PROJECT FINAL REPORT

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Project website: www.EuroDSD.eu

Table of Content

Section 1 – Final publishable summary report

EuroDSD

Logo:

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Website: www.EuroDSD.eu

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1.1 Executive summary

Disorders of Sex Development (DSD) constitute a group of rare to very rare congenital disorders affecting the genito-urinary tract and in most instances also the endocrine-reproductive system. DSDs are caused by chromosomal, genetic, and endocrine aberrations, which in consequence modulate the sexual phenotype of a given person. However, the exact aetiopathogenesis is not known in the majority of the cases. Children with DSD may be born with ambiguous genitalia and the decision-making of sex assignment has been perceived as extremely challenging for families and also to health care professionals. Often, multiple surgical interventions are performed for genital reconstruction to a male or female appearance. The gonads are often removed to avoid malignant development. Hormone replacement therapy is not studied well and usually follows common patterns of replacement used in other patient populations. Patients and their advocacy groups oppose the current use and management of surgical correction to adhere to morphological appearance with sex assignment and also demand specialized hormone therapy. Furthermore, the impact on gender with a distinction of genetic and endocrine "imprinting" versus cultural and social modulation of gender identity remains elusive and at present is the subject of controversy discussed by legal stakeholders, advocacy groups and professionals, addressing the need to political decision makers to accept a "third sex". Therefore, DSD paradigmatically illustrate the diagnostic, prognostic and ethical dilemmas encountered by humans affected by rare congenital disorders. To achieve the prospects of a healthy life and unhindered integration into society, patients with DSD need individualized diagnostic and therapeutic management that is based on a novel categorization approach with detailed clinical phenotyping including morphologic description and assessment of underlying genetic and metabolic abnormalities.

In the project *EuroDSD*, the partners created a unique data series on clinical, genetic, and endocrine features of patients with DSD of known and unknown cause. This was implemented as a security-oriented registry incorporating pseudoanonymized patient data with an associated communication platform supporting interactions and data sharing between clinical centres and researchers according to the consortium needs. The consortium named this tool "virtual research environment" and this platform approach has been replicated by several other collaborative projects on rare diseases, in part funded by FP7, in the meantime. A strong focus was put on standard operating procedures for biomaterials, on ethical reviews taking into account legal structures of participating countries, and the consent forms for patient participation. *EuroDSD* has achieved maintenance of the current database with long-term sustainability, including a 5-year MRC funded project (I-DSD). The initial European consortium has subsequently been broadened to clinical centres from Africa, Asia, South America and the USA. In its research approach, *EuroDSD* performed genetic, biochemical, and molecular studies on patient materials. The consortium was able to identify new copy number variants associated with abnormal sex development in a variety of syndromic and non-syndromic forms of DSD. *EuroDSD* also constructed a DSDGeneChip for rapid assessment of 36 known genes involved in DSD. This DSDGeneChip has now been validated in its sequence. The protocols that were created in the development of the GeneChip will be of tremendous importance in the application of new technologies such as next-generation sequencing approaches to rapidly target and sequence multiple genes involved in DSD. Furthermore, we performed epigenetic studies and demonstrated highly significant differences in methylation patterns between controls and patients with androgen insensitivity, a group of DSD patients where defective androgen action hinders male development. These results point towards a significant influence of sex hormones on gene methylation and, hence, gene expression that define differences between the sexes. Furthermore, we elucidated the diagnostic value of steroid hormone measurements by mass spectrometry techniques both in urine and in plasma. Reference intervals were defined for different age groups and also for different diseases entities. Novel steroid biosynthesis defects were identified employing these techniques and verified by definition of the underlying genetic aberration. We also described factors related to time-dependent modulation of androgen action as implementers of phenotype and carried out in-depth characterisation of defined mutations to explain the basis for phenotypic variation in certain forms of DSD.

EuroDSD partners have published their work in several highly ranked scientific journals already and evaluation of certain aspects is still on its way, so more scientific publications will be submitted in the near future. Thus, dissemination of research results was highly successful and will lead to sustainable knowledge in the scientific community. Moreover, as DSD is a highly sensitive, complex issue, we constructed an e-learning programme aimed at professionals of different levels of education. The *EuroDSD* consortium has contributed enormously to a very timely and pressing public debate on research and health care of patients with rare diseases affecting the genito-urinary tract. This will have effects on the implementation of research and health care standards for patients and will hopefully lead to the creation of Centres of Reference for these disorders throughout Europe.

1.2 Summary description of project context and objectives:

Background and Aims

Disorders of Sex Development (DSD) constitute an array of rare to very rare disorders affecting the genito-urinary tract and in most instances also the endocrine-reproductive system. DSDs are peculiar in that they may be caused by chromosomal, genetic, and endocrine aberrations, which in consequence modulate the sexual phenotype of a given person. Children with DSD may be born with ambiguous genitalia and the decision-making of sex assignment has been perceived as extremely disturbing and difficult to families and also to health care professionals. This is mainly due to generally poor information about the conditions and the exaggerated feelings of stigma and shame associated with genital abnormalities. Few long-term outcome studies on the various DSD entities have been performed and are needed in order to establish a basis for evidence-based medicine regarding sex assignment and conservative and surgical treatment options. Premature decisions leading to irreversible interventions before an accurate diagnosis has been found must be avoided and have led to severe disturbances in patients and families in the past. The poor understanding of the underlying pathophysiology as well as the historical use of mainly mythological terms for descriptive diagnostic purposes has led to a consensus conference in 2005 driven by the European Society for Pediatric Endocrinology (ESPE) in collaboration with the US-American Lawson-Wilkins Pediatric Endocrine Society (LWPES) and the Asian-Pacific Pediatric Endocrine Society (APPES). A new nomenclature and classification was developed based on the concept of precision and inclusion of genetic pathways. Starting from the chromosomal set, normally either 46,XX or 46,XY, further delineation is made for abnormalities of gonadal development versus disorders affecting hormone synthesis or action. Significant flexibility exists to incorporate so far unclassified or syndromic disorders. Cases of numerical chromosomal aberrations or disorders of development of both testicular and ovarian tissue are incorporated into this classification.

Table 1: Classification of DSD

Numerical chromosomal disorders	45,X
	47,XXY
	45,X/46,XY etc
46,XY DSD	Disorders of gonadal development (dysgenetic gonad)
	Disorders of hormone synthesis and action
	Non-classified (hypospadias, syndromic, etc)
46,XX DSD	Disorders of gonadal development (primary ovarian failure, etc.)
	Disorders of androgen excess (congenital adrenal hyperplasia, aromatase deficiency, etc.
	Non-classified (Mayer-Rokitansky-Küster-Hauser Syndrome), etc.

