# Characterization of 35 novel *NR5A1*/SF-1 variants identified in individuals with atypical sexual development: The *SF1next* study

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**Abstract**

**Context**

Steroidogenic factor 1 (*NR5A1*/SF-1) is a nuclear receptor that regulates sex development, steroidogenesis and reproduction. Genetic variants in *NR5A1*/SF-1 are common among differences of sex development (DSD) and associate with a wide range of phenotypes, but their pathogenic mechanisms remain unclear.

**Objective**

Novel, likely disease-causing *NR5A1*/SF-1 variants from the SF1next cohort of individuals with DSD were characterized to elucidate their pathogenic effect.

**Methods**

Different *in silico* tools were used to predict the impact of novel *NR5A1*/SF-1 variants on protein function. An extensive literature review was conducted to compare and select the best functional studies for testing the pathogenic effect of the variants in a classic cell culture model. The missense *NR5A1*/SF-1 variants were tested on the promoter luciferase reporter vector -*152CYP11A1*_pGL3 in HEK293T cells and assessed for their cytoplasmic/nuclear localization by Western blot.

**Results**

Thirty-five novel *NR5A1*/SF-1 variants were identified in the SF1next cohort. Seventeen missense *NR5A1*/SF-1 variants were functionally tested. Transactivation assays showed reduced activity for 40% of the variants located in the DNA binding domain and variable activity for variants located elsewhere. Translocation assessment revealed three variants (3/17) with affected nuclear translocation. No clear genotype-phenotype, structure-function correlation was found.

**Conclusions**

Genetic analyses and functional assays do not explain the observed wide phenotype of individuals with these novel *NR5A1*/SF-1 variants. In nine individuals, additional likely disease-causing variants in other genes were found, strengthening the hypothesis that the broad phenotype of DSD associated with *NR5A1*/SF-1 variants may be caused by an oligogenic mechanism.

**Introduction**

Differences of sex development (DSD) are rare, mostly genetic disorders and comprise a group of heterogenous conditions that lead to atypical chromosomal, gonadal, and/or anatomic sex development and related function (1). Since the Chicago consensus in 2005 (1), DSD are grouped into main categories of chromosomal, 46,XY and 46,XX DSD that are further divided in different subgroups. Still, genotypic and phenotypic characteristics of DSD are very broad and variable, and they may or may not be more specific for certain subgroups. For people with DSD, it is important to have an exact diagnosis at the molecular level for receiving specific information on health outcomes and treatment options as well as for genetic counselling (2, 3). Although advancements in genetics have enhanced the knowledge in the field of DSD significantly, current genetic approaches still fail to find the underlying molecular diagnosis in about half of individuals with a DSD. Chromosomal and monogenic DSD with a characteristic genotype-phenotype correlation such as Turner or Klinefelter syndrome and DSD associated with congenital adrenal hyperplasia or complete androgen insensitivity seem easy to diagnose (2-5). DSD caused by variants in genes manifesting with a broad phenotype like *NR5A1*/SF-1, *SOX9, SOX8* and *DHH* are more difficult to diagnose (6-9), and those that are most difficult to diagnose are when next-generation sequencing (NGS) approaches reveal multiple candidate gene variants classified as variants of unknown significance (VUS) by current guidelines (10).

Variants in *NR5A1*/SF-1 are reported causative in approximately 15% of all cases of 46,XY DSD (11). *NR5A1/*SF-1 is a transcription factor that regulates expression of

multiple genes and interacts with many proteins involved in sex and adrenal development, steroidogenesis and reproduction (12). The first human *NR5A1*/SF-1 gene variant was reported in a 46,XY DSD individual with adrenal failure and complete gonadal dysgenesis (13). Thereafter, the gonadal and reproductive phenotype associated with human *NR5A1*/SF-1 variants became predominant and encompassed a broad spectrum including 46,XY and 46,XX individuals with DSD, spermatogenic failure, primary ovarian insufficiency (POI) and even healthy carriers (14, 15). But an explanation for this broad phenotypic manifestation is still missing.

Reported *NR5A1*/SF-1 disease-causing variants are found throughout the whole gene without obvious hot spots and can be missense, nonsense, small insertions–deletions (indels), complete gene deletions or splice-site variants. They are mostly found in heterozygosis and only a few are compound heterozygous or homozygous (16).

To confirm pathogenicity, many *NR5A1*/SF-1 variants found in individuals with a DSD have been tested by *in vitro* cell-based studies. *NR5A1*/SF-1 variants located in the DNA binding domain (DBD) of the SF-1 protein revealed consistently impaired transactivation activity when studied on different gene promoters, whereas promoter studies testing variants located in the hinge region (HR) and the ligand binding domain (LBD) showed variable results (16, 17). For heterozygous *NR5A1*/SF-1 variants, a dominant negative effect where the mutated protein disrupts the function of the normal protein, even when present in only one copy, has never been found (16, 18-30), and also haploinsufficiency seems unlikely to explain the highly variable phenotype between individuals with the same *NR5A1*/SF-1 variant and even between family members (31).

Similarly, protein modelling and structure-function prediction attempts failed to explain pathogenicity of variants consistently (16, 19, 32, 33).

Thus, all these studies did not find a phenotype-genotype-function correlation.

More recently, oligogenic inheritance (2, 7, 17, 31, 34-38), genetic variants in non-coding regulatory elements (39), variable allelic expression (7, 35), epigenetic regulation and environmental factors (40) have been suggested as possible explanations for the broad manifestation of DSD associated with *NR5A1*/SF-1 variants.

Therefore, to gain further insight into DSD related to *NR5A1/*SF-1, we set up a large international collaboration in the *SF1next* study where we collected existing data on phenotype and genotype of the largest cohort to date of 197 individuals harbouring a *NR5A1/*SF-1 variant (41). In this cohort, 35 novel *NR5A1/*SF-1 variants were reported that had not been characterized previously. Here we provide the clinical, genetic and functional characterization of these novel variants. We used various bioinformatic methods and performed classic cell-based functional studies aiming at elucidating their disease-causing effects.

## Materials and Methods

### Literature search for functional studies of *NR5A1/*SF-1 variants

We used the Human Gene Mutation Database (HGMD, by April 2022) to search for publications, in which functional studies were performed to assess the pathogenicity of missense *NR5A1/*SF-1 variants; these included transactivation studies with promoter reporters in classic cell models (Supplementary table 1)(42) and other studies such as protein expression, nuclear transfer and DNA binding (Supplementary table 2)(42). In Supplementary table 3 we also collected clinical and genetic data from patients harbouring the corresponding variants(42).

### Ethical approval

Written informed consent was obtained from all participants and/or their parents. The study was approved by the I-DSD registry (UKCRN ID12729) and the local ethical committees responsible for the participating clinicians, for Switzerland Swiss Ethics (BASEC ID 2016-01210).

### Case reports and genetic analyses

The 39 patients with a DSD carrying 35 novel *NR5A1/*SF-1 variants included in this work are part of the *SF1next* study cohort (41). Clinical and genetic data were provided

anonymized by the responsible clinicians through REDCap (Research Electronic Data Capture). To classify the severity of the DSD phenotype of the patients, we used a modified external genitalia score (EGS) based on the karyotype and characteristics of the external genitalia at birth or before genital surgery (41). We considered the identified *NR5A1*/SF-1 variants as novel when not reported before and/or when *in vitro* functional studies hadn't been performed. In these patients we also assessed the possible pathogenicity of additional gene variants reported through REDCap.

### *In silico* analyses and variant classification

We searched for previously reported clinical associations in ClinVar and HGMD databases and the literature (e.g. PubMed). Among the variants considered as novel for this study, 29/35 had not been reported before and 6/35 had been reported in the literature but no *in vitro* functional testing was done.

We predicted the possible effect of identified novel nonsynonymous genetic variants on the structure and function of the protein using Polyphen-2, (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/), Panther (Protein ANalysis THrough Evolutionary Relationships, http://www.pantherdb.org/tools/csnpScore.do), SNPs and Go (https://snps-and-go.biocomp.unibo.it/snps-and-go/), CADD (Combined Annotation Dependent Depletion, https://cadd.gs.washington.edu/) and the calibrated scores given by VarSome (43) for Revel (Rare Exome Variant Ensemble Learner), SIFT (Scale-invariant feature transform), Provean (Protein Variation Effect Analyzer), Mutation taster and M-CAP (Mendelian Clinically Applicable Pathogenicity) (see supplementary table 4)(42). Variants were classified for pathogenicity according to the standards and guidelines of the American College of Medical Genetics and Genomics (ACMG) (10) using VarSome (43).

### *In vitro* testing of transactivation activity

Promoter luciferase reporter vector of human *-152CYP11A1*_pGL3, HA-tagged wild-type (WT) cDNA of *NR5A1*/SF-1 in pcDNA3, empty control vector pcDNA3, and *Renilla*-TK

(pRL-*TK*) were all available from previous work (16). The HA-tagged human *NR5A1*/SF-1 cDNA (NM 004959.5) containing pcDNA3 vector was used as a template to generate the novel *NR5A1*/SF-1 variant expression vectors by PCR-based site directed mutagenesis using specific primers (Supplementary table 5)(42) and the QuickChange protocol by Stratagene (Agilent Technologies Inc., Santa Clara, CA, USA). Only the variant *NR5A1*/SF-1 expression vector containing c.977G>T was custom made (GenScript, Piscataway, NJ, USA). The coding sequences of all mutant expression vectors were confirmed by direct sequencing.

