Clinical and genetic characteristics of a large international cohort of individuals with rare *NR5A1*/SF-1 variants of sex development

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Summary
Background Steroidogenic factor 1 (SF-1/*NR5A1*) is essential for human sex development. Heterozygous *NR5A1*/SF-1 variants manifest with a broad range of phenotypes of differences of sex development (DSD), which remain unexplained.

Methods We conducted a retrospective analysis on the so far largest international cohort of individuals with *NR5A1*/SF-1 variants, identified through the I-DSD registry and a research network.

Findings Among 197 individuals with *NR5A1*/SF-1 variants, we confirmed diverse phenotypes. Over 70% of 46, XY individuals had a severe DSD phenotype, while 90% of 46, XX individuals had female-typical sex development. Close to 100 different novel and known *NR5A1*/SF-1 variants were identified, without specific hot spots. Additionally, likely disease-associated variants in other genes were reported in 32 individuals out of 128 tested (25%), particularly in those with severe or opposite sex DSD phenotypes. Interestingly, 48% of these variants were found in known DSD or SF-1 interacting genes, but no frequent gene-clusters were identified. Sex registration at birth varied, with <10%

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undergoing reassignment. Gonadectomy was performed in 30% and genital surgery in 58%. Associated organ anomalies were observed in 27% of individuals with a DSD, mainly concerning the spleen. Intrafamilial phenotypes also varied considerably.

Interpretation The observed phenotypic variability in individuals and families with *NR5A1*/SF-1 variants is large and remains unpredictable. It may often not be solely explained by the monogenic pathogenicity of the *NR5A1*/SF-1 variants but is likely influenced by additional genetic variants and as-yet-unknown factors.

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Introduction

Human sex development is complex and may be disturbed at any stage of gonadal sex determination and/or sex differentiation and maturation. A critical factor in this multistep process is Steroidogenic Factor 1/Nuclear Receptor Subfamily 5 Group A Member 1 (SF-1/*NR5A1*), which plays a crucial role in the development and function of sex organs, in steroidogenesis and beyond.[1] SF-1 was initially identified as a transcription factor that regulates gene expression of steroidogenic enzymes.[2,3] Further research in the mid-1990s showed its importance in the typical development of the adrenal glands and gonads, as the *Nr5a1* (*Ftzf1*) knock-out mouse presented with adrenal and gonadal agenesis, male-to-female sex reversal, persistent Müllerian structures, absence of the ventromedial hypothalamus, and a small spleen.[4–7] The first human disease-causing *NR5A1*/SF-1 variant was described in 1999.[8] This phenotypically female patient presented with primary adrenal failure and 46, XY complete sex reversal, similar to the *Nr5a1* knock-out mouse. Since then, numerous patients with *NR5A1*/SF-1 variants have been reported, but their phenotypic manifestation has been vast.[9] Disease-causing *NR5A1*/SF-1 variants are common among individuals with mild to severe differences of sex development (DSD).[9–11] However, *NR5A1*/SF-1 variants may also be found in healthy carriers (individuals with a *NR5A1*/SF-1 variants who do not have DSD), infertile men or in women with primary ovarian insufficiency (POI).[12–15] Variability in DSD phenotypes exist between individuals with different *NR5A1*/SF-1 variants, but also among those with the same variant, even within families.[9,10,16,17] This variability complicates genetic counselling, prognostic prediction, and preventive health care for carriers of *NR5A1*/SF-1 variants.

Even less is known beyond the DSD phenotype for those with *NR5A1*/SF-1 variants. It has been suggested that these individuals may have other associated organ anomalies that may lead to adverse (metabolic) health outcomes later in life. Spleen anomalies have, for instance, been described in several patients[18,19] and in the mouse model.[20] Whether *NR5A1*/SF-1 variants affect blood pressure and late-onset obesity remains in question.[21–23]

So far, more than 218 *NR5A1*/SF-1 variants have been reported according to the Human Gene Mutation Database (HGMD).[24] They are distributed throughout

the entire gene with no apparent hot spots, and they can manifest as missense, nonsense, frameshift, insertions, deletions, or complex variants. Heterozygous inheritance is most common, but the broad phenotype cannot be attributed to a dominant negative mechanism of action.[14] Haploinsufficiency has also been considered, but so far not confirmed.[25] Numerous studies have attempted to understand the mechanism of disease-causing *NR5A1*/SF-1 variants using various *in vitro* tools and bioinformatic approaches[14,26–30]; however, the reason for the lack of genotype–phenotype correlation remains unclear. Therefore, it remains a challenge to anticipate how different *NR5A1*/SF-1 variants influence the expression of SF-1 target genes and the levels of proteins, regulators, and modulators in the complex networks of adrenal and gonadal development, as well as steroidogenesis,[1,15] leading to the ultimate phenotype.

Recent studies have suggested that oligogenic inheritance may contribute to the variability of the phenotype observed with *NR5A1*/SF-1 variants,[16,17,27,31,32] and other forms of DSD.[33–35] Oligogenic inheritance refers to the interaction of multiple genetic variants, each of which may contribute to the phenotype.[36] This concept is particularly relevant in the context of complex genetic conditions such as DSD, where the presence of multiple genetic variants may explain the variability and severity of the phenotype, when a single gene variant does not suffice. To identify oligogenicity in complex conditions such as DSD, next-generation sequencing (NGS) technologies and advanced bioinformatic pipelines are necessary and may help solve unexplained genotype–phenotype correlations. Although demonstrating the impact of multiple gene variants will remain a challenge, investigation of a large cohort may be the first step towards identifying common genes of a core network for a complex condition.[37] However, as DSD comprises a large group of individually rare genetic conditions, such large cohorts have not existed until recently when international registries and collaborations have been established.[38–40]

To address gaps in understanding the broad variability of rare DSD associated with *NR5A1*/SF-1 variants, a worldwide collaborative project called SF1next was initiated to collect data on the largest cohort to date. By employing a standardized dataset and adhering to international recommendations for optimal clinical care of DSD individuals,[41] we aimed to phenotype and genotype individuals and families with *NR5A1*/SF-1 variants from existing data retrospectively. We particularly focused on describing the precise DSD phenotype, intrafamilial DSD, additional genetic variants related to DSD, and other associated organ anomalies. The findings of this study provide insights into the complex genotype–phenotype correlations of individuals with *NR5A1*/SF-1 variants and contribute to the development of better diagnostic and treatment strategies for individuals with DSD.

