMLD3 was present in our 46,XX patient. The analysis of gene/protein network interactions per patient gives an idea of the complexity of the interactions among genes related to sex development. The more variants detected in DSD-related genes, the better to build an interaction network searching for clues on genetic relationship(s) for sex development. In our DSD individuals carrying MAMLD1 variants, three genes seemed prominent in the network analysis, NOTCH1, NOTCH2 and GLI3. NOTCH signaling is a highly conserved signaling pathway and comprises 4 transmembrane receptors. It is essential for the regulation of embryonic development of multiple organ systems including gonadal development (Windley and Wilhelm, 2016). NOTCH signaling is implicated in Leydig cell differentiation in an inhibitory regulatory fashion (Windley and Wilhelm, 2016). Autosomal dominant mutations in NOTCH1 cause the Adams–Oliver syndrome (OMIM 616028), while autosomal dominant mutations in NOTCH2 are reported in the Agalille syndrome 2 (OMIM 610205) and in the Hajdu–Cheney syndrome (OMIM 102500). By contrast, GLI3 is a zinc-finger transcription factor belonging to the desert hedgehog (DHH) signal transduction pathway. DHH signaling is essential for driving Leydig cell differentiation (Windley and Wilhelm, 2016). Thus, NOTCH and DHH signaling work together to regulate Leydig cell development (Windley and Wilhelm, 2016). Autosomal dominant mutations in GLI3 are described in the Pallister–Hall syndrome (OMIM 146510) or in the Greig cephalopolysyndactyly syndrome (OMIM 175700).

Taken together, our results expand the landscape of genes possibly involved in DSD by revealing both new and old players. Genetic platforms for DSD diagnostics currently consider about 270 genes that have been identified with monogenetic forms of DSD in (mostly) several independent individuals (Cools et al., 2018). Our eight MAMLD1 individuals share variants in 19 genes.
comprised in such DSD panels, including ATV3, BNC2, CUL4B, EVC, FLNA, FRAS1, FREM2, GLI3, HOXA13, HSD3B2, IRX5, NOTCH2, PROP1, PTPN11, REClQA, RET, RIPK4, WDR11 and ZBTB16. By contrast, through our work 22 new genes are now added for considering with differences in sex development: CDH23, COL9A3, CYP1A1, DAPK1, EME2, EYA1, FGF10, GLI2, GRID1, IGFBP2, IRX6, MAML1, MAML2, MAML3, MYO7A, NOTCH1, NRPI, PIK3R3, PPARGC1B, TGFBI, WNT9A and WNT9B.

Ideally, genetic variants are tested functionally for proof of their disease-causing effect in model systems. However, when finding multiple variants, which may all contribute only partially, such testing is no longer feasible. Therefore, the likelihood of disease-causing effect of identified variants was assessed in our study by established bioinformatic tools for genetics and by assessing the genotype-phenotype correlation in each patient with current knowledge from literature and databases in the field. In future studies with bigger sample size, next-generation statistical genetic analyses may be employed to identify associations between a group of variants and the complex trait of sex development (Weissenkampen et al., 2019).

In summary, HTS analysis indicates that the broad DSD phenotypes of MAMLD1 patients may be due to additional variants in other DSD-related genes. We found up to 55 additional genetic hits that may contribute to the DSD phenotype making an oligogenic causation plausible. Bioinformatic network analysis can help in interpreting complex genetic data and put identified single candidage genes into a greater perspective to understand their possible role in DSD biology.

DATA AVAILABILITY

The datasets generated for this study are publicly available in dbSNP (Sherry, 2001): https://www.ncbi.nlm.nih.gov/projects/SNP/snp_viewBatch.cgi?sbid=1063030.

ETHICS STATEMENT

The study was approved by the Ethics Committee of Hospital Universitari Vall d’Hebron (Barcelona, Spain) (CEIC: PR(IR)23/2016). Written informed consent was obtained from the patients for the publication of their cases.

AUTHOR CONTRIBUTIONS

CF: Conceptualization, funding acquisition, investigation, methodology, interpretation, project administration, resources, supervision, writing – original draft preparation, writing – review and editing. LA: Interpretation, supervision, resources, visualization, writing – review and editing. K-SS: Validation, interpretation, writing – review and editing. IM: Resources, interpretation, writing – review and editing. LC: Resources, writing – review and editing. IE: Resources, writing – review and editing. NC: Conceptualization, data-curation, formal analysis, investigation, methodology, interpretation, project administration, supervision, visualization, writing – original draft preparation, writing – review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2019.00746/full#supplementary-material

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