Non-steroidogenic, human embryonic kidney HEK293T cells were cultured as previously described (16). For promoter activity experiments, cells were cultured on 12-well plates and transiently transfected with 200 ng WT or mutant *NR5A1*/SF-1 expression vectors, 800 ng of the promoter luciferase reporter construct *-152CYP11A1*_pGL3, and 30 ng of the pRL-TK vector as an endogenous control using Lipofectamine 2000™ (Invitrogen, Glasgow, UK) in Opti-MEM (1X)-reduced serum medium (Gibco, Thermo Fisher Scientific, US). Forty-eight hours after transfection, cells were washed with PBS, lysed and assayed for luciferase activity with a dual- luciferase assay using a microplate Luminometer reader (Fluoroskan Ascent® FL & Fluoroskan Ascent®, Thermo Fisher). Specific *Firefly* luciferase readings were standardized against *Renilla* luciferase control readings. Experiments were repeated two to four times in duplicates and data were summarized giving the mean ± standard error of the mean (SEM). Statistical significance was examined by the Student's t-test (GraphPad Prism, GraphPad Software, Boston, MA, USA).

**Assessment of nuclear transfer of wild-type and variant *NR5A1*/SF-1**

HEK293T cells were cultured on 6-well plates and transiently transfected with WT or mutant *NR5A1*/SF-1 expression vectors using Lipofectamine 2000™ (Invitrogen) in Opti-MEM (1X)-reduced serum medium (Gibco). 48 hours after transfection, cells were collected with trypsin and washed with PBS, and then immediately collected for preparing cytoplasmic and nuclear extracts using the NE-PER™ nuclear and cytoplasmic extraction

reagents according to the manufacturer's instructions (Thermo Fisher Scientific). Protein concentrations were measured by the DC protein assay kit (Bio-Rad, Hercules, CA, USA). Nuclear and cytoplasmic protein fractions of WT and variant SF1 cell extracts were then analysed by Western blot with an antibody against HA-tag (RRID: AB_390918) for HA tagged-NR5A1/SF-1, Lamin B1 (RRID: AB_11002649) and Rab11 (RRID: AB_397984) as nuclear and cytoplasmatic markers, respectively. Expression of β-actin protein (RRID: AB_476692) was used as control. HA tagged-*NR5A1/*SF-1 and β-Actin band intensity on Western blots were quantified by the FUSION FX6 software program of the FUSION FX EDGE Imaging System (Witec AG, Sursee, Switzerland). For exact information on antibodies used, see Supplementary table 6(42).

## Results

### Review of reported promoter transactivation studies of *NR5A1/*SF-1 variants in cell models

To find the most successful functional assay system in a cell model for assessing pathogenicity of novel *NR5A1/*SF-1 variants, we reviewed the corresponding literature. Overall, we found 313 experiments performed on 98 different missense *NR5A1/*SF-1 variants (Supplementary table 1)(42). Non-steroidogenic cells were used in 280/313 (89.4%) experiments, with the HEK293T cell line being used most often (181/313, 57.8%). In promoter transactivation assays, we found that the *CYP11A1* promoter reporter was employed in 108/313 (34.5%) experiments, followed by promoter reporters of *AMH* (45/313, 14.3%), *CYP17A1* (39/313, 12.4%) and TESCO (40/313, 12.8%) (Supplementary table 1) (42). In total, 63 transactivation experiments using a *CYP11A1* promoter reporter in HEK293T cells were performed for 57 different *NR5A1/*SF-1 variants. In 38 out of 63 (60.3%) experiments performed on *NR5A1/*SF-1 variants located in the DBD, a significantly reduced activity was found. By contrast, variants located in the HR or LBD of the SF-1 protein showed reduced activity in only 22% or 22.4%, respectively (Supplementary table 1)(42). No dominant-negative effect was observed in

26 studies that tested the combined impact of the variant together with the WT human *NR5A1/*SF-1 expression vector (Supplementary table 1)(42).

In addition to transcriptional activation experiments, other *in vitro* studies were performed using different methods and techniques (Supplementary table 2)(42). SF-1 protein expression was assessed by Western-blot (WB) for 50 *NR5A1/*SF-1 variants, and most variants (66%) showed similar protein expression to WT. Furthermore, 62 *NR5A1/*SF-1 variants were tested for nuclear translocation using immunofluorescence (IF). Generally, variants located in the DBD impaired nuclear translocation more likely compared to variants located elsewhere. In addition, *NR5A1/*SF-1 variants' binding to target gene promoters such as steroidogenic enzymes, was tested with Electrophoretic Mobility-Shift Assays (EMSA) in 18 studies (Supplementary table 2)(42). *NR5A1/*SF-1 variants located in the DBD and the LBD showed 75% and 67% reduced binding to their responsive elements, respectively (Supplementary table 2)(42). Finally, structure predictions for 44 *NR5A1/*SF-1 variants were performed using different *in silico* tools, and almost all studies (89%) showed structural defects indicating that amino acid substitutions might affect DNA, ligand and/or cofactor interactions (Supplementary table 2)(42).

Taken together, a correlation between genotypes and phenotypes has not been found so far. For illustration: The DBD located *NR5A1/*SF-1 variant c.43G>A, (p.Val15Met), classified as pathogenic, was described in a patient with a severe 46,XY DSD phenotype but also in a 46,XX female with typical genitalia and POI (20, 33). Similarly, variant c.634G>A (p.Gly212Ser), also classified as pathogenic, was found in a 46,XY male without DSD but with a low sperm count, and a 46,XY female with sex reversal (38, 44).

**Clinical characteristics of patients harbouring novel *NR5A1/*SF-1 variants**

A summary of the clinical features of the 39 subjects harbouring novel *NR5A1/*SF-1 variants is given in Table 1. Most of the patients had a 46,XY karyotype (36/39, 92.3%). None of the subjects had an adrenal phenotype. Concerning DSD, 66.7% (26/39) were classified as having a severe DSD phenotype. An opposite sex phenotype was found in 10/39 (25.6%), a mild in 2/39 (5.1%) and a typical phenotype for karyotype in 2/39

(5.1%). Subjects classified as severe manifested with ambiguous genitalia at birth or in early infancy, and were registered either as male (17/26, 65.4%) or female (8/26, 30.8%). Patients 33 and 38, who were initially registered as female were reassigned to male at age ten years and less than one year, respectively. Patient 7 was registered male few months after birth. All patients classified as opposite sex presented with typical female external genitalia and were registered female at birth. Two males (patients 13 and 20) were classified as having mild DSD with micropenis, mild hypospadias and scrotal gonads. Patient 14 presented with typical male external genitalia but developed gynecomastia at age 11 years; he was classified as typical.

Two patients had a 46,XX karyotype (2/39). Patient 25 was referred at 12 years as a typical female with amenorrhea and abnormal uterus on magnetic resonance imaging (MRI), while patient 38 presented with ambiguous genitalia and small ovotestes at age 34 years. Patient 4 was a 47,XXY phenotypic female and presented with amenorrhea at the age of 16 years; she had a normal uterus, but gonadal biopsy revealed testicular tissue. Fourteen patients (33.3%) had anomalies in other organs, half of whom had spleen and associated blood system anomalies (7/14, 50.0%) (Table 1). So far, none of the patients has had any kind of cancer reported, but the median age of the study group was only 10 years (range 0-32 years).

Family history of our studied individuals revealed DSD or reproductive disorders in 11 individuals from 10 unrelated families (Table 1). These were mostly 46,XY males with either isolated hypospadias, hypospadias and cryptorchidism or micropenis (4/11, 36.4%). A 46,XY female with complete gonadal dysgenesis was also reported. Two affected 46,XX females presented with POI at the age of 39 years or needed assisted reproductive technology (ART) to achieve pregnancy. Genetic testing was not performed in four relatives who presented with POI, menstrual irregularities, hypospadias and cryptorchidism or isolated hypospadias. Abnormalities such as hyperextensibility, T1D (type 1 diabetes), left ventricular non compaction and developmental delay were reported in relatives of four index cases from four different families.

**Genetic characteristics of patients harbouring novel *NR5A1*/SF-1 variants**

Thirty-five novel *NR5A1*/SF-1 variants were reported in 39 patients from the *SF1ne*xt study cohort (Table 2, Figure 1) (41). These were mostly missense variants (19/35, 54.3%), followed by frameshift insertions or deletions (8/35, 22.8%), nonsense or intronic variants (3/35, 8.6% each), synonymous and non-frameshift (2/35, 5.7% each) and one big deletion (1/35, 2.8%).

We classified the identified novel variants according to the ACMG guidelines (10) (Figure 1). Among the novel *NR5A1*/SF-1 variants, 64.1% (25/39) classified as (likely) pathogenic, the rest were either VUS (10/39, 25.6%) or (likely) benign (4/39, 10.3%). All novel *NR5A1*/SF-1 variants were found in heterozygosis, and only patient 16 was a compound heterozygote for two variants. Genetic analysis had been performed by NGS in 27 patients (27/39, 69.2%) (Table 2), either by targeted gene panels (16/39, 41.0%) or whole exome sequencing (WES) (11/39, 28.2%) (Figure 1). In 16 patients (15/39, 38.5%) a single gene analysis was performed for the molecular diagnosis, together with an array in two patients.

Results of genetic testing of relatives was available from 21 families (21/39, 53.8%). *NR5A1*/SF-1 variants were found *de novo* in six patients (patients 9, 10, 14, 18, 26 and 28; 6/21, 28.6%); in the other 15, a heterozygous carrier was identified in the family, although genetic analysis of both parents was only performed in 14 families (Table 2).