The aim of the *EuroDSD* consortium was to provide as much information about the underlying biological aberration, but also about the possible outcome of given distinct disorders. The project combined unique strengths by linking a European patient-based data collection and analysis tools (WP01) with research on development of novel diagnostic strategies to identify new causes of DSD (WP02, WP04 and 05) in conjunction with a strong programme on functional molecular biology of the androgen receptor (WP03), thereby allowing for an in-depth analysis of a key factor in the pathogenesis of DSD. This was accompanied by the development of an e-learning programme (WP06) to teach professionals at various levels of education on the conditions.

The main scientific and technological objectives of this highly focused project were:

- To carry out well-integrated biochemical, molecular, and genome-wide research in order to identify patients with monogenic and non-defined DSD
- To stratify the patients according to clinical data
- To identify biochemical profiles related to specific disorders
- To allow for rapid genetic analysis in the patients
- To identify new genetic abnormalities in a whole genome screen
- To define factors related to time-dependent modulation of androgen action as implementers of phenotype
- To search for methylation patterns as “footprints” of hormone-dependent genetic alterations
- To disseminate study results and to provide implementations and guidelines
- To establish a European consortium on the basis of genuine and equal partnership between leading clinical centres, academic groups, cell biologists, and geneticists to provide further knowledge about the physiology and pathophysiology of sex development for the benefit of members of the European Community and

supported by a tailored virtual research environment targeted to the needs of DSD research.

EuroDSD facilitated the use of modern and novel technologies to assess the physiology of sex development, employing

- The creation of a novel and highly specific DSD gene chip for high throughput medical sequencing
- Ultra high-resolution comparative genomic hybridization approaches for the identification of gene anomalies associated with DSD
- Mass spectrometry based techniques (LC/MS/MS and GC/MS) for utmost scrutiny in the characterization of disease-specific steroid profiles
- DNA-methylation analysis with whole human genome promoter microarrays and custom promoter chips comprising approximately 100 newly identified sex-dimorphic methylation targets
- Yeast-two-hybrid screening for the identification of human homologues of mouse embryo-tissue derived co-factors of the androgen receptor and functional assessment of selected co-factors in cell models.

Information strategies for health care professionals and the public on DSD had been mostly provided by patient support groups in a structured fashion, although there is an ongoing enormous interest both in normal and abnormal sex development in the public. The creation of platforms incorporating both biological information as well as support strategies for those affected is desperately needed. We believe that communication, knowledge transfer and general information is of utmost importance between researchers, health care providers, patients and families, as well as the public.

EuroDSD was the first structured international consortium incorporating a critical mass of European experts in the field of disorders of sex development, acquiring basic knowledge derived from human-based and model studies in order to alleviate the negative impact a disorder of sex development has on the affected individuals and their families. *EuroDSD* aimed for three communication platforms:

- A virtual research environment (VRE) in WP01 for communication of clinical and research data within the consortium
- A structured e-learning programme in WP06 that disseminates the knowledge to health care professionals of different levels and thus aims at improving care of patients with DSD in Europe and world-wide.
- A website that distributes the information gained from this collaborative project to the public.

Work strategy and general description

EuroDSD proposed a parallel genomic and biochemical human-based approach, supplemented by functional molecular models to characterize thoroughly the pathophysiology and natural course of DSD. We aimed to characterize new genes, variants, proteins and regulatory pathways contributing to normal and abnormal sex development. The functional studies focussed on the time-dependent expression of relevant co-factors of androgen action, which play a pivotal role in the development of the sex phenotype as well as the development of adequate in-vitro models for investigation. All data acquired in parallel were communicated through the creation of a virtual research environment (VRE). The overall scheme of the research activity is depicted in the figure below.

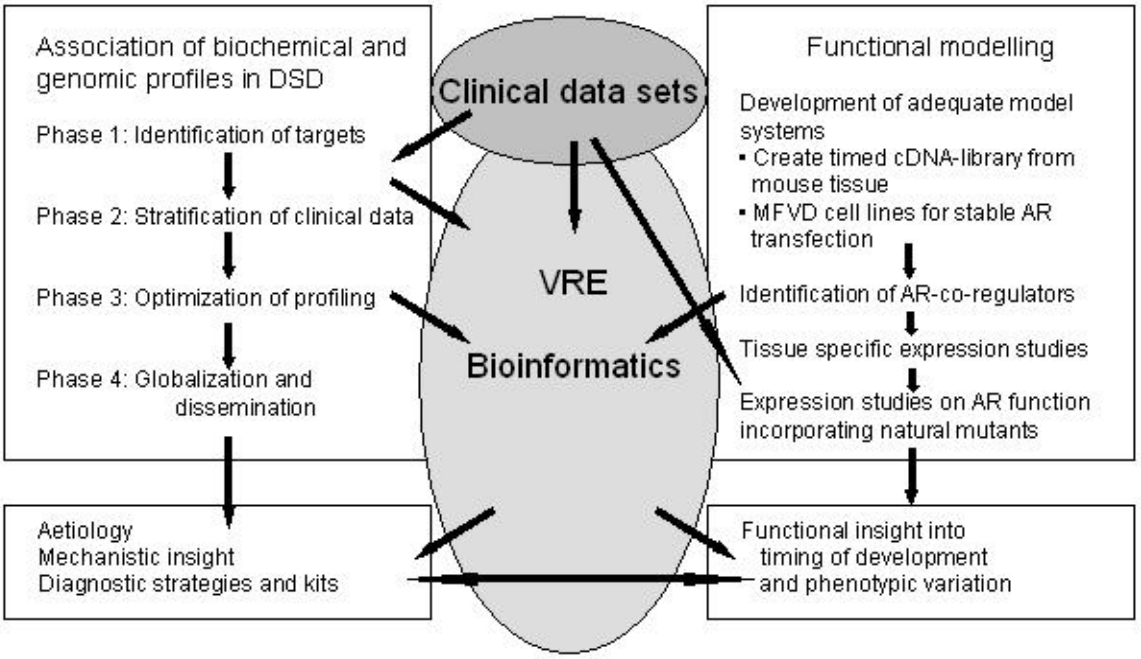


Figure 1: Stepwise and coordinated identification of genomic and biochemical profiles in DSD as well as interaction with functional studies through the VRE.

The interdependency of the work packages in order to allow a structured scientific approach is demonstrated in the Pert diagram below. The clinical data base and VRE developed in WP01 was the core activity of the project, linking the results of the research results within WP02 to WP05 together with the clinical data sets created from the clinical partners shown below. While there is no direct connectivity to the e-learning programme, the results of the project were to a maximal extent already implemented in the educational activities. The project management steered the whole project towards a stringent achievement of the goals.