Methods

Identification of eligible individuals and study design

Individuals were eligible if they had a genetically confirmed *NR5A1*/SF-1 variant. We excluded individuals with 46, XX disorders of androgen excess, disorders of Müllerian development or cloacal exstrophy, and 46, XY DSD individuals with Leydig cell defects, persistent Müllerian duct syndrome or cloacal exstrophy as these defects are caused by other specific genetic defects. We identified individuals with *NR5A1*/SF-1 variants through the I-DSD registry (https://sdmregistries.org/), by contacting clinicians and researchers who had previously reported data of individuals with *NR5A1*/SF-1 variants, or through the DSD research community. All individuals who were identified consented to participate in international studies. We also included family members with confirmed *NR5A1*/SF-1 variants. We collaborated with clinical partners in 55 centers from 18 countries (Fig. 1a). We excluded individuals with insufficient data in mandatory variables (e.g., precise genetic description of the *NR5A1*/SF-1 variant at DNA and protein level, karyotype, phenotypic details allowing categorization of DSD severity, etc.).

Data collection and phenotype

Data collection took place between September 2019 and August 2022. We collected standardized variables at predefined age intervals based on current international recommendations for optimal clinical care of DSD individuals[41] and developed a Research Electronic Data Capture (REDCap) database for data entry (Supplementary Table S1). Each clinical partner entered anonymized data on the phenotype and on available results of genetic analyses of individuals with *NR5A1*/SF-1 variants in the REDCap database.[42,43] If available, we used existing data from the I-DSD registry on DSD diagnosis and karyotype; otherwise, we surveyed the clinicians and captured these data in the REDCap database. We described the phenotype of overall health according to the terminology of the Human Phenotype Ontology (HPO) vocabulary.[44,45] We asked for associated organ anomalies of the adrenal glands, urinary system, metabolism and homeostasis, endocrine system, blood system, immune system, spleen, abdomen, respiratory system, cardiovascular system, vasculature, integument, connective tissue, musculature, head and neck, skeletal system, central nervous system (CNS), peripheral nervous system (PNS) and psychosocial disorders. We defined abnormalities of the endocrine system as not associated with a DSD, if the individual had received growth hormone, thyroid hormone or insulin. Individuals with more than 50% of missing data with respect to information on associated organ anomalies were not included in this analysis. Collected data (basic data) of family members with *NR5A1*/SF-1 variants for
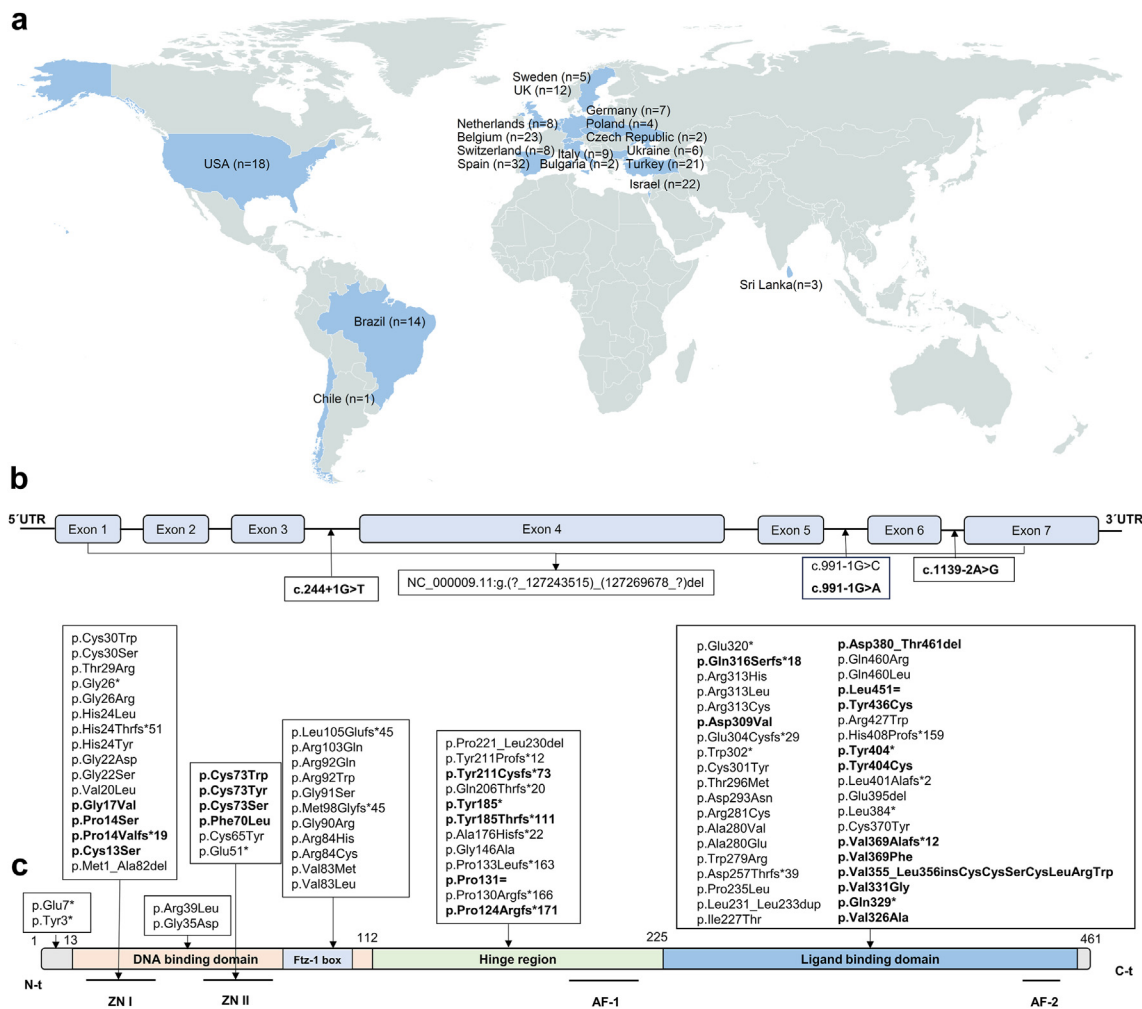
Fig. 1: Overview of the participants and summary of the *NR5A1*/SF-1 variants in the international SF1next study cohort. (a) Number of individuals collected from each country comprising the SF1next study cohort are shown (n = 197). (b and c) Identified variants in the *NR5A1* gene are shown with respect to the gene and protein sequence. (b) Location of four intronic variants and one whole gene deletion of *NR5A1*/SF-1 (NC_000009.11). (c) Location of 87 *NR5A1*/SF-1 variants identified in the SF1next cohort, shown at their protein level (NM_004959.5). Novel variants are shown in bold, while previously reported variants are shown in normal font. The SF-1 protein comprises the DNA-binding domain, which contains two zinc fingers (Zn1 and Zn2), a FTZ-F1 box, the accessory hinge region, and the ligand-binding domain. It harbors two activation functional domains, AF-1 and AF-2. *NR5A1*, nuclear receptor subfamily 5 group A member 1; UTR, untranslated region.

whom we did not have informed consent included the degree of relationship to index patient, genetic details of their *NR5A1*/SF-1 variant and a brief description of overall health and DSD phenotype as routinely assessed by physicians in patient's family history during consultations with index patients.