In nine (23%) individuals with a *NR5A1*/SF-1 variant, additional genetic variants were reported in a total of 28 different genes, with one to 16 additional variants per individual (Table 2, Figure 1). The majority of these additional variants were classified as VUS (11/28, 39.3%) and likely benign (LB) (8/28, 28.6%), followed by benign (B) (6/28, 21.4%), likely pathogenic (LP) (1/28, 3.6%), pathogenic (P) (1/28, 3.6%) and one undetermined variant (1/28, 3.6%). Pathogenicity prediction of these with respect to the associated DSD phenotype was similarly poor as for the related, specific *NR5A1*/SF-1 variants. Of the eight individuals (46,XY or 47,XXY) with an opposite sex or severe DSD phenotype, two presented with at least one (likely) pathogenic additional variant, three

individuals had VUS and three had (likely) benign variants. Only patient 25 with a typical phenotype carried a VUS and a likely benign additional variant.

**Protein structure prediction and *in vitro* functional testing of novel *NR5A1*/SF-1 variants**

We tested 17 novel missense *NR5A1*/SF-1 variants originating from 18 DSD patients of the *SF1next* cohort for their impact on protein structure and function (Table 2). Identified variants were located throughout the SF-1 protein; ten were located in the DBD, six in the LBD and one at the C-terminus. Comparison of SF-1 protein similarity across species revealed that all 17 variants and the surrounding regions are highly conserved (Figure 2). Structure prediction programs suggested structural defects in all. Novel *NR5A1*/SF-1 gene variants were thus classified as (likely) pathogenic or VUS (Table 2).

After literature review (Supplementary table 1)(42), we decided to use HEK293T cells transfected with WT or mutant *NR5A1*/SF-1 expression vectors and with the *CYP11A1* promoter reporter for the functional studies of our 17 novel missense *NR5A1*/SF-1 variants (Figure 3). Four out of ten *NR5A1*/SF-1 variants located in the DBD showed severely impaired reporter activity (p.Cys13Ser, p.Arg39Leu, p.Cys73Tyr and p.Cys73Trp), while the other variants had similar activity as WT (Figure 3A). Six out of seven variants located in the LBD and C-terminus showed 50% or more transactivation activity on the *CYP11A1* promoter reporter compared to WT, except Ala280Glu (Figure 3A).

We also assessed nuclear translocation of WT and variant *NR5A1*/SF-1 in transfected HEK293T cells. Only three variants located in the DBD (p.Cys13Ser, p.Cys73Tyr and p.Cys73Trp) affected nuclear translocation compared to the WT protein, which showed about 80% nuclear localization. None of the variants contained in the LBD or the C-terminus differed from the WT (Figure 4A).

Relating our functional study results to the clinical phenotype of the patients, only two out of nine variants of patients with a severe phenotype showed impaired transactivation activity (p.Cys13Ser and p.Arg39Leu), and only one (p.Cys13Ser) affected the nuclear

translocation. Similarly, only two of six variants of patients with opposite sex had severely impaired transactivation activity (Figure 3B) and affected nuclear translocation (p.Cys73Tyr and p.Cys73Trp) (Figure 4B). By contrast, *NR5A1/*SF-1 variants of individuals with typical female or mild phenotypes showed similar transactivation activity and nuclear translocation as WT (Figure 3B and 4B).

Taken together, we found that the DSD phenotypes of the individuals, pathogenicity prediction and ACMG classification of the related *NR5A1/*SF-1 variants and results of the *in vitro* functional assessments aligned only in four out of the 17 studied variants. *NR5A1*/SF-1 variants p.Cys13Ser, p.Arg39Leu, p.Cys73Tyr and Cys73Trp harboured by patients with a severe or an opposite sex phenotype were all classified as either pathogenic or likely pathogenic (Table 2), and *in silico* tools predicted them as either pathogenic, probably damaging or disease causing (Supplementary table 4)(42). In addition, these predictions were confirmed by both functional *in vitro* assays. By contrast, variants p.Pro14Ser, p.Gly17Val, p.Phe70Leu and p.Cys73Ser, also found in patients with a severe or an opposite sex phenotype, were also all classified and predicted as (likely) pathogenic (Table 2 and Supplementary table 4)(42), but in these cases functional assays failed to confirm a disease-causing effect. Furthermore, variants located in the LBD were almost all classified as VUS and *in silico* as well as *in vitro* studies showed diverse results not aligning to each other (Figures 3A and 3B).

## Discussion

*NR5A1*/SF-1 variants are associated with unexplained broad DSD phenotypes (14, 15, 17, 31). This is also reflected in the *SF1next* study cohort comprising 197 individuals with novel and known *NR5A1*/SF-1 variants (41). Here, we characterized the novel *NR5A1*/SF-1 variants identified in this cohort by established *in silico* and *in vitro* methods and reviewed the corresponding literature for known variants. Our review revealed that although most reported variants were classified as (likely) pathogenic and were predicted to disrupt SF-1 protein structure, only some variants, mostly located in the DBD, had impaired transactivation activity on different promoter reporters in several cell models

(Supplementary tables 1 and 2) (16, 19, 32, 42, 45). Similarly, only a few of the 17 novel *NR5A1*/SF-1 variants tested in our study showed impaired transcriptional activation activity and affected SF-1 nuclear translocation. These few variants were located in the DBD and the corresponding phenotype was severe or opposite sex. However, for most of the individuals with DSD who had *NR5A1*/SF-1 variants, *in silico* predictions and results from *in vitro* testing did not align with the phenotype. Thus, a clear genotype-phenotype, structure-function correlation remains elusive for *NR5A1*/SF-1 variants.

So far more than 260 *NR5A1/*SF-1 variants located in all regions of the SF-1 protein have been described in 46,XY and 46,XX individuals, presenting healthy or with variable severity of DSD (15). In our study, clinical characteristics of the individuals with novel heterozygous *NR5A1*/SF-1 variants were also variable, but most had a 46,XY karyotype and a severe DSD. In line with other reports (15, 16), severity of the phenotype did not correlate with specific *NR5A1/*SF-1 variants. Missense, frameshift, or synonymous *NR5A1*/SF-1 variants were observed in individuals with a severe DSD phenotype (Figure 1). It is important to realize that the reported *NR5A1*/SF-1 variants in our studied patients may not explain the DSD phenotype at all or only in combination with other genetic variants. Thus, further genetic testing in such patients is advised.

ACMG classification (10) of the novel *NR5A1*/SF-1 variants identified in the *SF1next* study cohort suggested a pathogenic or likely pathogenic impact for 2 out of 3 variants (23/35). Novel variants located in the DBD were predicted (likely) pathogenic, while variants located in the LBD were mostly predicted VUS and B. However, corresponding functional experiments revealed mixed results and an alignment of data was only found for variants p.Cys13Ser, p.Arg39Leu, p.Cys73Tyr and p.Cys73Trp located in the DBD. Similar results have been reported in the literature indicating that there is no clear genotype-phenotype correlation for *NR5A1*/SF-1 variants (19, 20, 32, 38, 46). Guidelines recommend assessing pathogenicity of missense variants by *in silico* prediction methods and functional tests (10), but currently used test methods may not reveal a clear answer.

In our study, prediction software programs for gene variants classified most *NR5A1*/SF-1 variants more accurately as (likely) pathogenic, while functional assays were less predictive.

Similar results were obtained by protein structure-based prediction of pathogenicity for the 17 novel missense *NR5A1/*SF-1 variants where almost all variants located in the DBD were suggested pathogenic or VUS, but aligned with the *in vitro* functional assays in less than 1 in 3 cases (5/17).

Functional testing is recommended for variant classification (10), but after reviewing the related literature originating from numerous research groups, we conclude that established *in vitro* assays for assessing the activity of *NR5A1*/SF-1 variants are in doubt (Supplementary table 1 and 2) (19, 20, 25, 32, 38, 42, 46-48). In some studies, functional studies were able to provide clear experimental evidence for a disease-causing effect of tested *NR5A1*/SF-1 variants, while others were inconclusive showing mixed correlative results between and within studies for different variants for no obvious experimental reasons. Thus, false-negative or false-positive results could be suspected for maybe missing factors in the experimental models used. Overall, in reported studies and in our study, functional tests were most predictive for *NR5A1*/SF-1 variants located in the DBD of the SF-1 protein (Supplementary tables 1 and 2)(42). The DBD of SF-1 is a highly conserved domain among species which comprises two zinc finger (ZNI and ZNII) domains essential for the recognition of the DNA target sequences (16, 49). In our study, the novel *NR5A1/*SF-1 variants p.Cys13Ser, p.Cys73Tyr and p.Cys73Trp located in the ZN finger domains had reduced activity and these variants affect important cysteine residues involved in the *NR5A1/*SF-1 binding to the recognition sites. The p.Arg39Leu variant also showed activity loss and is located in the hinge region that links the zinc fingers and is involved in stabilizing the non-specific contacts across the DNA minor groove (16, 49). However, the novel *NR5A1*/SF-1 p.Pro14Ser, p.Gly17Val and p.Cys73Ser variants, which are also located in the zinc finger domains and also manifested with a severe or opposite sex phenotype, showed unaffected transactivation

activity and nuclear translocation, and these results remain unexplained. In our study, we only used the *-152CYP11A1* promoter luciferase reporter construct in non-steroidogenic HEK293 cells. SF-1 targets many genes during sex determination and differentiation, therefore using additional promoters, such as SOX9 or AMH, and different cell lines, might be helpful to explain a particular phenotype caused by a concrete *NR5A1*/SF-1 variant. The appropriate functional study should be chosen based on the phenotype of the patient to obtain an accurate genotype-phenotype correlation. However, previous studies including several promoters and cell lines also show heterogeneous results (16).