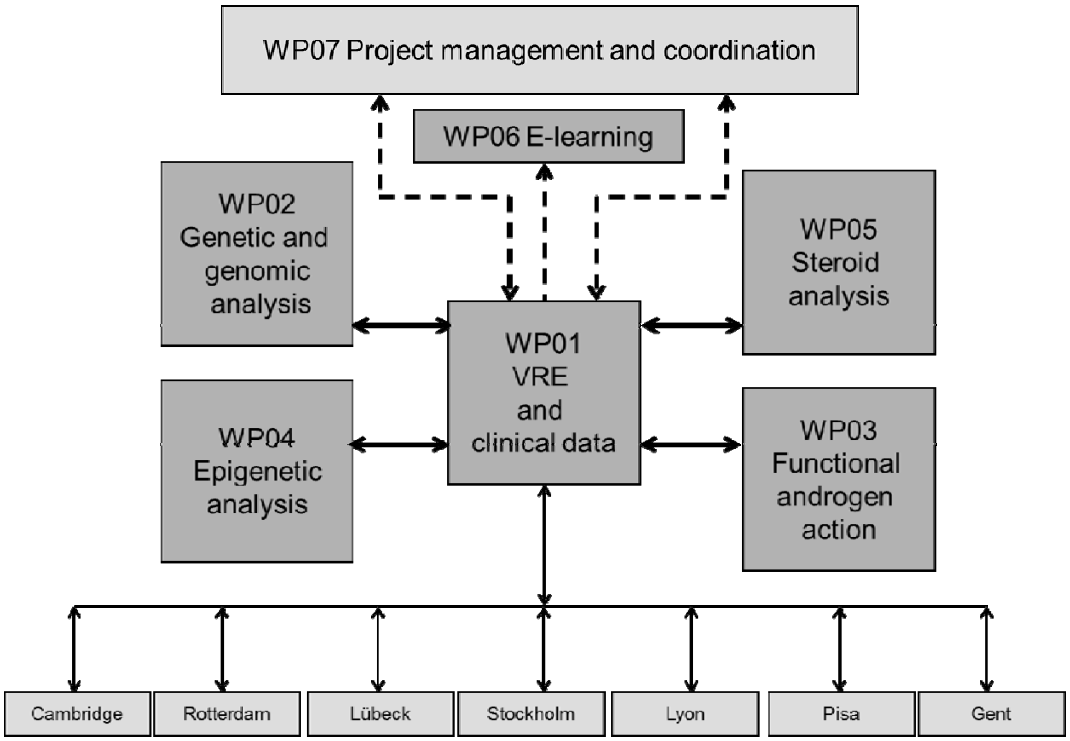


Figure 2: Graphical structure of the interdependencies of the work packages

Management structure and procedures

The Project Coordinator ensured the smooth operation of the project and guaranteed that all efforts were focused towards the objectives. He submitted all required progress reports, deliverables, financial statements to the European Commission, and, with the assistance of GABO he was responsible for the proper use of funds and their transfers to participants. The *EuroDSD* office was established by and based at the coordinator in Lübeck and at GABO in Munich. The Project Office at the Coordinator was concerned with the scientific management and the co-ordination of all research activities. The Project Office at GABO was responsible for administrative, financial and contractual management and the organisational co-ordination of the project activities.

The Project Governing Board was in charge of the political and strategic orientation of the project and acted as the arbitration body. It met once a year unless the interest of the project required intermediate meetings. The Project Coordination Committee consisted of all work package leaders and the Coordinator and was in charge of monitoring all activities towards the objective of the project in order to deliver as promised, in due time and in the budget. The Project Coordination Committee met every six months during the funding period. Furthermore, a scientific advisory board was implemented to ensure a high standard of research and monitor the progress of the project by taking part in the annual Governing Board Meetings. It was chaired by Prof. Laura Audi, Barcelona, Spain

Further panel members were
Dr. Christa Flück, Bern, Switzerland
Prof. Yehia Gad, National Research Council, Cairo, Egypt
Prof. Larry Jameson, NW Illinois, Chicago, USA
Prof. Hanns Lochmüller, Newcastle upon Tyne, Great Britain
Prof. Marek Niedziela, Poznan, Poland

Objectives of *EuroDSD*

We hypothesized that a stringent stepwise analysis of individuals with Disorders of Sex Development (DSD) would result in a systematic and reliable discovery of DSD-relevant biochemical, genetic, epigenetic, and functional profiles. This will allow for the detection of new diagnostic markers, both in steroidogenesis as well as in genetics and epigenetics, and it provides the basis for explaining the natural course of these disorders. Characterization of the functional aspects of androgen action further improves the understanding of the pathophysiology of DSD and we utilized a novel model system on the basis of the embryonal mouse genital tubercle. Furthermore, as DSD is a highly sensitive, complex issue, we constructed an e-learning programme aimed at professionals of different levels of education. Our project was aimed to allow in a translational research process for better decision-making in gender assignment and therapeutic approaches to DSD.

1.3 Description of the main S&T results/foregrounds of *EURODSD*

Implementation of communication and management structures

The *EuroDSD* consortium began its work on May 1st, 2008 with the implementation of its management structures and procedures to start scientific work. The first project coordination committee meeting comprising all the work package leaders and the first project governing board meeting took place on May 30th to 31st 2008 in Paris. At this time, a start-up structure of the *EuroDSD* office was established and management structures between the coordinator in Lübeck and GABO:mi in Munich were constructed. The meeting in Paris as well as the second project governing board meeting from 27th May to 29th May 2009 in Pisa, Italy were complemented by our international advisory board on scientific matters as well as the international advisory board on ethical matters. The reports by our advisory boards verified our progress and our achievements. Their suggestions were implemented into our working programme. At the third project governing board meeting from 20th May to 22nd May 2010 in Stockholm, Sweden, only the Chair of the Scientific Advisory Board was present to discuss with the consortium and report to the Board members.

It was our aim to create a European data series on clinical features of patients with DSD of known and unknown cause. To achieve this, we started in work package 01 to create a registry incorporating pseudoanonymized patient data and construct a virtual research environment according to the consortium needs and with a very high security level. This has been achieved and both the registry set-up as well as the research matters were approved by the respective national and local ethical committees. We created a very special internet-based communication structure (Figure 3) with three components: First, a public website (www.*EuroDSD*.eu) was created to inform about our goals

and achievements and to put our project into the perspective of current research. Here, also public meeting reports and news are placed. Also, this website has information on our standard operating procedures on biomaterials, our ethical reviews and the consent forms for patient participation. Second, we have used the internet-based programme :millarium of our partner GABO:mi, to create a tool for the administrative purposes. Here, all achievements within the project are documented and internal protocols are kept to inform all partners timely of the progress of all work packages and the overall project. Third, we have created a security-driven, internet-based data base on patients with DSD. Patient consent given, a mandatory data set can be created which can be supplemented by additional clinical and scientific information as well as information on the availability of biomaterials (see also Figure 4).