We used a modified external genitalia score (EGS), similar to the one described previously[46] to classify the severity of the DSD. This classification was based on the karyotype and external genitalia phenotypic features at birth or before genital surgery. For the modified EGS, we assessed phenotypic features of five anatomical landmarks of the external genitalia: degree of

labioscrotal fusion, length of the genital tubercle, position of the urethral meatus, and locations of the right and left gonads. We defined four categories based on the results of the modified EGS assessment: typical for karyotypic sex (typical female-type phenotype, typical male-type phenotype), mild DSD, severe DSD, and opposite sex karyotype (see Supplementary Table S2). We classified family members in whom only minimal information (basic data) was available into these four groups, based on DSD or related conditions reported by the clinicians. We categorized pubertal development into three groups: normal, abnormal, or unknown. Individuals who did not experience a spontaneous start of

puberty, received sex hormones, had a Tanner stage outside the expected range for the given age, or did not have menarche by the age of 15 were classified as having abnormal puberty. For family members, we asked clinicians to directly indicate the puberty category without asking for further details. Individuals who were too young at last follow up to draw conclusions about pubertal development (aged <13 years for 46, XX karyotype or <14 years for 46, XY karyotype) were not included in the descriptive analyses of puberty.

Genetic data collection and analysis
Genetic data were provided by collaborators. The methods used for genetic analysis included candidate gene analysis (CGA), gene panels, whole exome sequencing (WES)/whole genome sequencing (WGS) approach and array comparative genomic hybridization (aCGH). We assessed the *NR5A1*/SF-1 gene variants for each individual according to the NM_004959 reference sequence. We then reanalysed all genetic data available for their disease-causing effect using various *in silico* webtools: Polyphen-2 (Polymorphism Phenotyping v2), Panther (Protein Analysis Through Evolutionary Relationships), SNPs and GO, CADD (Combined Annotation Dependent Depletion), and the calibrated scores given by VarSome[47] 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). These webtools, provide insights on the impact of identified genetic variants on structure and functionality of the corresponding proteins. We assessed potential pathogenicity using ACMG (American College of Medical Genetics and Genomics) criteria[48] using VarSome and searched previously reported clinical associations in ClinVar and HGMD[49] databases. Pathogenicity of variants in other genes found in these individuals were assessed using a similar pipeline. In addition, we tested, if reported additional variants were included in our previously reported algorithm searching for oligogenic disease related to DSD with *NR5A1*/SF-1,[16] as well as previously described in the literature.[31] We also tested variants classified as VUS, LP or P found in combination with *NR5A1*/SF-1 variants for oligogenic pathogenicity using the Oligogenic Resource for Variant AnaLysis (ORVAL) online platform.[50]

Ethics
All data included in the SF1next study cohort were collected in anonymized form. We had ethical approval and informed consent from all study participants providing comprehensive data. All study participants had a family history taken, and this information included the reported genetic information of family members. If we were able to contact such family members and receive their study participation informed consent, they became study participants with comprehensive study data included. In family members that we were not able to contact and receive informed consent, we were allowed to include basic data as of our ethical approval. The University of Glasgow has ethical approval and provides guidelines to international partner centers for collecting routine data of individuals with a DSD in the I-DSD registry (UKCRN ID 12729). International clinical partners had approval from their respective ethical committees.

Statistics
Descriptive analysis included median with interquartile ranges (25th percentile P25 and 75th percentile P75) for continuous variables and frequencies and percentages for categorical variables. For anthropometric variables (birth weight at gestational age, height and BMI), SDS (standard deviation score) were calculated based on karyotype, using reference data of the general population from the INTERGROWTH-21st study[51] and the World Health Organisation (WHO).[52] Standard deviation scores are the z scores representing the number of standard deviations by which an individual's anthropometric variables differ from the population mean at a certain age. We conducted one-sample Wilcoxon signed-rank tests to assess the median of differences between the SF1next study cohort and the general population, with the assumption that the median of differences equals zero. We also tested differences of anthropometric variables (SDS) between the DSD phenotype groups using Kruskal–Wallis tests. We used Stata 16.1 statistical software for data cleaning and analyses and R software (version 4.0.3, 2020-10-10) for graphs and figures.

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Results
Description of the SF1next study cohort
In the SF1next study, a total of 222 eligible individuals with *NR5A1*/SF-1 variants were identified, 74 (33%) through the I-DSD registry and 148 (67%) by directly contacting DSD researchers (Fig. 1a and Supplementary Fig. S1). After excluding 23 individuals (10%) for incomplete data, the final SF1next study cohort included 197 individuals with verified *NR5A1*/SF-1 variants, consisting of 113 (57%) index cases with comprehensive data, and 84 (43%) family members. Among the 84 family members, we had comprehensive data on 15 and basic data on 69 individuals. Of all 197 individuals, 131 (67%) had a DSD phenotype and seven (4%) women had

POI. The majority had a 46, XY karyotype (79%, 155/197), 20% (40/197) had a 46, XX karyotype, one individual had a 47, XXY and another a 47, XYY karyotype. Almost all individuals (94%, 185/197) were heterozygous for *NR5A1*/SF-1 variants, only eleven (6%) were homozygous, and one was compound heterozygous; mosaic variants were found in four (2%). In total, 93 different *NR5A1*/SF-1 variants were found, with 29 (31%) being variants that, to the best of our knowledge, were not previously reported (Fig. 1b and c and Supplementary Table S3). Most variants were located in the DNA-binding domain (DBD) (38%, 35/93) and the ligand binding domain (LBD) (41%, 38/93) of the SF-1 protein (Fig. 1c). The predicted pathogenicity of these variants varied considerably: 47% (44/93) were likely pathogenic (LP), 31% (29/93) were pathogenic (P), 17% (16/93) were variants of unknown significance (VUS) and 4% (4/93) were benign (B) or likely benign (LB) variants (Supplementary Table S3). The majority of the variants were substitutions (70%, 65/93), followed by deletions (22%, 20/93), insertions or/and duplications (3%, 3/93) and delins (2%, 2/93).