Thus, something is clearly missing when it comes to understanding the mechanism of disease related to *NR5A1/*SF-1 variants and the broad spectrum of DSD. Several hypotheses have been put forward over the years including genetic and environmental factors affecting *NR5A1/*SF-1 expression, activity and degradation, as well as overlooked co-factors of SF-1 or other genes working in networks together with *NR5A1/*SF-1 to reveal a DSD phenotype by oligogenic mechanisms (15, 40, 50, 51). Evidence for involvement of several of these factors in SF-1-related DSD has been reported by many studies. Here we mention only few. Recently, variants in non-coding promoter regions of *NR5A1/*SF-1 have been reported in 3 patients with 46,XY DSD (39). *In vitro* analyses showed that promoter activity was affected in all cases. WES in two of the patients also revealed additional variants in *SRA1, WWOX*, and *WDR11* genes with potential impact on the DSD phenotype (39). Dominant negative effect was presumed initially, as most DSD individuals carry heterozygous *NR5A1/*SF-1 variants, but corresponding *in vitro* experiments did not confirm a dominant negative effect (16, 18). Variable allelic expression due to imbalanced cis-regulation of mutant versus wild-type alleles could also explain variable expressivity of a phenotype when mutations are present in a heterozygous state (15). In fact, it has been shown that complex modes of allelic expression are implied in development and pathologies, including autosomal dominant disorders (52) but to our knowledge, this gene-specific theory has not been tested in *NR5A1*/SF-1 variants so far.

By contrast, many studies have reported oligogenicity as a mechanism to explain the broad and inter-individual and intra-familial variable DSD phenotypes associated with *NR5A1/*SF-1 variants (2, 31, 50). Newer parallel sequencing strategies have facilitated the identification of gene variants in individuals with DSD, but they have also revealed that healthy individuals carry many variants and informed that the genetics explaining an atypical sex development might be complex (2, 3, 50). While some DSD may be explained by monogenic variants, others may be caused by oligogenic variants in interacting genes. Accordingly, we and others have described several patients with a DSD who have *NR5A1*/SF-1 variants in whom other likely disease-causing additional gene variants were found (17, 53-55). However, so far mechanistic confirmation in these cases is missing, as it is very difficult to assess the disease-contribution of each variant contained in a complex, multi-gene network where the effect of the single variant might be mild. Thus, identification of additional genetic hits in individuals with a DSD poses large challenges for distinguishing between disease-causing variants and variants that do not contribute to the phenotype. In the future, human tissue-derived models such as organoids or *in vitro* cellular reprogramming of pluripotent stem cells (iPSC) may enable studies of oligogenic mechanism as the patient-derived material contains the individual genetic background (56-58).

In this study, nine patients were reported to have additional gene variants (Table 2 and Methods). Five patients (patients 4, 8, 21, 35 and 39) harboured additional genetic hits in genes related to sex development and differentiation (e.g. *AMH, SRD5A2*, *DHX37*), steroidogenesis (e.g. *PO*R) and hypogonadotropic hypogonadism (e.g. *SOX10*) (8, 17, 59-62). On the other hand, patients 10 and 25 were found to have 16 and 2 additional variants, respectively, in genes that have not been related to DSD so far. The clinical relevance of copy number variants in patients 15 and 16 has been described before (63). Additional gene variants reported in this study have been achieved through different NGS methods, in different laboratories using different algorithms to annotate the variants of interest. Therefore, subsequent data analysis depended on the criteria of the researcher from the corresponding laboratory and clarification of the role of some of these gene

variants in the pathogenesis of DSD is missing. With the increasing use of NGS methods for the molecular diagnosis of individuals with a DSD, it is expected that more patients with multiple gene variants will be identified, which may not be deleterious alone but may contribute to the observed DSD phenotype when occurring in combination with a heterozygous *NR5A1*/SF-1 variant. If so, the genotype−phenotype correlation will depend on the specific combinatory effect of the involved genetic variants and will be unique in many cases. In addition, this will not only be true in DSD patients carrying *NR5A1*/SF-1 variants but also for DSD related to other genetic variants.

In conclusion, we characterized 35 novel *NR5A1*/SF-1 variants identified in individuals with a DSD and their family members of the international *SF1next* study cohort. Protein structure analyses and functional studies were performed for 17 novel missense variants. We found that current genetic analyses and functional assays for studying novel variants of *NR5A1/*SF-1 frequently do not explain the observed phenotype. In nine individuals, additional likely disease-causing variants in other genes were found, strengthening the hypothesis that the broad phenotype of DSD with *NR5A1*/SF-1 variants may be caused by an oligogenic mechanism.

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## Data Availability Statement

Access to basic data is possible through the international I-DSD registry; general rules apply (https://sdmregistries.org/about/). Additional data were collected in a project-specific REDCap database governed by the Clinical Trials Unit (CTU) at University of Bern, Switzerland. Genetic data are also stored on servers of the University of Bern. These data can also be accessed upon reasonable request according ethical and informed consent.

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## Tables

**Table 1.**

Clinical characterization of the DSD patients harbouring novel *NR5A1/*SF-1 variants identified by the SF1next study.

**Table 2.** Genetic characterization of the novel *NR5A1/*SF-1 gene variants and of additional variants identified in related genes in combination.

## Figure legends

**Figure 1.** Novel *NR5A1/*SF-1 variants identified in 39 DSD patients included in the *SF1next* study. For each patient, pathogenicity prediction of the novel *NR5A1/*SF-1 variant, method of genetic workup and information on additional gene variants that have been identified, is represented. Severity of the DSD phenotype of each patient harbouring a novel *NR5A1/*SF-1 variant is also indicated on the y-axis.

**Figure 2**. Protein localization and conservation across species of the newly identified human *NR5A1/*SF-1 missense variants. Shown is a multiple alignment of parts of the SF-1 protein sequences across different species. Localization of the newly identified human amino acid variants are given in bold and seem highly conserved across different species.

**Figure 3.** Transcriptional activity studies of 17 novel missense *NR5A1*/SF-1 variants. The ability of wild-type (WT) and mutant *NR5A1/*SF-1 to activate the promoter of the *CYP11A1* gene was tested in non-steroidogenic HEK293T cells. Cells were transiently transfected with *NR5A1/*SF-1 expression vectors and the *-152CYP11A1* promoter luciferase reporter construct. **A.** Activity of *NR5A1/*SF-1 variants is shown with respect to the ACMG pathogenicity classification (43) and the localization of the variants in the protein structure. **B.** Activity of the *NR5A1/*SF-1 variants is shown with respect to the ACMG pathogenicity classification (43) and according to the phenotype of affected individuals. Luciferase activity was measured with the Dual Luciferase assay system

(Promega). Results are shown as the mean ± standard error of the mean (SEM) of three to four independent experiments, all performed in duplicate. *, p<0.05; **, p<0.005; ***, p<0.001. RLU, relative light units.

**Figure 4.** Nuclear translocation studies of 17 novel missense *NR5A1*/SF-1 variants. Western blot showing cytoplasmic and nuclear localization of wild-type (WT) and mutant HA tagged-SF-1 proteins. HEK293T cells were transiently transfected with WT and mutant *NR5A1/*SF-1 expression vectors for 48 hours. Cytoplasmic and nuclear protein fractions were separated and probed by Western blots. An anti-HA antibody was used to detect the *NR5A1/*SF-1 protein at 53kDa. β-actin (42 kDa) was used as loading control, Rab11(24 kDa) was used as a cytoplasmic protein marker and Lamin B1 (66 kDa) as a nuclear protein marker. The intensity of the HA tagged- *NR5A1/*SF-1 and β-actin bands was quantified by using the FUSION FX6 EDGE Imaging System. Data are expressed as percentage of HA tagged- *NR5A1/*SF-1 cytoplasmic (in white) and nuclear (coloured) fraction of total protein, normalized to β-actin. Results from two independent experiments are presented as mean ± SEM. **A.** Results are shown according to localization of variants in the *NR5A1/*SF-1 protein. **B.** Results shown with respect to the corresponding DSD phenotypes.