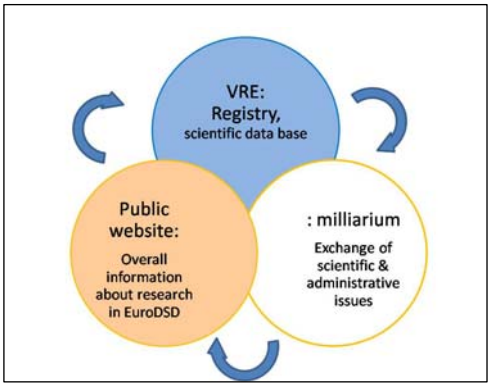


Figure 3: Internet-based communication structures of *EuroDSD*

Construction of a virtual research environment

Critical to unravelling the biochemical and genetic basis of DSD is the assessment of the clinical phenotype in standardized precise manners. A core data set was developed in several sessions (Table 2) which identifies centres entering data as well as secures the data to the highest level. The data collection and creation of the clinical data entry and compilation of the VRE provided the foundation for the work plan and logical phases of *EuroDSD*. Data collection was built on key inclusion criteria. The diagnostic criteria and clinical data were verified by the center entering the data. The clinical centers agreed to include all patients into the study who fulfil the key inclusion criteria. Biomaterial exchange depends on the written informed consent given by the patient or the legals as demanded by the ethics approval.

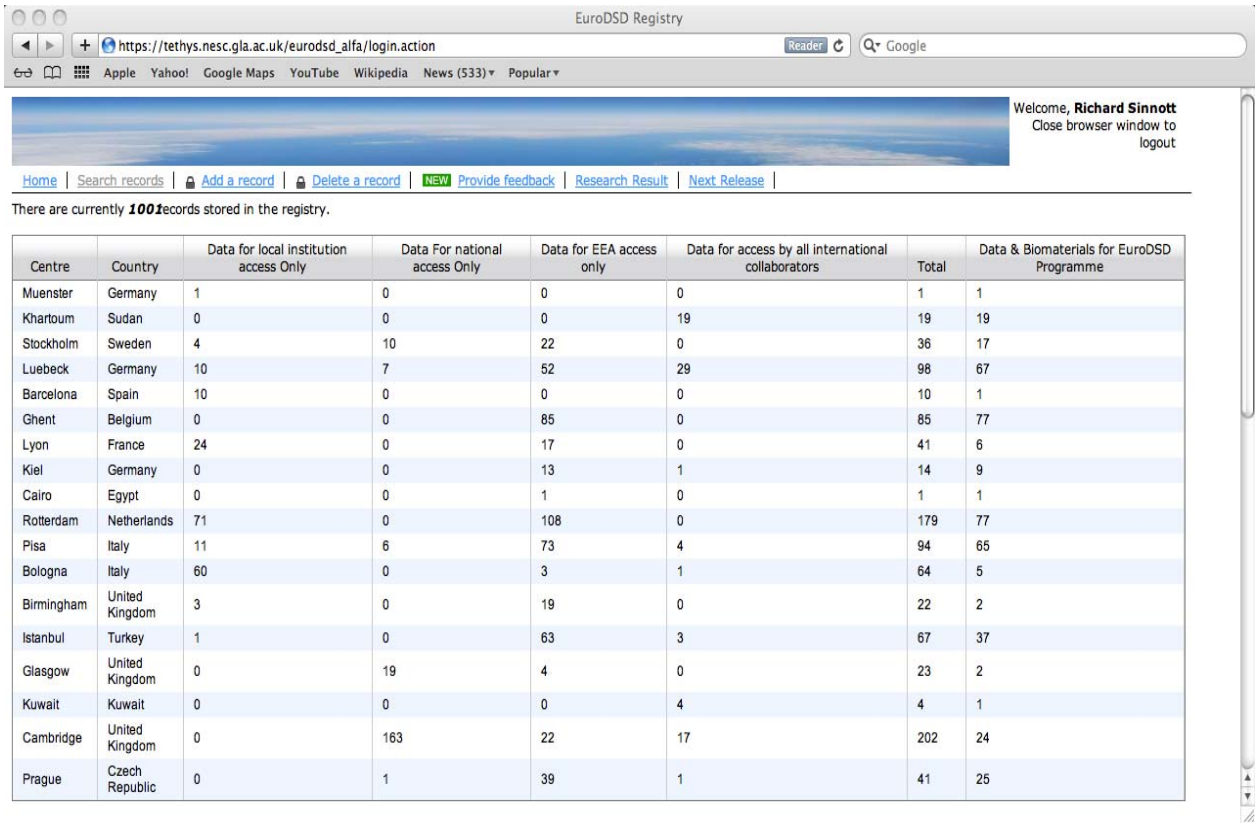
Identifiers	Diagnosis according to Classification	Certainty of Diagnosis	Genetic Analysis	Clinical presentation	Associa-ted Malforma-tion	Material available
▪ ESPE Register ID ▪ Country code ▪ Centre code ▪ Local ID ▪ Date of Birth ▪ Date of 1st presentation ▪ Sex assigned ▪ Clinician ▪ Contact	▪ Primary root ▪ Secondary root ▪ Tertiary root	▪ Clinical Certain Clinical Uncertain ▪ Genetic Certain ▪ Genetic Uncertain	▪ Performed: Yes/No ▪ Gene analysed and mutation identified: Yes/No ▪ Functional studies	▪ At presentation ▪ Follow-up ▪ Date of assessment ▪ Phallus length ▪ Meatus site ▪ Labioscrotal folds ▪ Right gonad ▪ Left gonad ▪ Uterus ▪ Prostate ▪ Masculini-zation score ▪ Puberty at presentation ▪ Date of assessment ▪ Tanner stages	▪ According to key features	▪ Clinical information ▪ Growth data ▪ Puberty data ▪ DNA ▪ Tissue ▪ Cell line ▪ Urine ▪ Serum

Table 2: Data available in the core data set for DSD

We have set out to meet the highest ethical standards, because DSDs are a very sensitive issue and patients might be highly disturbed if security and ethical issues are not addressed in an appropriate manner. All data were entered with respect to the individual consent level given by the patients and thus are available only to a subset of partners, to all partners or even to international collaborators (Figure 4). All data are pseudoanonymized and personal information is not available. Biomaterials are kept in a decentralized fashion and were only sent upon request after a positive search of the data base.

Due to high professional international interest, the consortium had discussed extensively to open up the registry to other international institutions that fulfil high security and ethical guidelines that were established in *EuroDSD*. At the time of the midterm report, the University of Ghent in Belgium was established as an additional partner to *EuroDSD* to incorporate clinical data of DSD patients into the registry after the ethical review and the consent papers had been demonstrated to the PCC (project coordination committee) and were published on our website. Until the end of the project, the data base was used by eighteen clinical centres mostly from Europe and from Arabian countries for patient entry. Overall, 1001 index cases could be entered into the data base until the end of the reporting period. Furthermore, the data base was used by centres from other continents to study their own patients in a similar fashion as in the *EuroDSD* project. This will be particularly valuable, because it will allow in the future a comparable design of clinical descriptive studies on DSD.