DSD phenotype and genotype classification

We classified all individuals into four groups based on the severity of the DSD phenotype as detailed in the methods section and in Supplementary Table S2. Using this classification, 41% revealed a severe DSD phenotype, 35% had a typical phenotype for their karyotype, 19% an opposite sex DSD phenotype and 5% had a mild DSD phenotype (Fig. 2a and b). For five (3%) 46, XY male individuals, complete data were unavailable, but DSD was not reported, therefore we classified them as having a typical male-type phenotype. Among the 155 individuals with a 46, XY karyotype, 49% (76/155) had a severe DSD phenotype, followed by 23% (36/155) with an opposite sex phenotype, 21% (33/155) with a typical male-type phenotype and 7% (10/155) with a mild phenotype. Among the 40 individuals with a 46, XX karyotype, 90% (36/40) had a typical female-type phenotype and 10% (4/40) a severe DSD phenotype. Both individuals with a 47, XYY or 47, XXY karyotype had an opposite sex DSD phenotype.

For all individuals, identified *NR5A1*/SF-1 variants were newly tested for predicted pathogenicity using different bioinformatic tools, incorporating the ACMG classification and data related to DSD phenotypes. Variants classified as P, LP or VUS (95%, 187/197) were found in individuals across all four DSD phenotype groups. LB and B variants were found in individuals who had severe DSD phenotype (90%, 9/10) or typical male-type phenotype (10%, 1/10) (Fig. 2c). Thus, no association between the predicted pathogenicity of the *NR5A1*/SF-1 variants and the severity of the DSD phenotypes was observed.

Additional characteristics of individuals with *NR5A1*/SF-1 variants

Table 1 and Supplementary Figs. S2 and S3 provide detailed characteristics of 128 individuals with comprehensive data. Of 121 individuals with DSD, 45% (54/121) of individuals with a 46, XY karyotype were registered female at birth, while 2% (3/121) of individuals with 46, XX karyotype were registered male. Eleven individuals with a 46, XY karyotype had sex reassignment, nine from female to male, one from male to female, and one from other to male; eight before and three after the age of two years (at 4, 10 and 19 years, respectively). At their last consultation, 30% of individuals were reported to have undergone gonadectomy at a median age of 17 years (P25 13, P75 22), and all of these had an opposite sex or severe DSD phenotype (Supplementary Fig. S2f).

Moreover, 58% were reported to have undergone genital surgery at a median age of 12 years (P25 6, P75 16), most of them with a severe phenotype (Supplementary Fig. S2g and h). Out of the 74 individuals with genital surgery, 70 had a 46, XY karyotype. Of these, 38 (54.3%) underwent masculinizing genitoplasty, 13 (18.6%) feminizing genitoplasty, 11 (15.7%) had both masculinizing genitoplasty and orchidopexy and seven (10%) had only orchidopexy. One individual had orchidopexy in combination with phalloplasty. In the four individuals with a 46, XX karyotype, three had masculinizing and one had feminizing genitoplasty.

Pubertal development was assessed in 109 individuals (54 with a DSD, 5 with POI) as described in the methods section. Seventy-one individuals were too young to assess puberty, and 17 individuals had missing data. In 62 individuals, puberty was normal, of whom 81% did not have a DSD (24 46, XX and 26 46,XY) and 19% had a 46, XY DSD (seven with a mild and five with a severe DSD phenotype, Supplementary Fig. S4). Forty-seven individuals (43%) had abnormal puberty, of whom 42 had a DSD phenotype (35 46,XY, 5 46,XX, two with 47, XYY or 47,XYY); 57% showed an opposite sex DSD, 38% a severe DSD, and 5% a typical female-type phenotype (46,XX). Thirty of these 42 individuals had gonadectomy, 12 at an early age, thus spontaneous pubertal development could not be assessed; in two individuals, data were not available. Sixteen individuals underwent gonadectomy at later age, and prior to the procedure, they experienced abnormal puberty (five with streak gonads). After gonadectomy, 28 individuals received pubertal induction and/or hormonal replacement therapy with normal pubertal development thereafter observed in three, while in 17 individuals the treatment response assessed by Tanner stage progression was insufficient. Four individuals had gonadectomy in adulthood and in four data were missing. Of eight 46, XY individuals with a severe DSD without gonadectomy, normal pubertal development was
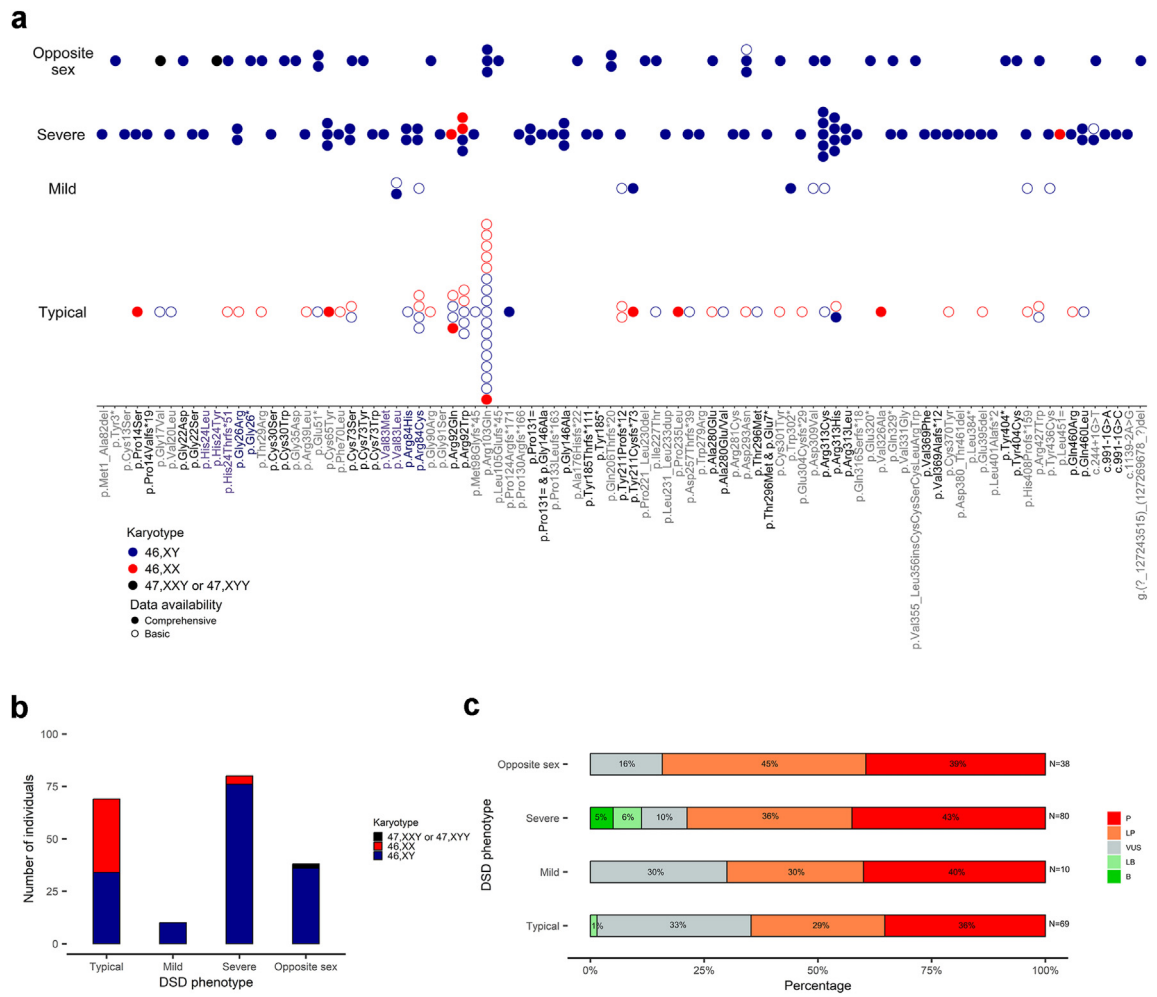
Fig. 2: Characterization of NR5A1/SF-1 variants identified in the SF1next study cohort. (a) Genotype-phenotype correlation. Each dot represents one individual (n = 197) with the corresponding DSD phenotype of the external genitalia and karyotype, stratified by karyotype and type of data. Filled dots show individuals who required medical care while white filled dots show individuals who came to medical attention because of the genetic workup of their NR5A1/SF-1 positive relatives (i.e., family members with basic data who did not require medical care because of DSD). NR5A1/SF-1 variants affecting the same amino acid residue/intronic region are highlighted in black, purple or blue colour, while others are shown in light grey. (b) Karyotype and DSD phenotype of individuals in the cohort. (c) Summary of the pathogenicity of the 93 different NR5A1/SF-1 variants according to ACMG classification, stratified by DSD phenotype of the identified individuals in the cohort. P, pathogenic, LP, likely pathogenic, VUS, variant of unknown significance, LB, likely benign, B, benign.