Table 1. Clinical characterization of the DSD patients harbouring novel *NR5A1*/SF-1 variants identified by the SF1next study.

| Patient | Karyotype | DSD phenotype classification[a] | Sex assignment/ reassignment | Current or last age of assessment/Clinical description | Other organ anomalies/Cancer | Family history |
|---|---|---|---|---|---|---|
| 1 | 46,XY | Severe | M/No | 1y, micropenis (10-20mm), perineal hypospadias, labioscrotal testes, posterior fused labioscrotum. Cytoscopy: Mullerian remnants. Masculinizing genitoplasty and orchidopexy. 6y, penis (>30mm), coronal hypospadias. 10y, testes 4mL, gynecomastia, Tanner: Genitals II, pubic hair II. | No/No | |
| 2 | 46,XY | Severe | F | 1y, perineal hypospadias, labioscrotal gonads, posterior fused labioscrotum. | Flat nasal root; epicanthus/No | |
| 3 | 46,XY | Severe | M | 1y, micropenis (10-20mm), scrotal hypospadias, labioscrotal testes, posterior labioscrotum fusion. Masculinizying genitoplasty (1y, 1y, 5y, 6y). | No/No | Mother[b]: pregnancy achieved by ART |
| 4 | 47,XXY | Opposite sex | F | 16y, female external genitalia. MRI: normal uterus and gonads. Spontaneous start of puberty, no menarche, Tanner: genitals III, breast IV, pubic hair IV. 17y, biopsy of right gonad, at histology: atrophic seminiferous tubules and testicular tissue, Leydig cell proliferation. | No/No | Father[b]: azoospermia; Cousin: menstrual irregularities. |
| 5 | 46,XY | Severe | M | 6m, perineal hypospadias, inguinoscrotal gonads. 3y, orchidopexy and masculinizing genitoplasty. 14y, penis (21-30mm), coronal hypospadias, testes (4-5mL), Tanner: genitals I, pubarche I. Spontaneous start of puberty. | No/No | |
| 6 | 46,XY | Severe | M | At birth, micropenis (<10mm), penoscrotal hypospadias, labioscrotal gonads. 2y, coronal hypospadias. Masculinizing genitoplasty (1y, 2y). | No/No | Mother[b]: POI (39y) |
| 7 | 46,XY | Severe | Other/M | 1m, penis (<10mm), penoscrotal hypospadias, labioscrotal testes. US: rudimentary uterus. Reassigned sex to male. 2y, orchidopexy. 5y, typical male meatal opening and 10-20mm penis. 12y, testes 2-4mL, Tanner: genitals IV and pubarche IV. US: uterus is absent. | Brachymetatarsia and brachymetacarpia; long-jointed hands and feet; decreased muscle mass/No | Mother[b]: hyperextensibility; sister[b]: CGD. |

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|---|---|---|---|---|---|---|
| 8 | 46,XY | Severe | M | 2m, micropenis (10-20mm), penoscrotal hypospadias, labioscrotal testes. Masculinizing genitoplasty (2m, 2y). 8y, penis >30mm. 11y, Testes 6-8mL, Tanner: genitals II and pubarche I. 16y, Tanner: genitals V and pubarche V. Testes 15-20mL. Spontaneous start of puberty. | No/No | |
| 9 | 46,XY | Opposite sex | F | 7y, inguinoscrotal gonads. US: normal uterus and streak gonads. 10y, bilateral gonadectomy. | No/No | |
| 10 | 46,XY | Severe | F | 2y, perineal hypospadias, unfused labioscrotum. US: rudimentary uterus. | Urogenital sinus; 2y, development delay; 4y, epilepsy, severe mental retardation/No | |
| 11 | 46,XY | Severe | M | 2y, perineal hypospadias, impalpable and inguinal testes. Masculinizing genitoplasty. | No/No | Father[b]: urogenital sinus, hypospadias, cryptorchidism; brother[b]: hypospadias, unilateral cryptorchidism; grandfather: hypospadias. |
| 12 | 46,XY | Severe | M | 3y, micropenis (21-30mm), penoscrotal hypospadias, labioscrotal and inguinal testes. 3y, masculinizing genitoplasty. 9y, penis >30mm, coronal hypospadias. 12y, testes 2mL, Tanner: genitals III, pubarche III. | Hb and reticulocytes above normal range, thrombocytosis/No | |
| 13 | 46,XY | Mild | M | 2y, coronal hypospadias, labioscrotal testes, 21-30mm penis, fused labioscrotum. US: uterus is absent. Masculinizing genitoplasty (1y, 2y). 7y, typical male meatal opening, inguinoscrotal gonads, penis >30mm. | No/No | Father[b]: hypospadias |
| 14 | 46,XY | Typical | M | 11y, penis (>30mm), scrotal testes (2mL), gynecomastia, Tanner: genital I, pubic hair II. | Elevated insulin/No | |
| 15 | 46,XY | Severe | M | 9m, micropenis (21-30mm), penoscrotal hypospadias, inguinal testes, unfused labioscrotum. Masculinizing genitoplasty (1y, 2y, 3y). 6y, penis (>30mm), labioscrotal gonads. | No/No | |
| 16 | 46,XY | Severe | M | 1y, micropenis (10-20mm), penoscrotal hypospadias, labioscrotal testes, unfused labioscrotum. Masculinizing genitoplasty (1y, 2y). | Ventricular septum defect/No | |

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|---|---|---|---|---|---|---|
| 17 | 46,XY | Severe | M | 11m, micropenis (21-30mm), penoscrotal hypospadias, labioscrotal testes. Masculinizing genitoplasty (1y, 2y). 8y, penis >30mm. Masculinizing genitoplasty. Orchidopexy. 10y, testes 3mL, Tanner: genital III, pubic hair IV. | No/No | |
| 18 | 46,XY | Severe | F | 1y, micropenis (10-20mm), perineal hypospadias, labioscrotal testes, posterior labioscrotal fusion. Feminizing genitoplasty. Bilateral gonadectomy, at histology: normal for Karyotype. 16y, Tanner: breast V. Induction of puberty. | No/No | |
| 19 | 46,XY | Severe | M | 11m, micropenis (21-30mm), perineal hypospadias, labioscrotal and inguinoscrotal testes. Orchidopexy and masculinizing genitoplasty (2y). | No/No | |
| 20 | 46,XY | Mild | M | At infancy, orchidopexy and masculinizing surgeries for hypospadias, cryptorchidism and micropenis. 4y, penis (21-30mm), labioscrotal testes. Masculinizing genitoplasty. 11y, penis (>30mm), testes 4.5mL, Tanner: genital II and pubic hair II. | No/No | |
| 21 | 46,XY | Opposite sex | F | 16y, typical female external genitalia MRI: hypoplastic uterus and testes. Tanner: Breast II; pubarche V. Sponaneous start of puberty: Bilateral gonadectomy, at histology: testis with primitive seminiferous tubules. Sertoli cells only and occasional spermatogonia. Vaginal hypoplasia. | No/No | |
| 22 | 46,XY | Opposite sex | F | 15y, external female genitalia, clitoromegalia (>30mm). Tanner: breast I, pubarche III. At imaging: hypoplastic uterus and small testes. Vaginal hypoplasia. 17y, induction of puberty. Bilateral gonadectomy, at histology: bilateral testicular structures and fallopian tubes. | No/No | Father[b]: micropenis, hypospadias, left ventricular non compaction. |
| 23 | 46,XY | Opposite sex | F | 1y, female external genitalia. US: normal uterus. | No/No | Father[b]: hypospadias, oligospermia, ART for conception. |
| 24 | 46,XY | Severe | F | 3y, micropenis (10-20mm), penoscrotal hypospadias, labioscrotal testes, posterior labioscrotal fusion. Feminizing genitoplasty. | No/No | |

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| 25 | 46,XX | Typical | F | 12y, typical female external genitalia. MRI: abnormal uterus. Tanner: breast III; pubic hair IV. 20y, spontaneous start of puberty. Tanner: breast V; pubic hair V. No menarche. US: normal uterus and gonads. | Anemia; skoliosis, dislocation of the hip; muscle weakness, spastic tetraparesis; wheelchair bound, ataxia; pachygyria; epilepsy, tetraparesis/No | |
| 26 | 46,XY | Opposite sex | F | 1y, penis (21-30mm), typical female meatal opening, impalpable testes, unfused labioscrotum. Laparoscopy: mullerian remnants. | No/No | |
| 27 | 46,XY | Severe | M | 10m, micropenis (21-30mm), perineal hypospadias, inguinal gonads, posterior labioscrotal fusion. | No/No | |
| 28 | 46,XY | Severe | M | 8m, micropenis (10-20mm), perineal hypospadias, labioscrotal testes, posterior labioscrotal fusion. | Abnormal morphology spleen, poikilocytes, giant thrombocytes, thrombocytosis, no HJB, no PRBC/No | |
| 29 | 46,XY | Severe | M | At birth, micropenis (10-20mm), penoscrotal hypospadias, labioscrotal testes. Masculinizing genitoplasty. | No/No | ND |
| 30 | 46,XY | Opposite sex | F | 12y, typical female external genitalia. US: hypoplastic uterus and abnormal testes. Bilateral gonadectomy, at histology: Left gonad with atrophic testis tissue and small amount of Sertoli cells; right gonad only epididymis. Induction of puberty. 17y, Tanner: breast V and pubic hair V. | No/No | |
| 31 | 46,XY | Severe | F/M | 10y, penoscrotal hypospadias, impalpable gonads. US: right teste 5mL; left is unknown. Spontaneous start of puberty, Tanner: genitals II; pubic hair IV. Biopsy (right gonad): Leydig cell hyperplasia, focal testes atrophy, mature spermatogenesis. Masculinizing genitoplasty (1y, 2y). Reassigned sex to male. 13y, labisocrotal testes (7-9mL) , Tanner: breast III; pubic hair V. | Accelerated bone age; pain while micturation/No | |
| 32 | 46,XY | Severe | M | 3y, micropenis (21-30mm), scrotal hypospadias, labioscrotal testes. US: Mullerian remnants. 5y, penis (>30mm), perineal hypospadias. Masculinizing genitoplasty (5y, 6y). 12y, testes 4-5mL, Tanner: breast | Abnormal morphology spleen, mild thrombocytosis, | |