With continuous evolvement of the data base, the surrounding Virtual Research Environment was implemented according to the requirements of partners. As more research results were obtained on individual patient data sets, they were uploaded to complement the clinical data set. Thus, a true Virtual Research Environment was created, which is continuously adapted to the need of the partners. This approach has been copied by other FP7 funded research projects such as *EURO-WABB, Ensat-Cancer,* and *DiPAR*. The data base will continue as I-DSD under the auspices of the European Society for Paediatric Endocrinology (project leader S. Faisal Ahmed, Glasgow) and has received funding from the Medical Research Council of the UK for the next five years.



Centre	Country	Data for local institution access Only	Data For national access Only	Data for EEA access only	Data for access by all international collaborators	Total	Data & Biomaterials for EuroDSD Programme
Muenster	Germany	1	0	0	0	1	1
Khartoum	Sudan	0	0	0	19	19	19
Stockholm	Sweden	4	10	22	0	36	17
Luebeck	Germany	10	7	52	29	98	67
Barcelona	Spain	10	0	0	0	10	1
Ghent	Belgium	0	0	85	0	85	77
Lyon	France	24	0	17	0	41	6
Kiel	Germany	0	0	13	1	14	9
Cairo	Egypt	0	0	1	0	1	1
Rotterdam	Netherlands	71	0	108	0	179	77
Pisa	Italy	11	6	73	4	94	65
Bologna	Italy	60	0	3	1	64	5
Birmingham	United Kingdom	3	0	19	0	22	2
Istanbul	Turkey	1	0	63	3	67	37
Glasgow	United Kingdom	0	19	4	0	23	2
Kuwait	Kuwait	0	0	0	4	4	1
Cambridge	United Kingdom	0	163	22	17	202	24
Prague	Czech Republic	0	1	39	1	41	25

Figure 4: Overview on the entry of clinical data of the DSD data base, showing the individual partners, the number of patients entered, and the level of consent reached. Also the availability of biomaterials is shown in this context.

Identification of new genetic markers

Diagnostic sequencing of genes known to be involved in development of the genitourinary tract in the human is both time-consuming and laborious using conventional Sanger sequencing technology. High-throughput, high-density

sequencing using microarray technology offers the possibility to rapidly and accurately sequence large portions of the genome. We aimed to design and validate a novel microarray GeneChip dedicated to DSD in work package 02. The design of the DSD GeneChip representing 36 genes known to be involved in gonadal development was accomplished and completed. The selection of genes was performed from an initial set of 69 candidate genes that were proposed by agreement amongst the consortium members. The decision to reduce this number was made according to the array format (max 100kb in triplicates) and a reasonable number of PCR amplifications in the experimental strategy. At the end, a final list of 36 genes was selected representing 3 different functional groups (Table 3).

Functional Group	Gene	Gene Symbol	UCSC ID	Gene Name
Gonadal	SOX9 (TES)	-	-	Gonad specific enhancer element for SOX9
	AMH	AMH	uc002lvh.2	anti-mullerian hormone
	AMHR2	AMHR2	uc001scx.1	anti-mullerian hormone receptor, type ii
	AR	AR	uc004dwu.1, uc004dwv.1	androgen receptor
	CBX2	CBX2	uc002jxb.1, uc002jxc.1	chromobox homolog 2 (pc class homolog, drosophila)
	DHH	DHH	uc001rtf.1	desert hedgehog homolog (drosophila)
	DMRT1	DMRT1	uc003zgv.1	doublesex and mab-3 related transcription factor 1
	FGF9	FGF9	uc001uog.1	fibroblast growth factor 9 (glia-activating factor)
	FKBP4	FKBP4	uc001qkz.1	fk506 binding protein 4, 59kda
	GATA4	GATA4	uc003wuc.2	gata binding protein 4
	MAMLD1	MAMLD1	uc004fee.1	mastermind-like domain containing 1
	NR5A1	NR5A1	uc004boo.1	nuclear receptor subfamily 5, group a, member 1
	RSPO1	RSPO1	uc001cbl.1	r-spondin homolog (xenopus laevis)
	SOX9	SOX9	uc002jiw.1	sry (sex determining region y)-box 9 (campomelic dysplasia, autosomal sex-reversal)
	SRD5A2	SRD5A2	uc002rnw.1	steroid-5-alpha-reductase, alpha polypeptide 2
	SRY	SRY	uc004fqg.1	sex determining region y
	TSPYL1	TSPYL1	uc003pwp.2	tspy-like 1
	WT1	WT1	uc001mtn.1	wilms tumor 1
Hypothalamic-pituitary	FSHB	FSHB	uc001msl.1	follicle stimulating hormone, beta polypeptide
	FSHR	FSHR	uc002rww.1	follicle stimulating hormone receptor
	GNRH	GNRH1	uc003xen.2	gonadotropin-releasing hormone 1
	GNRHR	GNRHR	uc003hdn.1	gonadotropin-releasing hormone receptor
	KISS1	KISS1	uc001har.1	kiss-1 metastasis-suppressor
	GPR54	KISS1R	uc002lqk.2	kiss1 receptor
	LHB	LHB	uc002plt.1	luteinizing hormone beta polypeptide
	LHCGR	LHCGR	uc002rwu.2	luteinizing hormone/choriogonadotropin receptor
	NR0B1	NR0B1	uc004dcf.2	nuclear receptor subfamily 0, group b, member 1
	PROK2	PROK2	uc003dpa.2	prokineticin 2
	PROKR2	PROKR2	uc010gbj.1	prokineticin receptor 2
	PROP1	PROP1	uc003mif.1	prophet of pit1, paired-like homeodomain transcription factor
	TAC3	TAC3	uc001smn.1	tachykinin 3 (neuromedin k, neurokinin beta)
	TACR3	TACR3	uc003hxe.1	tachykinin receptor 3
Steroid hormone synthesis	CYP17A1	CYP17A1	uc001kwg.1	cytochrome p450, family 17, subfamily a, polypeptide 1
	CYP21A2	CYP21A2	uc003nze.1	cytochrome p450, family 21, subfamily a, polypeptide 2
	HSD17B3	HSD17B3	uc004awa.1	hydroxysteroid (17-beta) dehydrogenase 3
	HSD3B2	HSD3B2	uc001eht.1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2
	STAR	STAR	uc003xkv.1	steroidogenic acute regulator

Table 3: Genes included in the *EuroDSD* GeneChip. The amplicons also include the TESCO motif that is required for gonad specific expression of Sox9 in the mouse.