reported in five, while three were noted to have abnormal puberty.

Germ cell tumors of the gonads were reported in two individuals at the age of three and 21 years, respectively (2/128, 1.6%).

Based on available anthropometric data (Table 1 and Supplementary Fig. S3), median birth weight SDS of newborns with NR5A1/SF-1 variants was - 0.7 SDS (P25–1.3, P75–0.1) (p < 0.0001, Wilcoxon signed-rank test). At their last consultation (median age 14 years (P25 9, P75 17)), median height SDS was - 0.2 (P25 -1, P75 0.6) (p = 0.181, Wilcoxon signed-rank test) and median body mass index (BMI) SDS was 0.6 (P25–0.5,

P75 1.7) (p = 0.021, Wilcoxon signed-rank test). Adult height SDS (available in 14 individuals) was - 0.8 SDS (P25–1.8, P75 0.6) (p = 0.064, Wilcoxon signed-rank test). We did not find a difference in birth weight, height or BMI between the four DSD phenotype groups (birth weight p = 0.443, height p = 0.715, BMI p = 0.519, Kruskal–Wallis test).

Associated organ anomalies

We had information on associated organ anomalies for 173 individuals (88%); 116 had DSD and six had POI. Out of 57 individuals without DSD or with POI, eight (14%) had associated organ anomalies. Of the 116

	Total cohort		46,XY		46,XX		47, XXY or 47,XYY	
	n	%	n	%	n	%	n	%
Year of birth								
<1989	13	10	8	7	4	36	1	ND
1990–<1999	22	17	18	16	4	36	0	0
2000–<2009	43	34	41	36	1	9	1	ND
2010–<2021	50	39	48	42	2	18	0	0
Age at last follow-up (years)								
<5	28	22	27	24	0	0	1	ND
5–<10	29	23	26	22	3	27	0	0
10–<15	27	21	27	24	3	27	1	ND
15–<25	32	25	28	24	5	46	0	0
25–<55	12	9	7	6	0	0	0	0
Sex registration at birth								
Male	61	48	58	50	3	27	0	0
Female	64	50	54	47	8	73	2	ND
Other	1	1	1	1	0	0	0	0
Unknown	2	2	2	2	0	0	0	0
Sex reassignment	11	9	11	10	0	0	0	0
No reassignment	117	92	104	90	0	0	0	0
DSD phenotype								
Typical	9	7	2	2	7	64	0	0
Mild	3	2	3	3	4	36	0	0
Severe	79	62	75	65	0	0	0	0
Opposite sex	37	29	35	30	0	0	2	ND
DSD related surgery								
Biopsy of gonads								
Yes	30	23	25	22	4	36	1	ND
No	95	74	87	75	7	64	1	ND
Unknown	3	2	3	3	0	0	0	0
Gonadectomy								
Yes	39	30	37	32	1	9	1	ND
No	87	68	76	66	10	91	1	ND
Unknown	2	2	2	2	0	0	0	0
Genital surgery								
Yes	74	58	70	61	4	36	0	0
No	50	39	41	36	7	64	2	ND
Unknown	4	3	4	3	0	0	0	0

	n*	Median (P25,P75)	n*	Median (P25, P75)	n*	Median (P25, P75)	n*	Value (P25,P75)
Year of birth	128	2008 (1999–2012)	115	2008 (2001–2013)	11	1994 (1982–2009)	2	1992 (ND)
Age at last follow-up (years)	128	12 (6–18)	115	12 (5–16)	11	25 (10–35)	2	10 (ND)
Gestational age (weeks)	93	38.7 (38–40)	87	38.7 (38–40)	5	39.6 (39–40)	1	32 (ND)
Birth weight (SDS)	90	–0.7 (–1.3 to –0.1)	84	–0.7 (–1.3 to –0.2)	5	–0.8 (–0.1 to –0.3)	1	1.7 (ND)
BMI at last follow-up (SDS)	67	0.6 (–0.5 to 1.7)	60	0.6 (–0.6 to 1.7)	6	–0.02 (–0.8 to 1.5)	1	2.3 (ND)
Height at last follow-up (SDS)	69	–0.2 (–1 to 0.6)	60	–0.2 (–0.9 to 0.6)	8	–0.5 (–2 to 0.8)	1	0.2 (ND)

n*, Number available for analysis; ND, not determined, median (P25, P75), median with interquartile ranges (25th percentile P25 and 75th percentile P75).