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| | | | | I, genital III, pubic hair I. Spontaneous start of puberty. | elevated reticulocytes/No | |
| 33 | 46,XY | Severe | F | 1y, micropenis (10-20mm), typical female meatal opening, inguinoscrotal gonads, posterior labioscrotal fusion. Feminizing genitoplasty and bilateral gonadectomy. 11y, vaginal hypoplasia, Tanner: breast III, pubic hair III. Spontaneous start of puberty. | Abnormal morphology spleen/No | |
| 34 | 46,XY | Severe | M | 3y, penoscrotal hypospadias, inguinoscrotal testes. Masculinizing genitoplasty (1y, 2y, 3y). | No/No | |
| 35 | 46,XY | Opposite sex | F | 12y, typical female meatal opening, inguinal testes, penis (>30mm), hirsutism. Tanner: pubic hair V. Spontaneous start of puberty. | Accessory spleen/No | |
| 36 | 46,XY | Opposite sex | F | 13y, typical female external genitalia. MRI: hypoplastic uterus and streak ovaries. Spontaneous start of puberty. Tanner: breast III; pubic hair V. | No/No | Mother: POI (25y), premature menopause (37y). |
| 37 | 46,XY | Severe | F | 4m, micropenis (10-20mm), scrotal hypospadias, labioscrotal testes. Orchidopexy and masculinizing genitoplasty (1y, 2y). | No/No | Father[b]: micropenis |
| 38 | 46,XX | Severe | M | 34y, penoscrotal hypospadias, inguinal (left, 20mL) and labioscrotal (right, 4mL) testes, posterior labioscrotal fusion, gynecomastia, Tanner: breast III, genitals V. MRI: abnormal uterus, US: abnormal/small testes. Genitoplasty. Left gonad biopsy, at histology: Sertoli only, Leydig cell hyperplasia, ovarian tissue with corpora albicantia and follicles in epididymis. | No/No | |
| 39 | 46,XY | Severe | M | 1y, micropenis (10-20mm), penoscrotal hypospadias, inguinal gonads. Masculinizing genitoplasty (3y) and orchidopexy (4y). 5y, micropenis (21-30mm), coronal hypospadias, labioscrotal gonads, . | Intracranial cyst/No | Father[b]: T1D; brother: penoscrotal hypospadias, labioscrotal/inguinal gonads, developmental delay, quadriplegic cerebral palsy |

M, male: F, female; ART, assisted reproductive technology; CGD, complete gonadal dysgenesis; m, month; MRI, magnetic resonance imaging; POI, primary ovarian insufficiency; T1D, type 1 diabetes; US, ultrasound; y, years. [a]DSD classification according to the severity of the phenotype of external genitalia[b]. [41]
Relatives with a confirmed *NR5A1*/SF-1 gene variant.

Table 2. Genetic characterization of the novel *NR5A1*/SF-1 gene variants and of additional variants identified in related genes in combination.

| Patient | Chromosome position | Gene | Variant | Zygosity | Previously reported | ACMG classification (criteria) (43) | Family studies | Method |
|---|---|---|---|---|---|---|---|---|
| **1** | 9:127265637 | *NR5A1* | c.35_38dup; p.Pro14Valfs*19 | Het | ND | LP (PVS1, PM2) | F: ND; M: wt | WES |
| 2* | 9:127265638 | *NR5A1* | c.37T>A; p.Cys13Ser | Het | ND | LP (PP3, PM1, PM2) | ND | SGA |
| 3* | 9:127265635 | *NR5A1* | c.40C>T; p.Pro14Ser | Het | ND | VUS (PM1, PM2, PP3) | F: ND; M: het | SGA |
| **4*** | 9:127265625 | *NR5A1* | c.50G>T; p.Gly17Val | Het | ND | LP (PP3, PM1, PM2) | F: het; M: wt | TGP |
| | 19:2249631 | *AMH* | c.300C>T; p.Phe100= | Het | ND | B (BS1, BS2, BP4, BP6, BP7) | F: wt; M: het | |
| **5*** | 9:127265486 | *NR5A1* | c.116G>T; p.Arg39Leu | Het | 46,XY DSD (64) | LP (PP3, PM1, PM5, PM2) | F: ND; M: het | TGP |
| **6*** | 9:127265394 | *NR5A1* | c.208T>C; p.Phe70Leu | Het | ND | VUS (PM1, PM2, PP3) | F: ND; M: het | WES |
| 7* | 9:127265385 | *NR5A1* | c.217T>A; p.Cys73Ser | Het | ND | P (PM5, PP3, PM1, PM2) | F: wt; M: het; S: het | SGA |
| **8*** | 2:31805706 | *SRD5A2* | c.265C>G; p.Leu89Val | Hom | Prostate cancer (65) | B (BA1, BP6, BP4) | ND | TGP |
| | 9:127265384 | *NR5A1* | c.218G>C; p.Cys73Ser | Het | ND | P (PM5, PP3, PM1, PM2) | F: het; M: wt | |
| **9*** | 9:127265384 | *NR5A1* | c.218G>A; p.Cys73Tyr | Het | ND | P (PP3, PM1, PM5, PM2, PP5) | F: wt; M: wt | WES |
| **10*** | 1:43895417 | *SZT2* | c.4039C>T; p.Arg1347Cys | Het | ND | LB (BP4, BP1, PM2) | ND | WES |
| | 2:73677990 | *ALMS1* | c.4207A>G; p.Thr1403Ala | Het | ND | LB (BP4, BP1, PM2) | ND | |
| | 3:15686753 | *BTD* | c.1330G>C; p.Asp444His | Het | Biotinidase activity (66) | VUS (PP5, PM2) | ND | |
| | 3:48508260 | *TREX1* | c.206T>C; p.Leu69Pro | Het | ND | VUS (PP3, PM2) | ND | |
| | 3:58415529 | *PDHB* | c.701-3C>T | Het | ND | LB (BP4, PM2) | ND | |
| | 5:82815537 | *VCAN* | c.1412C>T; p.Thr471Met | Het | ND | B (BS1, BS2, BP1, BP4) | ND | |
| | 6:152650875 | *SYNE1* | c.14732G>A; p.Arg4911His | Het | ND | LB (BP4, BP1, PM2) | ND | |
| | 6:157099607 | *ARID1B* | c.369_392dup; p.Gln124_Gln131dup | Het | ND | B (BS1, BS2) | ND | |
| | 7:103341394 | *RELN* | c.865A>G; p.Asn289Asp | Het | ND | VUS (PM2, BP1) | ND | |
| | 9:127265383 | *NR5A1* | c.219C>G; p.Cys73Trp | Het | ND | P (PP3, PM1, PM5, PP5, PM2) | F: wt; M: wt | |
| | 10:28250492 | *ARMC4* | c.1386+5G>A | Het | ND | VUS (PP3, PM2) | ND | |
| | 11:103029673 | *DYNC2H1* | c.4295T>C; p.Ile1432Thr | Het | ND | VUS (PP3, PM2) | ND | |
| | 11:124794912 | *HEPACAM* | c.139G>A; p.Val47Met | Het | ND | VUS (PM1, PM2, BP4) | ND | |
| | 14:88407888 | *GALC* | c.1685T>C; p.Ile562Thr | Het | Krabbe disease (67) | B (BA1, BP6, BP4) | ND | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 15:28259941 | *OCA2* | c.1025A>G; p.Tyr342Cys | Het | Ocular albinism (68) | LP (PP3, PM2, PP5) | ND | |
| | 17:78087041 | *GAA* | c.2065G>A; p.Glu689Lys | Het | Alpha-glucosidase activity (69) | B (BA1, BP6, BP4) | ND | |
| | 21:44837615 | *SIK1* | c.1784G>A; p.Arg595Gln | Het | ND | LB (BP1, BP4, PM2) | ND | |
| **11** | 9:127265357 | *NR5A1* | c.244+1G>T | Het | ND | P (PVS1, PM2, PP5) | F: het; M: wt; Br: Het | WES |
| **12*** | 9:127262992 | *NR5A1* | c.247G>A; p.Val83Met | Het | 46,XY DSD (70, 71) | VUS (PM1, PP3, PM2) | ND | TGP |
| **13*** | 9:127262992 | *NR5A1* | c.247G>T; p.Val83Leu | Het | 46,XY DSD (72) | VUS (PM1, PP3, PM2) | F: het; M: wt | TGP |
| 14 | 9:127262866 | *NR5A1* | c.370_373del; p.Pro124Argfs*171 | Het | ND | LP (PVS1, PM2) | F: wt; M: wt | SGA |
| 15 | | | 2p16.3p16.3 (50732444-50894316)x1 | | 46,XY DSD (63) | VUS (PM2) | ND | SGA/Array |
| | 9:127262846 | *NR5A1* | c.393G>A; p.Pro131= | Het | ND | LB (BP4, BP7, PM2) | ND | |
| | | | 16p13.11p13.11(15830681-16270149)x3 | | 46,XY DSD (63) | VUS (PM2) | ND | |
| 16 | 9:127262846 | *NR5A1* | c.393G>A; p.Pro131= | Het | ND | LB (BP4, BP7, PM2) | ND | SGA/Array |
| | 9:127262802 | *NR5A1* | c.437G>C; p.Gly146Ala | Het | Adrenal disease (26) | B (BA1, BP6, BS3, BP4, PM1) | ND | |
| | | | Xq13.3.3q13.3(74380482-74567915)x2 | Het | 46,XY DSD (63) | VUS (PM2) | F: wt; M: het | |
| 17 | 9:127262846 | *NR5A1* | c.393G>A; p.Pro131= | Het | ND | LB (BP4, BP7, PM2) | ND | SGA |
| **18** | 9:127262687 | *NR5A1* | c.552del; p.Tyr185Thrfs*111 | Het | ND | LP (PVS1, PM2) | F: wt; M: wt | WES |
| 19 | 9:127262684 | *NR5A1* | c.555C>A; p.Tyr185* | Het | ND | LP (PVS1, PM2) | ND | SGA |
| 20 | 9:127262607 | *NR5A1* | c.632_668del; p.Tyr211Cysfs*73 | Het | ND | LP (PVS1, PM2) | F: ND; M: het | SGA |
| **21*** | 7:75612866 | *POR* | c.859G>C; p.Ala287Pro | Het | Disordered steroidogenesis (73) | P (PS3, PP5, PP3, PM2, BP1) | ND | TGP |
| | 9:127262559 | *NR5A1* | c.680T>C; p.Ile227Thr | Het | 46,XY DSD (72) | VUS (PM1, PM2) | F:het; M: ND | |
| **22*** | 9:127262400 | *NR5A1* | c.839C>A; p.Ala280Glu | Het | 46,XY DSD (72, 74) | LP (PP3, PM1, PM2) | F: mosaic; M: ND; S: het | TGP |
| **23** | 9:127255373 | *NR5A1* | c.926A>T; p.Asp309Val | Het | ND | LP (PP3, PM1, PM2) | F: het; M: wt | WES |
| **24** | 9:127255353 | *NR5A1* | c.946del; p.Gln316Serfs*18 | Het | ND | LP (PVS1, PM2) | ND | WES |
| **25*** | 4:126329821 | *FAT4* | c.686A>G; p.Tyr229Cys | Het | ND | VUS (PM2, BP1) | ND | WES |
| | 4:126372555 | *FAT4* | c.5278A>G; p.Ile1760Val | Het | ND | LB (BP4, BP1, PM2) | ND | |
| | 9:127255322 | *NR5A1* | c.977T>C; p.Val326Ala | Het | ND | VUS (PM1, PM2, PP3) | ND | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 26 | 9:127255314 | *NR5A1* | c.985C>T; p.Gln329* | Het | ND | LP (PVS1, PM2) | F: wt; M: wt | SGA |
| **27** | 9:127255314 | *NR5A1* | c.985C>T; p.Gln329* | Het | ND | LP (PVS1, PM2) | ND | TGP |
| **28** | 9:127253508 | *NR5A1* | c.991-1G>A | Het | ND | LP (PVS1, PM2) | F: wt; M: wt | WES |
| **29*** | 9:127253506 | *NR5A1* | c.992T>G; p.Val331Gly | Het | ND | VUS (PM1, PP3, PM2) | ND | TGP |
| 30 | 9:127253415 | *NR5A1* | c.1065_1066insTGCTGCAGCTGCTTGCGCTGG;p.Val355_Leu356insCysCysSerCysLeuArgTrp | Het | ND | LP (PM1, PM4, PM2) | ND | SGA |
| 31* | 9:127253393 | *NR5A1* | c.1105G>T; p.Val369Phe | Het | ND | VUS (PM1, PP3, PM2) | ND | SGA |
| 32 | 9:127253389 | *NR5A1* | c.1106_1109del; p.Val369Alafs*12 | Het | ND | LP (PVS1, PM2) | ND | SGA |
| 33 | 9:127245036 | *NR5A1* | c.(1138+1_1139-1)_(1386+1_1387-1)del; p.Asp380_Thr461del | Het | ND | P (PVS1, PM2) | ND | SGA |
| **34** | 9:127245286 | *NR5A1* | c.1139-2A>G | Het | ND | LP (PVS1, PM2) | ND | TGP |
| **35** | 9:127245211 | *NR5A1* | c.1157_1211dup; p.Tyr404* | Het | ND | LP (PVS1, PM2) | ND | TGP |
| | 12:125460041 | *DHX37* | c.904G>A; p.Gly302Ser | Het | ND | VUS (PP3, PM1, PM2) | ND | |
| **36*** | 9:127245212 | *NR5A1* | c.1211A>G; p.Tyr404Cys | Het | ND | LP (PP3, PM1, PM5, PM2) | ND | TGP |
| **37** | 9:127245116 | *NR5A1* | c.1307A>G; p.Tyr436Cys | Het | ND | VUS (PM1, PP3, PM2 ) | F: het; M: wt | TGP |
| **38** | 9:127245070 | *NR5A1* | c.1353G>A; p.Leu451= | Het | ND | B (BS1, BS2, BP6, BP6, BP7) | ND | TGP |
| **39*** | | | 2p16.3 dup | | | ND | F: wt; M: het; Br: het | TGP |
| | 9:127245044 | *NR5A1* | c.1379A>T; p.Gln460Leu | Het | 46,XY DSD (75) | LB (BP4, PM1, PM2) | F: het; M: wt; Br: het | |
| | 22:38369662 | *SOX10* | c.1241A>C; p.His414Pro | Het | | LB (BS2, PP3) | ND | |
| | 22:38369619 | *SOX10* | c.1284G>T; p.Met428Ile | Het | | LB (BS2) | ND | |