The next step consisted on the development of a bioinformatics tool to identify and define the sequences of interest belonging to the 36 genes (and the TESCO enhancer region) to be interrogated by the GeneChip. A bioinformatic tool was developed based on UCSC genome browser (Human genome version hg18). It identified the 213 genomic regions of interest (79kb) to be interrogated on the array. For each gene, targeted sequences were: promoter (1kb), all coding exons flanked by 50pb of surrounded introns (no 3'UTR sequences). The following step was the design of PCR primers and the development of common PCR protocols for all amplicons. Conventional Sanger sequencing was performed on all PCR amplicons from one patient sample to confirm that each amplicon corresponded correctly to the gene fragment of interest. This showed that all amplified products corresponded to the appropriate genic region. The GeneChip was manufactured by Affymetrix and 15 DNA samples were amplified for the 213 amplicons. The pooled DNA amplicons were then subjected to fragmentation, labelling and hybridization. Array data was analyzed and base calls were made using the Affymetrix GSEQ software package version 4.1. Data analysis in GSEQ software was performed at three 'Score' levels. The 'Score' level is a base calling GSEQ parameter that determines the stringency of the base calling algorithm. A Score of 12 results in conservative base calling (more ambiguous N base calls) while a Score of 1 is the most liberal (less N calls, but potential miscalls). For the validation of the array, base calls were made with a score of 1. As shown in table 10, in the example of 10 patients, on average 94.73 of all sample amplicons proceeded to sequence analyses and base calling.

QC using Centered replicate (13)	SDS_01	SDS_02	SDS_03	SDS_04	SDS_05	SDS_06	SDS_07	SDS_09	SDS_10	SDS_11
Total Regions of interest	213	213	213	213	213	213	213	213	213	213
Failed Regions	11	10	11	8	6	6	14	14	19	19
Failure Rate	5,2%	4,7%	5,2%	3,8%	2,8%	2,8%	6,6%	6,6%	8,9%	8,9%
Total positions on array	82 526	82 526	82 526	82 526	82 526	82 526	82 526	82 526	82 526	82 526
Total positions on good QC regions	76 277	77 793	77 341	78 135	78 385	79 079	74 184	73 291	71 276	71 309
Total Called position	73 929	74 875	74 470	75 402	75 742	76 150	71 542	70 976	68 650	69 248
Call Rate	96,9%	96,2%	96,3%	96,5%	96,6%	96,3%	96,4%	96,8%	96,3%	97,1%

Table 4: Summary of amplicon fail rate and the respective base pair call rates shown for 10 examples.

The average call rate is high with 96.5% of all bases successfully called. To determine the accuracy of the array compared to classical Sanger sequencing, a total of 30,841 bp was sequenced independently by the teams of IP, Paris, UCL, London and WWU, Munster using the sample SDS_05. This analysis of 55 independent amplicons revealed the presence of 27 single base pair variants. The sequence analyses of the identical amplicons by the GeneChip array revealed 22 false positive variants. In addition of the 27 known variants, 13 were successfully identified by the array. The major concern of sequencing arrays is the high false positive rate, which is generally in the range of 0.1-0.2%. These can arise through various mechanisms such as PCR failure, SNP interference, cross-hybridisation and repetitive sequences. The elimination of false positive calls is essential to reduce the need to perform confirmatory capillary resequencing. Based on other array platforms we would predict the number of false positives in a total of 30,841 bp to be in the range of 30-60. Here, we found that the false positive rate is very low compared to other array platforms with a total false positive rate of 0.0007. This is due to the fact that all sequences are interrogated in triplicate on the array. However, the high false negative rate is of some concern and this may be due to position of the SNP relative to one another (many of the variants that were not called are very closely linked). The array did not detect insertion duplication/deletion events but this was to be expected from this type of platform.

There is a considerable body of evidence in the literature that errors in human gonadal development are often associated with duplication and deletion events in the human genome Therefore, work package 02 aimed to identify deletion/duplication events in the human genome that are associated with DSD using high resolution microarray comparative genomic hybridisation (CGH) and thereby identifying new genetic markers of DSD to be tested on defined patient samples. The array CGH was performed in platforms in London, Munster and Paris on a total of 363 patients, of which a total of 86 patients were selected primarily from the *EuroDSD* data base.

Phenotype	UCL, London	IP, Paris	WWU, Munster	Total Cases
46,XY gonadal dysgenesis	39 (8 syndromic)	39 (12 syndromic)	113 (24 syndromic)	191 (44 syndromic)
46,XX ovotesticular or testicular DSD	17	21	2	40
46,XY undermasculinization	24 (6 syndromic)	25 (2 syndromic)	55 (14 syndromic)	104 (22 syndromic)
MKRH syndrome	1	2	2	5
Anorchia	2	6	0	8
Leydig cell hypoplasia	1	0	0	1
46,XX GD	0	2	1	3
DSD with known chromosomal anomalies	0	0	11	11
Total	84	95	184	363 patients

Table 5: A summary of DSD patients included in the high resolution CGH analyses from each of the three participating research centres.

Prior to CGH analyses these patients were sequenced for mutations in known candidate genes. This analyses revealed mutations in *NR5A1* associated with simple hypospadias or male infertility (Allali et al., PLoS One. 2011 6:e24117; Bashamboo et al., 2010 Am J Hum Genet. 87:505-12) and mutations in *GATA4* as a novel cause of 46,XY DSD (Lourenço et al., Proc Natl Acad Sci U S A. 2011 108:1597-602).

The CGH analyses identified known genetic causes and in addition novel genetic markers of DSD. An example of the latter is a rearrangement of the SOX9 locus. The aCGH identified a duplication located upstream of SOX9 associated with 46,XX testicular DSD in two brothers. The rearrangement falls within a minimal critical region associated with 46,XX DSD that was recently defined and further reduces the minimal region to 46 kb. Further analyses using qPCR

with markers located within this region on a larger series of patients has identified an additional two cases of DSD carrying rearrangements. These cases significantly reduce the minimal interval associated with DSD and will be of tremendous value in identifying the key regulatory elements and represent a novel genetic marker of DSD.

Additionally, we identified a number of chromosomal rearrangements that encompassed or were located within the DMRT1 gene on 9p24. Deletions of 9p24 are associated with 46,XY gonadal dysgenesis and DMRT1 is an excellent candidate gene as it determines sex in some other vertebrate species and the mouse knockout shows postnatal testicular regression. However, attempts to conclusively demonstrate that DMRT1 is the gene responsible for the testicular dysgenesis associated with monosomy 9p24 are lacking. Here, using the CGH approach we identified deletions within the DMRT1 gene associated with gonadal dysgenesis convincingly associating this gene with the phenotype. The complete findings of the consortium with CGH array analysis are summarized in table 6.