Table 1: **Characteristics of 128 individuals with comprehensive data in the SF1next study cohort.**

individuals with DSD, 38 (33%) had associated organ anomalies (Fig. 3a): 55% had a severe DSD phenotype, 32% had an opposite sex DSD phenotype, 5% had a mild DSD phenotype, and 8% had a typical female-type phenotype. Among the 38 individuals with associated organ anomalies, one organ system was affected in 39% (15/38), two organ systems in 47% (18/38), three organ systems in 8% (3/38), and more than three organ systems in 6% (2/38) (Fig. 3b and c). The spleen and blood system were most frequently affected (45%),

Fig. 3: Associated organ anomalies found in individuals with a DSD and *NR5A1*/SF-1 variants (n = 116). (a) Heatmap depicts frequency of organ anomalies found with specific *NR5A1*/SF-1 variants. Sufficient information on organ anomalies was available in 116 out of 131 individuals with a DSD. *NR5A1*/SF-1 variants affecting the same amino acid residue/intronic region are highlighted in blue or purple colour, while others are shown in light grey. The colour of a single cell provides the information on how many individuals are affected (light red to dark red (≥1)) or unaffected (green (≤–1)) by the specific organ anomaly (see value scale). Organ systems with reported anomalies are shown in bold. Number of individuals with reported anomaly divided by the number of individuals with sufficient information per organ system is shown on the right side of the heatmap. Individuals with organ anomalies and without DSD (n = 8) are not reported in the figure; in those, anomalies were found in the blood system and spleen (3/8), metabolism and homeostasis (2/8), endocrine system (1/8), head and neck (1/8), skeletal system (1/8), musculature (1/8), CNS (1/8), cardiovascular system (1/8), connective tissue (1/8), or abdomen (1/8). For white coloured cells no data were available. (b and c) Range of anomalies reported in each organ system according to karyotype (b) or (c) DSD phenotype (n = 38). CNS, Central nervous system; PNS, Peripheral nervous system.

followed by the central nervous system (16%). Interestingly, only five individuals (13%) had adrenal anomalies, with three of them having homozygous *NR5A1*/SF-1 variants, one a compound heterozygous and another a heterozygous variant. Among the 38 individuals with reported organ anomalies, 31 different *NR5A1*/SF-1 variants were identified. Pathogenic variants were most frequent (42%), followed by LP variants (35%), VUS (13%), LB (7%) and B (3%) variants. We also searched for correlations between specific *NR5A1*/SF-1 variants and associated anomalies in these individuals. Apart from the *NR5A1*/SF-1 variant p.Arg103Gln (c.308G > A), which was identified in five individuals of four unrelated families with spleen anomalies, we did not observe any other significant correlations.

Intrafamilial DSD

In 48 families (42%), we had data of at least one relative with the same *NR5A1*/SF-1 variant as the index case. Among these 48 families, we identified 84 relatives, of whom 21 (25%) individuals from 16 families had a DSD phenotype. The intrafamilial DSD phenotype in these 16 families varied significantly, with 12 family members (57%) presenting the same phenotype, seven (33%) a less severe phenotype, and two relatives (10%) presenting a more severe phenotype than their respective index family member (Supplementary Fig. S5).

Genetic findings - additional genetic variants

Information on genetic testing used for the identification of *NR5A1*/SF-1 variants was available from 128 individuals. Of these, 58 were tested with CGA, 42 with

gene panels, 22 with WES, two with WGS and four with aCGH (Supplementary Fig. S6a).

Additional genetic variants (n = 81) were reported for 32 individuals using different genetic approaches, such as CGA (6%, 2/32), aCGH (9%, 3/32), gene panels (53%, 17/32) and WES (31%, 10/32). Of the 81 variants in 62 genes, 42 variants (52%) were identified in 34 DSD-related or SF-1-related genes (Supplementary Table S4). Each individual had between one to 16 additional variants (see Supplementary Fig. S7). We classified all the reported additional variants for their pathogenicity according to ACMG guidelines and found that most variants were VUS (29/81, 36%), followed by LB (23/81, 28%), B (20/81, 25%), P (6/81, 7%), and LP variants (3/81, 4%) (Supplementary Fig. S6b and c). Testing of potential disease-causing additional gene variants (VUS, LP, P), together with the specific combined NR5A1/SF-1 variants in 21 individuals using ORVAL, indicated oligogenic pathogenicity in eight individuals (38%) (Supplementary Table S5). Of the 32 individuals with additional variants, 59% (19/32) had a severe DSD phenotype, 34% (11/32) had an opposite sex DSD phenotype and only 6% (2/32) had a typical female-type phenotype (Supplementary Fig. S7). Ten of 32 individuals (31%) with additional genetic variants and DSD were found to have associated organ anomalies. No obvious correlations were found between the additional gene variants and the associated organ anomalies.

Discussion

NR5A1/SF-1 is among the three genes for which variants are most often identified in individuals with a 46, XY DSD.[53] Unlike variants in the androgen receptor (AR/NR3C4) and 5α-reductase type 2 (SRD5A2), which affect androgen synthesis and action and therefore sex differentiation exclusively,[54,55] NR5A1/SF-1 variants affect both early sex determination (e.g. gonad formation) and sex differentiation. Therefore, NR5A1/SF-1 variants can also lead to (ovo)testicular DSD in 46, XX individuals,[11,56,57] while X-linked AR mutations and autosomal recessive variants in SRD5A2 cause a DSD phenotype in 46, XY individuals only. Overall, the mechanism of DSD associated with NR5A1/SF-1 variants is more complex and there are still large gaps in our understanding of this disorder, including the very broad phenotype, lack of genotype–phenotype correlation, as well as the mostly heterozygous mode of inheritance. This study provides new insights into DSD through a comprehensive description and analysis of the largest international cohort to date, consisting of 197 individuals carrying NR5A1/SF-1 variants. In these individuals, we confirmed the very broad range of possible phenotypes, ranging from severe or even opposite sex DSD in more than 70% of individuals with a 46, XY karyotype, to healthy carriers predominantly in 46, XX females (90%). Our study also highlights that

the DSD phenotype varies within the families studied. We identified both novel and known, mostly heterozygous variants scattered throughout the whole NR5A1/SF-1 gene without hot spots. Pathogenicity testing using ACMG guidelines[48] revealed no genotype–phenotype correlation for specific NR5A1/SF-1 variants. Genetic testing using gene panels or WES discovered additional gene variants in 25% of 128 individuals with NR5A1/SF-1 variants tested, more likely in those with a severe or opposite sex DSD phenotype. Almost half of the identified additional variants were found in DSD-related and/or SF-1-related genes. The various additional genes did not point to recurrent, preferred gene partners; and some individuals had several additional variants, with 36% of variants classified as VUS. Overall, our data indicate that the observed phenotypic variability in individuals with identified NR5A1/SF-1 variants cannot be explained only by the NR5A1/SF-1 variants, and that additional genetic hits or other unknown factors may be involved.