B, benign; Br, brother; F, father; Het, heterozygous; Hom, homozygous; LB, likely benign; LP, likely pathogenic; M, mother; ND, not determined; P, pathogenic; S, sister; SGA, single gene analysis; TGP, targeted-gene panel; VUS, variant of unknown significance; WES, whole-exome sequencing. Individuals in which next-generation sequencing (either TGP or WES) was used as the genetic approach are highlighted in bold. *SF-1/*NR5A1* variants tested for functionality in this study. Sequence information is based on the following reference sequences or transcripts: ALMS1: ENST00000409009; AMH: NM_000479.3; ARID1B: NM_020732.3; ARMC4: NM_018076.2; BTD: NM_001281723.3; DHX37: NM_032656.3; DYNC2H1: ENST00000398093; FAT4: ENST00000335110; GAA: NM_000152. 3; GALC: NM_001201401.1; HEPACAM: NM_152722.4; NR5A1: NM_004959.4; OCA2: NM_000275.2; PDHB: NM_001173468.1; POR: NM_000941.2; RELN: NM_005045.3; SIK1: NM_173354.3; SOX10: NM_006941.3; SRD5A2: NM_000348.4; SYNE1: ENST00000448038.1; SZT2: NM_015284.3; TREX1: NM_033629.3; VCAN: NM_004385. ACMG criteria for classification of variants pathogenicity: PVS1, very strong evidence of patho genicity; PS1/2, strong evidence of pathogenicity; PM1-6, moderate evidence of pathogenicity; BA1, stand-alone evidence of benign impact; BP1/2; supporting evidence of benign impact; BS1-4; strong evidence of benign impact.