Phenotype		Pathogenic variant	Pathogenic rearrangements	Candidate variants	Examples of candidate variants
46,XY gonadal dysgenesis	Non-syndromic (n=147)	15 (10.2%)	7 rearrangements involving DMRT1 including exon deletions. 5 AZFc deletions. 2 Xp21 duplications. 1 deletion of SRY.	7	1 case with 120 kb duplication of 9q34.2 including RXRA. 3 cases with ~ 11kb deletion of RXRA. 1 case with 11kb duplication of RXRA 1 case with 100 kb duplication of 22q13.31 including WNT7B, 1 case with 176 kb duplication including KAAG1 and DCDC2 (6p22.2), 1 case with a deletion affecting SPATA16 (3q26.31), 1 case with a duplication affecting MTNR1A and FAT1 (4q35.2), 1 case with a duplication affecting CTNNA3 (10q21.3), 1 case with a duplication including GKAP1 (9q21.32), 1 case with a duplication including bMSMB and NCOA4 (10q11.23)
	Syndromic (n=44)	6 (13.6%)	2 deletions of 9p24. 1 deletion of regulatory region of SOX9. 1 interstitial deletion of Ch. 17. 1 duplication of Xp21 including DAX-1. 1 l deletion of 10q24-pter	2	1 case with deletion of MAPKBP1. 1case with ~ 11kb deletion of RXRA
46,XX ovotesticular or testicular DSD	N=40	1 (2.5%)	1 microduplication in upstream regulatory region of SOX9.	5	1 case with 10 kb deletion located 100 kb 3' to SOX13. 1 case with 2.21 Mb duplication of 12q32.1 including HMGA2. 1 case with deletion 5' to CBX gene cluster. 1 case with 429 kb duplication of 10q26.13 including CTBP2 and an independent 400 kb deletion 9 kb 5' to MAP2K2. 1 case with deletion of WNT9A and WNT3A at Ch.1q42.13.
46,XY undermascu-linization	Non-syndromic (n=83)	0	-	-	
	Syndromic (n=22)	2 (9.1%)	1 deletion of 19 Mb within Ch. 2q31.3-q33.1. 1 deletion of 5.2 Mb in Ch. 7p21.2-p15.3.	4	2 cases with 100 kb duplications of 9q34.2 including RXRA. 1 case with 270 kb duplication of 22q13.31 including WNT7B, 1 case with duplication affecting SPECC1 (17p11.2), 1 case with

					duplication including ADCYAP1 (18p11.32),
MKRH syndrome	N=5	0	-	-	-
Anorchia	N=8	0	-	1	1 duplication of ch 20p12.3 including BMP2.
Leydig cell hypoplasia	N=1	0	-		
46,XX GD	N=3	0		2	2 sisters with duplication of PYGO1, 1 syndromic case with 12 Mbp deletion 5p15.3-p15.2 and adjacent duplication of ~25,95 Mbp in 5p15.2-p13.1
DSD with chromosomal anomalies	N=11	-	7 cases with 45X/46,XY, 2 with AZFc microdeletions. 1 case 45,X/46,Xi(Y)(p/o) 3 cases 46,XX/46,XY	-	-

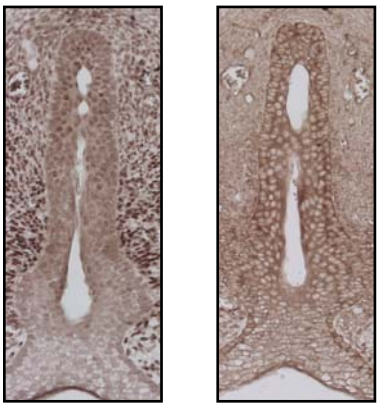
Table 6: Summary of CGH results on 363 patients with DSD. The number of patients with each phenotype is indicated. Examples of candidate pathogenic variants are shown.

Functional assessment of androgen action

In most cases of DSD there is a wide phenotypic variability even in the event of the same underlying molecular abnormality. Sex phenotype depends on the facilitation of androgen action via the androgen receptor (AR) during sensitive periods of development. In work package 03, we aimed at identifying modulators of androgen action influencing embryonic and pubertal development. A cDNA library derived from the male mouse genital tubercle as well as of the urogenital folds and the genital swellings was constructed for a yeast two hybrid screen to search for developmentally specific androgen receptor co-regulators using the activated androgen receptor ligand binding domain as bait. We have used this approach to search for AR binding partners in genital tissue of male mouse embryos at the embryonal development days E15 and E16. The clones selected from the cDNA-library were identified by sequencing. 71 different proteins were identified. Besides 14 already known androgen receptor co-regulators, we have found several new candidate genes for AR binding partners. 18 candidate genes that play a role in proliferation, cell cycle control and differentiation were analyzed in part by biochemical and cellular assays. Interestingly, in 10 of these genes we could identify FxxLF and LxxLL motifs in the corresponding protein sequence. These motifs might be important for the interaction with the ligand binding domain of the AR, as it has been shown for already known co-regulators of the receptor.

One newly identified coregulator, Rwdd1, was found in 9 independent clones from all three libraries and showed an enhancement of androgen receptor transcriptional activity at three different promoters in HeLa and Cos1 cells in transient transactivation assays. The cellular function of this protein is completely unknown so far. Cotransfection assays using a common minimal promoter with two glucocorticoid responsive elements, but different nuclear receptors (glucocorticoid receptor; GR, mineralocorticoid receptor MR) showed also a positive effect of this cofactor on GR and MR activity, indicating that this protein might be a general nuclear receptor coregulator. Although the newly identified coregulator acts as a coactivator, the protein showed no intrinsic activation domain. In-vitro protein binding assays demonstrated a physical DHT-independent interaction with the androgen receptor. With a TAP-tagged androgen receptor the coregulator could also be coimmunoprecipitated from nuclear extracts of P17 cells, confirming the interaction within the cell. Cotransfection of the coregulator with androgen receptor-L712F (a mutant found in three patients with partial androgen insensitivity with highly variable phenotype) lead to activation of the mutant androgen receptor activity and revealed an AF2-independent mechanism of action. Immunohistochemical staining of the coregulator in genital tubercles at E16 and E17 showed a mainly cytoplasmic localization with a more pronounced expression in the urethral plate epithelium of the developing male urethra. To get more information on the function of this new coregulator, we have knocked down the coregulator in androgen responsive LNCaP cells. Successful knock down and androgen responsiveness was confirmed by RT-PCR and Western blotting and the effect on genome-wide expression of androgen dependent target genes was compared with that of control siRNA treated cells by microarray hybridization. Full-length cDNAs of human orthologs of several other potential androgen

receptor coregulators identified in the screenings have been cloned into mammalian and bacterial expression vectors and have been confirmed as androgen receptor interacting proteins by GST-pulldown assays with in-vitro translated androgen receptor.



E17, AR E17, Rwdd1

Figure 5: Immunohistochemical stainings of transverse sections of E17 genital tubercle from male mouse embryos. Transverse sections were stained with an AR or Rwdd1 antibody. The lower pictures show an enlarged image section of the urethra. Scale bars = 200 µm (upper images) or 50 µm (image sections)

To get further insight into the developmental impact of androgen receptor mutations, we developed a murine MFVD cell line for functional analysis of androgen receptor mutants associated with partial androgen insensitivity syndrome (PAIS). Comparing AR co-regulator profiles in proliferating versus non-proliferating P17 cells, we identified 1196 AR associated proteins in FLAG purified extract from proliferating cells and 1456 AR associated proteins in FLAG purified extracts from non-proliferating cells. Of those 882 were common between those two groups, 314 were specific to proliferating and 574 specific to non-proliferating cells. Among the co-purified proteins were 58 known wild-type androgen receptor interacting proteins, identified in cells grown under proliferating and of 65 under non-proliferating conditions, 48 of those were common to both samples, suggesting differences of AR protein complex components in proliferating and proliferation inhibited cells from the mouse proximal caput epididymis.