The wide spectrum of phenotypes and possible genotype–phenotype correlation associated with NR5A1/SF-1 variants remain unsolved. Lack of correlation has been reported in several smaller studies[9,10,14,17] and can be confirmed by the data of this larger cohort study. Nevertheless, it is interesting that the p.Arg92 variants (p.Arg92Trp or p.Arg92Gln) manifest consistently in 15 46, XX individuals with a (ovo)testicular DSD, and in two 46, XY individuals with sex reversal, whether in heterozygous or homozygous state, although the variants have also been found in 14 unaffected individuals.[8,11,56–60] The specific mechanism of action of this variant has been studied, and it has been suggested that it downregulates the pro-ovarian Wnt4/β-catenin pathway.[11,56,58,59]

By contrast, many hypotheses have been formulated to explain the variability and complexity observed with NR5A1/SF/1 variants, including, a) haploinsufficiency and variable expressivity, e.g., by skewed allelic expression b) tissue-specific somatic reversion mechanisms, c) mosaicism, d) additional genetic hits (e.g., genetic modifiers), e) epigenetic regulation and other non-Mendelian genetic contributions, and f) gene–environment interactions. The genetic network comprising NR5A1/SF-1 is large and includes transcription factors, co-modulators, posttranslational modulators and signaling molecules.[1,61–63] NR5A1/SF-1 seems to be regulated by epigenetic modification, such as methylation,[64] and post-transcriptional mechanisms involving phosphorylation.[65] Thus, several mechanisms may contribute to the complexity of DSD associated with NR5A1/SF-1 variants. However, more recently, NGS approaches for genetic workup of individuals with DSD have revealed additional variants in several other genes, in combination with known and novel NR5A1/SF-1 variants, supporting the hypothesis that phenotypic variability might be due to oligogenic

inheritance.[17,26,31,32,37,66–68] In addition, a recent study suggested that non-coding *NR5A1*/*SF-1* variants may contribute to the DSD phenotype.[69]

Multilocus variant burden has been shown to impact the occurrence of rare diseases, both within and between families.[70] This has been reported for other rare endocrine disorders such as hypogonadotropic hypogonadism.[34,35] Our study points towards possible oligogenic causation of DSD associated with *NR5A1*/SF-1 variants, at least in some individuals. Additional variants in candidate genes possibly contributing to the disease phenotype were reported in 32 of the individuals with a DSD in our SF1next cohort. This number may even increase in the future with rising awareness of possible mode of oligogenic inheritance and the use of NGS approaches for genetic analysis,[31,37,71] although many of these identified variants may not be disease-causing and could be detected by chance as part of the genetic background. Confirmation of an oligogenic mode of inheritance is challenging, and calculating the occurrence of variants by chance is not feasible for small cohorts and non-standardized genetic methods. In our study, we assessed the reported variants using established bioinformatic tools following current guidelines, which are mostly designed for evaluating monogenic disorders.[16,72,73] So far, only very few bioinformatic tools are available to check for oligogenic pathogenicity, e.g., ORVAL including VarCoPP (Variant Combination Pathogenicity Predictor).[50,74] Using these tools, we found potential oligogenic pathogenicity in a third of tested individuals with a DSD. But, of course, final proof whether and how these variants in combination contributed to the DSD phenotype remains to be demonstrated. From a genetics perspective, trio family analysis and finding additional individuals with the same spectrum of gene variants can help. Experimental approaches using cell models or animal models to investigate pathogenicity are also challenging, as there are limitations to how many genes can be manipulated simultaneously, and simplified modelling might produce false negative results. In addition, classic cell experiments that investigated the promoter activation or nuclear translocation of different *NR5A1*/SF-1 variants have shown uncertain results.[8,14,21,26,28,75–77] Even mouse models do not consistently reflect the human phenotype, as only *Sf1* knock-out mice showed adrenal insufficiency and sex reversal, while heterozygous mice did not recapitulate the broad phenotype seen in humans.[78–80] However, these specific findings may also point towards an oligogenic mode of disease mechanism where a monogenic *NR5A1*/SF-1 variant is insufficient to explain the observed complex and individual phenotype.[34,35,81] In the future, experimental models of patient-derived biomaterials, which carry the individual's full genetic background and can be reprogrammed into tissue-specific cell lines, may help in evaluating the concerted impact of specific gene variants on the DSD phenotype and enhance our understanding of oligogenic disease mechanisms.

Our study aimed to investigate the SF1next cohort beyond genetics and the DSD phenotype. We found anomalies in other organ systems in 27% of individuals, which were more frequent in those with a DSD phenotype (33%), than in those without (14%). Moreover, our data indicated that individuals with a more severe DSD phenotype are more likely to have additional anomalies. We also found that 45% of individuals with a DSD had associated organ anomalies in more than one organ system. Most common organ anomalies observed were in the spleen, followed by the central nervous system. Previous studies in individuals with DSD[82] or in infants with atypical genitalia[83] have reported similar rates of associated anomalies (27% and 23%, respectively). The most common anomalies reported in these studies were small birth size, cardiac anomalies, CNS disorders, renal malformations, and extremity malformations, respectively.[82,83] By contrast, additional organ anomalies are not reported in cohorts of 46, XY DSD caused by *AR* or *SRD5A2* mutations.[84,85] With respect to comorbidities, little is known for specific DSD. Due to the retrospective study design and the young age of our study participants, we were not able to assess long-term health. This is certainly a field of interest to follow up on in future studies.

Spleen anomalies were seen in several individuals of the SF1next cohort. SF-1 plays a critical role in the development and function of the spleen,[1] and *NR5A1*/SF-1 variants have been shown to cause abnormal spleen development (e.g., asplenia).[18,19] As spleen anomalies might only be diagnosed when specifically searched for by imaging studies, we speculate that these anomalies are severely underreported. Given the potential serious consequences splenic dysfunction may have with increased susceptibility to serious infections (e.g., sepsis, meningitis), we strongly advise screening carriers of *NR5A1*/SF-1 variants for spleen anomalies. Early detection and management may prevent life-threatening complications.