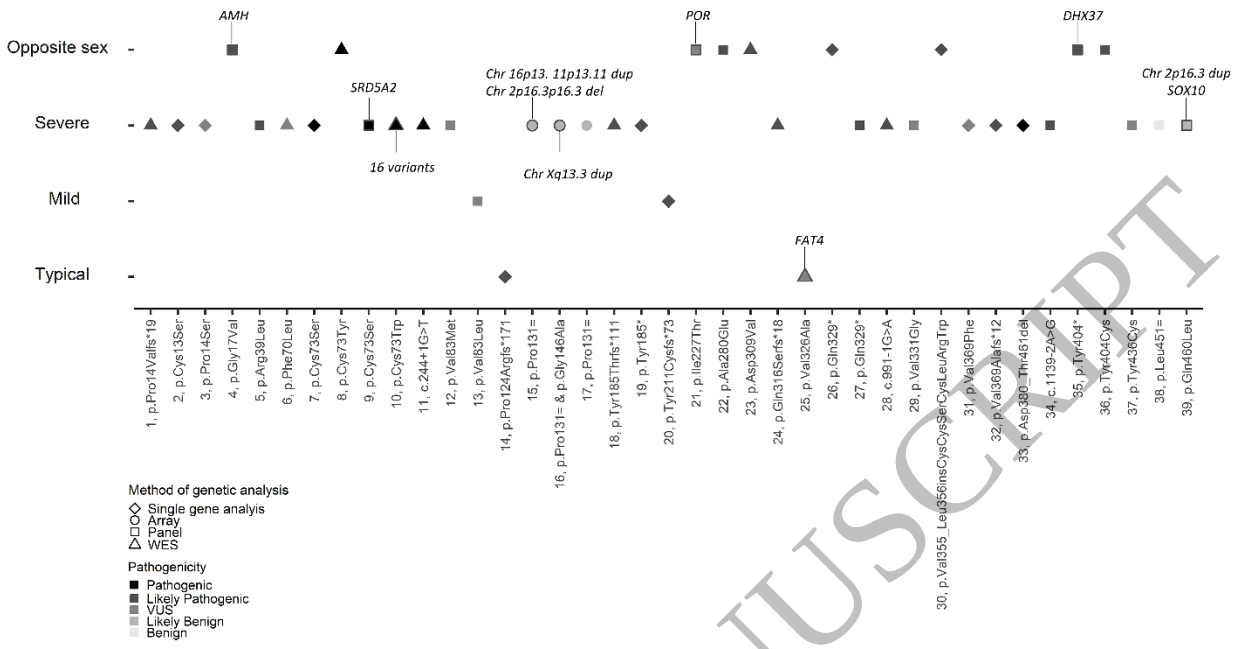


*Figure 1*
*210x112 mm ( x DPI)*

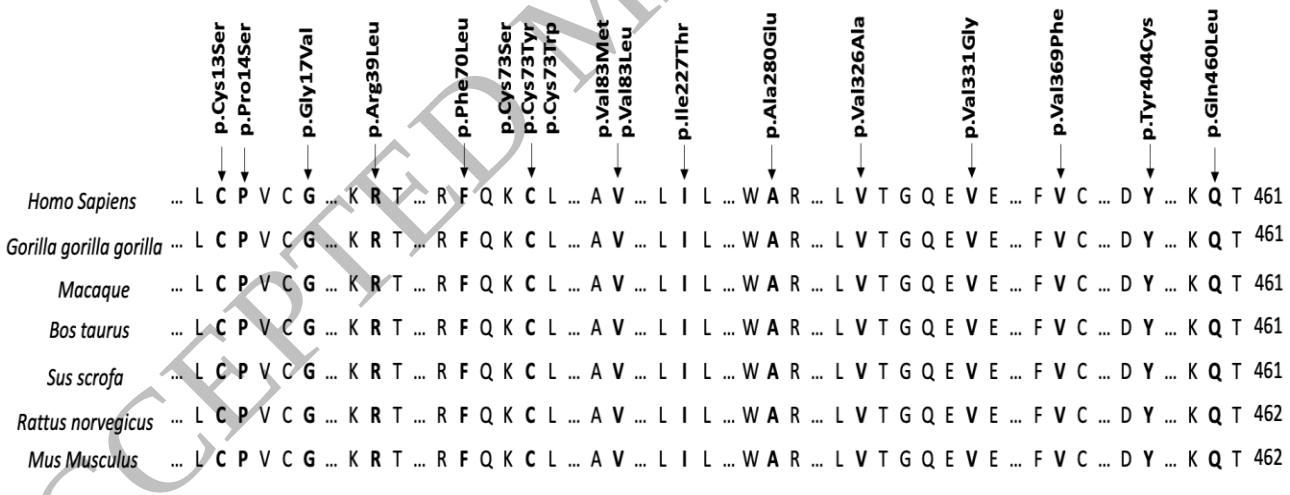


*Figure 2*
*81x23 mm ( x DPI)*

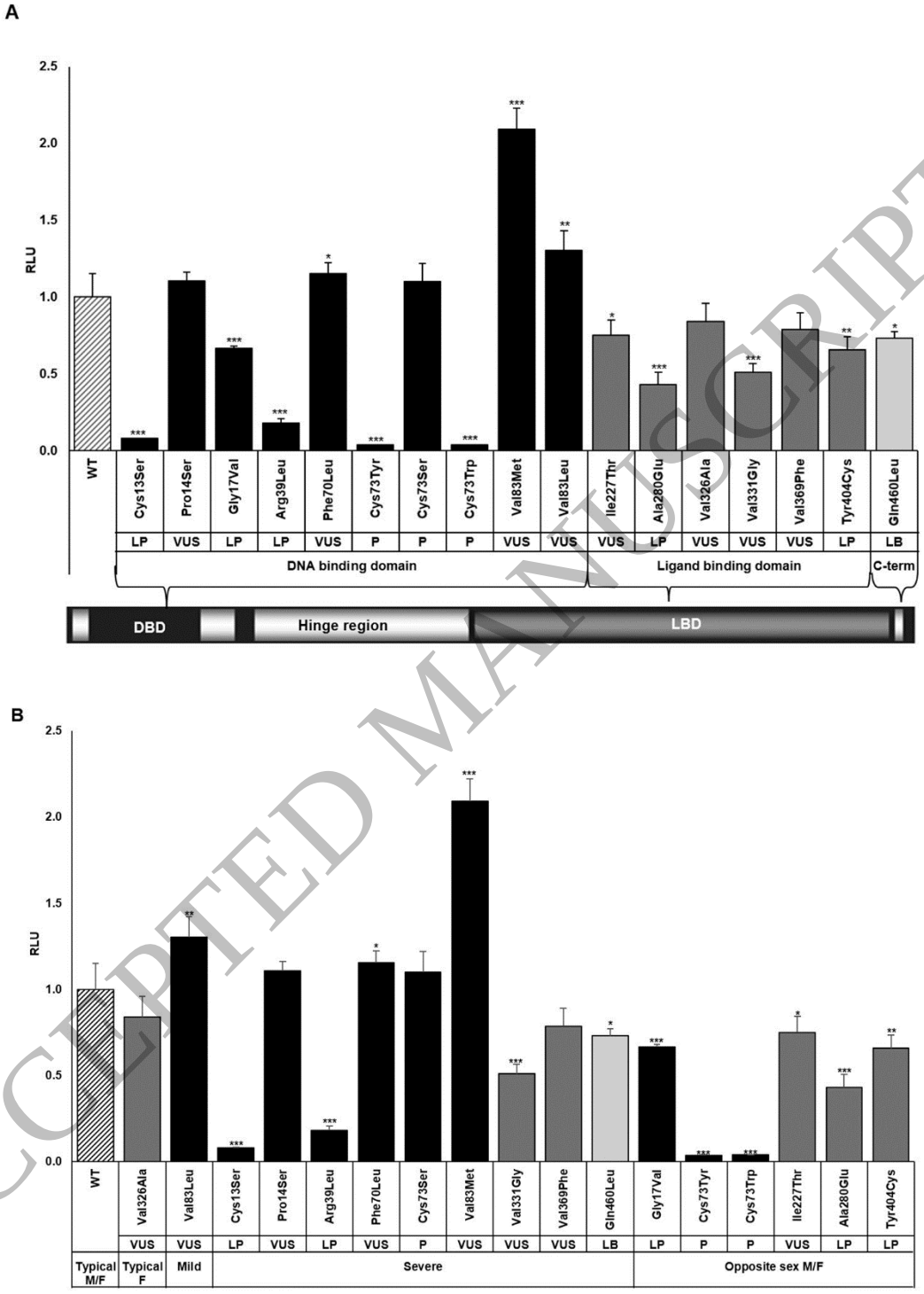


*Figure 3*
*147x200 mm ( x DPI)*

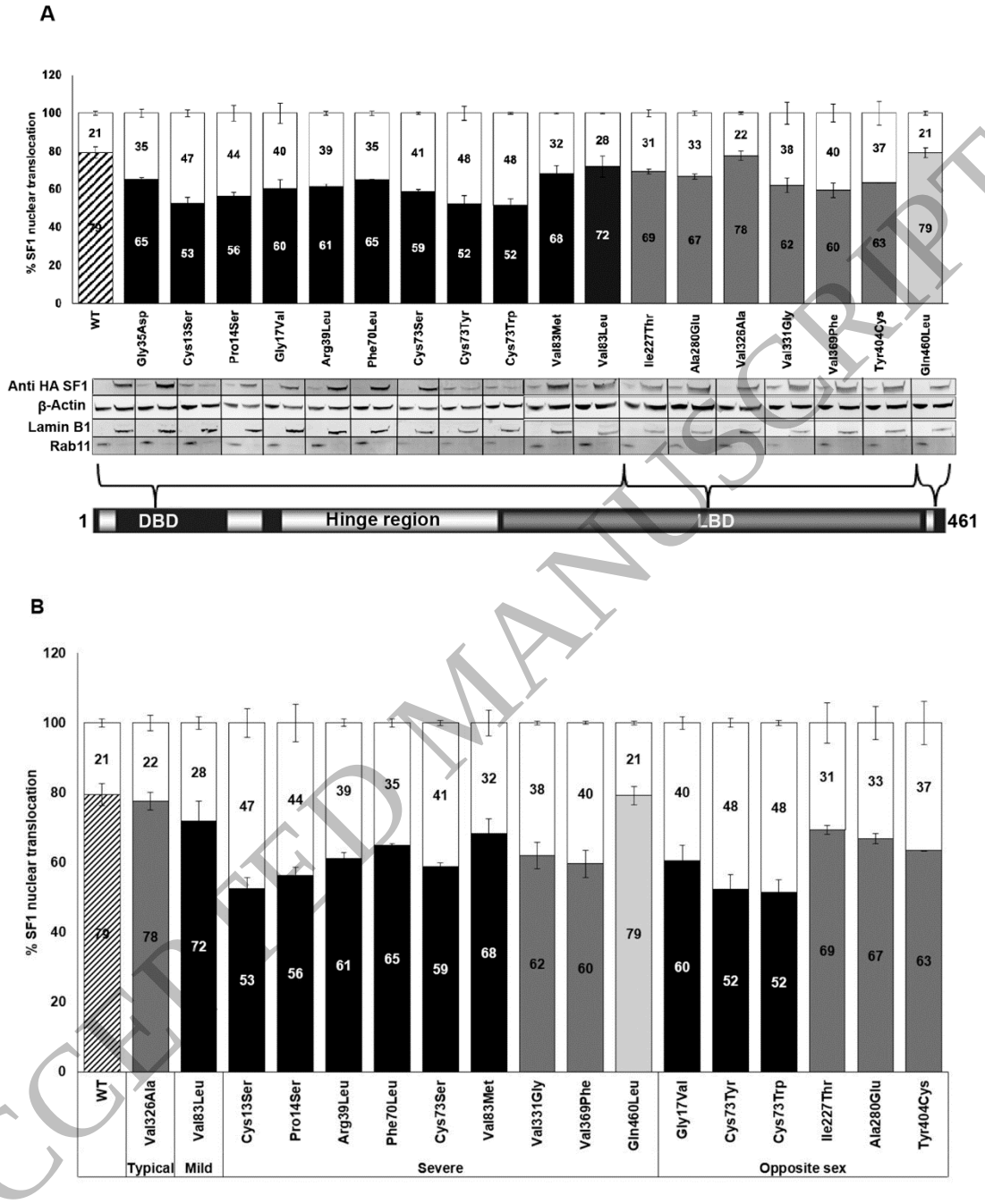


*Figure 4*
*147x200 mm ( x DPI)*