In our large cohort, only five individuals had adrenal insufficiency. While the first reported patient with a heterozygous *NR5A1*/SF-1 variant had sex-reversal DSD and adrenal insufficiency,[8] it appears that adrenal insufficiency is rare in individuals with *NR5A1*/SF-1 variants.[9,75,76,86] It may occur with monoallelic or biallelic *NR5A1*/SF-1 variants. However, adrenal insufficiency might only develop over time and detected by ACTH (adrenocorticotropic hormone) testing. More data are needed to advise whether screening or long-term follow up is necessary.

Moreover, our study found that individuals with *NR5A1*/SF-1 variants had lower birth weights compared to the normal population, but later in life these individuals gained in terms of growth with normal height and BMI. However, these results have to be interpreted

with caution as our cohort is still young. Conditional *Nr5a1* mutants in the mouse model have shown that Sf-1 deficiency affects the regulation of reproduction and energy balance.[22] Selective deletion of *Nr5a1* in the ventromedial hypothalamus in mice prenatally resulted in late-onset obesity, and postnatally to deregulated thermogenesis.[87] Thus, long-term studies including more individuals are needed to conclude whether *NR5A1*/SF-1 plays a role in growth and metabolism. Birth weight and size, as well as catch-up growth and final height seem to vary in patients with different types of DSD,[88,89] but little data are available.

Many 46, XY individuals with a DSD had a severe phenotype with female sex registration at birth, but only few were sex reassigned later in life. Notably, surgical procedures such as genitoplasty and gonadectomy were performed in a considerable number of individuals with *NR5A1*/SF-1 variants. Germ cell tumors were reported in only two individuals with a DSD in our cohort. Although this small number may suggest that tumor risk with *NR5A1/SF-1* variants is low, we caution to draw this conclusion prematurely because many individuals had gonadectomy at an early age with histological investigations of the gonads not performed uniformly; in addition, many study individuals are still young. Clearly, there are case reports in the literature of individuals with *NR5A1*/SF-1 variants and germ cell tumors.[26,90] Long-term studies are therefore needed to assess tumor risk, while meanwhile surveillance is recommended.

Normal pubertal development was rather seldom observed in individuals with a DSD and *NR5A1*/SF-1 variants, even with hormonal replacement therapy. Mostly, an opposite sex 46, XY DSD phenotype related negatively to normal puberty, while even with a severe 46, XY DSD phenotype both normal and abnormal pubertal development was possible. This finding is in line with other studies on pubertal development of patients with a DSD and *NR5A1*/SF-1 variants, which describe a wide spectrum of pubertal courses with considerable virilization at puberty in 46, XY individuals, irrespective of the degree of virilization of the external genitalia at birth.[91–93] Thus, the severity of the DSD phenotype based on the assessment of the external genitalia at birth may not be a reliable predictor of later pubertal development in individuals with *NR5A1*/SF-1 variants. Similarly, the severity of external genital undervirilization at birth does not necessarily reflect the potential of virilization in patients with a loss of 5α-reductase activity or partial androgen resistance syndromes.[94–96] A recent review on pubertal development in patients with a different DSD suggested that puberty may be absent, incomplete or atypical, and even timing and progression of puberty may be modulated by the specific type of DSD.[97] These facts should be considered when counselling parents of children with a DSD in decisions such as sex registration at birth, hormonal treatments, genital surgeries or even gonadectomy. This certainly appears to be true for children with a DSD and *NR5A1*/SF-1 variants and indicates the need for prospective standardized assessment of these individuals through puberty and beyond.

Our study has several limitations. Due to the retrospective study design and inherent issues with registry data, we had to rely on pre-existing data, which were not originally collected for the specific purpose of this study and sometimes were partially incomplete or inaccurate. For instance, information regarding specific phenotypes associated with *NR5A1*/SF-1 variants, such as spleen anomalies, may have been missing. The SF1next cohort only includes cases from centers that specialize in DSD, resulting in sampling bias and underreporting of individuals with less severe phenotypes. In addition, carrier status was tested in only a subset of family members, who were also phenotypically incompletely characterized. Our cohort predominantly consists of individuals with a 46, XY karyotype, which reflects the incidence of DSD reported in the general population,[98] but which limits the applicability of our findings to less common DSD individuals with a 46, XX karyotype and *NR5A1*/SF-1 variants. In particular, POI arises over time and may not have developed at the time of data collection in some index cases or family members. The young age of our cohort does not allow the assessment of long-term outcomes, and there is limited phenotypic data on family members without DSD. Finally, the analysis and interpretation of *NR5A1*/SF-1 and other gene variants identified in our study cohort through different genetic approaches in different genetic labs over a long time period, was a big issue, although we tried our best by using established bioinformatic tools and genetic guidelines. In addition, some reported (likely) disease causing *NR5A1*/SF-1 variants included in our study were identified by our bioinformatic reanalysis as (likely) benign (4/93) raising doubts whether they should have been included in the study. Finally, the descriptive nature of our study limits its ability to establish causal relationships or determine underlying disease mechanisms. Future research employing analytical techniques, such as functional studies, molecular investigations, and in-depth genetic profiling, will be necessary to close further gaps. Also, it is important to realize that sometimes genetic data generated with less advanced techniques need to be repeated with newer methods. Despite these limitations, our study has several strengths. It provides insight into the largest international cohort of individuals with *NR5A1*/SF-1 variants to date. It offers a comprehensive overview of various outcomes including precise phenotyping, genotyping, growth assessment, and identification of additional associated anomalies. It includes data of family members, which are often overlooked, but are important for understanding the complex phenotype and genotype of DSD. Ultimately, our study was an international effort, which contributed to increase the

diversity of the cohort, improved generalizability of findings, and provided further insights into the broad phenotypic variability of DSD for individuals carrying *NR5A1*/SF-1 variants. Importantly, it informs clinicians on considerations of caring for these people on an individual basis, specifically regarding sex registration and pubertal development, spleen anomalies and surveillance of adrenal insufficiency.

In conclusion, the SF1next cohort study has significantly enhanced our knowledge on genotypic and phenotypic characteristics of individuals with *NR5A1*/SF-1 variants. It strengthens the hypothesis that the broad variability of the disease might be due to oligogenic or other non-Mendelian pathogenicities, therefore encouraging advanced genetic testing in individuals with a DSD and *NR5A1*/SF-1 variants, and further mechanistic research. Furthermore, it emphasizes the need for special surveillance and management of associated clinical features to ensure optimal patient care and outcome.

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Data sharing 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. These data can also be accessed upon reasonable request.

Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104941.

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