These results were meant to define the molecular basis of androgen action during early sexual development with respect to assess the possibility for prognostic value in predicting the necessity for androgen treatment during puberty in patients with partial androgen insensitivity raised as males. Pubertal outcome data was obtained from a total of 20 patients, which between them had 14 different AR mutations. Only three out of 20 patients were given androgens to induce puberty, those with AR mutations S703G, F754L and R840C. A further two patients (AR mutations R840H and F754S) started spontaneous puberty but were given androgens in order to complete pubertal progression. It was interesting to note that while one patient with R840C was given androgens to induce puberty, a further three patients with the same mutation entered puberty spontaneously. This highlights the variable *in vivo* effects of some AR mutations.

A total of 19 androgen receptor mutations that were identified at the start of the project were functionally analysed. The transactivation ability of all 19 mutants was assayed using the GRE/COS1 and the PEM/Hela reporter/cell combinations. Ligand binding affinity, Ligand Dissociation rate and N-Terminal/C-Terminal interaction assays were also carried out on relevant mutations. The majority of the AR mutations displayed a loss of transactivation activity that would be consistent with a PAIS phenotype. The exceptions were mutations from the DNA binding domain and hinge region. Defective ligand binding or dissociation rates were identified in all but two of the ligand binding domain mutations. Greatly increased N-terminal/C-Terminal interaction was recorded for mutations F673C, R840C and R840H. These mutations are located in a poorly characterised region termed BF-3, which has been shown to allosterically modulate function of co-regulator binding surface AF-2. The in vivo effects of increased N-terminal/C-terminal interaction remain unclear.

The function of mutations associated with induced puberty was lower at physiological concentrations of androgen than those who went through puberty spontaneously. GRE assay 10 nM DHT, $p=0.05$, PEM assay 1 nM DHT, $p=0.14$. No relationship was seen between spontaneous puberty and ligand binding affinity, ligand dissociation or N-terminal/C-terminal interaction. Clinically defined Low External Masculinisation Score (EMS) as calculated in the registry and the presence of a micro penis at birth were associated with the need for pubertal induction ($p=0.05$ and $p=0.03$ respectively).

AR mutation	Activity at 1nM DHT(PEM)	Activity at 100nM DHT (PEM)	Activity at 1nM DHT(GRE)	Activity at 100nM DHT (GRE)	N/C interactio at 1nM DHT	N/C interactio at 10nM DHT	Ligand bindingK_d [nM	Ligand dissociation [min]
WT AR	55.3 ± 5.3	100.0 ± 4.3	22.1 ± 3.1	100.0 ± 10.5	101.7 ± 3.7	100.0 ± 4.7	0.31 ± 0.07	189.5 ± 33.0
G568V	1.1 ± 0.1	1.1 ± 0.2	4.4 ± 0.7	8.7 ± 1.0	no data	no data	N/A	N/A
A596T	24.0 ± 2.2	51.2 ± 8.0	37.1 ± 10.6	92.8 ± 20.2	no data	no data	N/A	N/A
R629W	40.7 ± 7.0	84.1 ± 15.1	35.4 ± 5.8	117.5 ± 16.2	105.8 ± 52.3	88.5 ± 40.2	0.21± 0.03	190.1 ± 38.5
I664T	27.6 ± 5.9	102.0 ± 5.2	7.9 ± 0.9	44.0 ± 6.5	90.14 ± 5.4	130.5 ± 18.7	1.02 ± 0.18	191.1 ± 17.1
F673C	3.5 ± 0.7	100.0 ± 15.2	7.1 ± 3.5	64.7 ± 7.6	80.0 ± 16.7	380.1 ± 28.0	1.30 ± 1.00	68.9 ± 2.5
A687V	63.1 ± 5.4	112.0 ± 4.2	3.9 ± 0.7	91.9 ± 13.7	5.5 ± 1.3	74.6 ± 1.3	0.63 ± 0.05	33.6 ± 1.5
D690E	7.7 ± 1.1	101.9 ± 13.2	2.1 ± 0.3	61.0 ± 4.7	24.8 ± 1.2	95.0 ± 6.6	0.44 ± 0.29	47.8 ± 9.9
S703G	5.9 ± 1.6	91.1 ± 6.8	6.1 ± 1.0	78.6 ± 11.0	no data	no data	0.46 ± 0.24	56.7 ± 7.1
L712F	40.8 ± 8.9	82.9 ± 3.5	11.1 ± 3.5	52.6 ± 6.2	2.7 ± 0.2	3.5 ± 0.6	0.37 ± 0.14	86.8 ± 6.7
F754L	6.7 ± 1.8	88.7 ± 11.4	4.7 ± 0.5	85.4 ± 8.1	65.7 ± 13.6	83.0 ± 22.5	0.95 ± 0.40	90.0 ± 23.5
F754S	20.6 ± 3.8	92.1 ± 19.2	4.3 ± 0.5	68.1 ± 8.6	58.1 ± 11.2	71.3 ± 17.2	0.49 ± 0.06	63.7 ± 2.6
Y763C	25.4 ± 8.8	74.6 ± 4.2	8.8 ± 4.1	72.8 ± 11.8	40.8 ± 10.8	82.4 ± 13.6	0.42 ± 0.04	56.0 ± 9.2
Q824H	23.6 ± 4.8	86.8 ± 12.9	10.8 ± 2.8	40.2 ± 8.6	71.4 ± 12.1	81.7 ± 5.0	0.47 ± 0.28	208.2 ± 14.5
Q824K	42.0 ± 7.1	86.9 ± 16.2	16.9 ± 7.5	48.2 ± 6.7	83.2 ± 13.3	95.2 ± 7.6	0.33 ± 0.19	108.6 ± 9.4
R840C	3.7 ± 1.0	69.4 ± 8.3	6.5 ± 1.2	101.7 ± 12.4	351.1 ± 125.4	574.3 ± 231.6	2.18 ± 1.73	200.9 ± 6.0
R840H	2.5 ± 0.6	55.9 ± 7.1	5.0 ± 1.2	43.2 ± 3.4	143.9 ± 49.1	323.6 ± 9.0	1.60 ± 0.21	117.9 ± 13.1
R855H	4.0 ± 0.4	98.6 ± 6.5	3.0 ± 0.6	39.1 ± 4.3	35.4 ± 11.0	75.3 ± 15.2	2.01 ± 1.59	123.4 ± 21.6
I869M	21.2 ± 7.6	102.5 ± 4.2	5.7 ± 0.8	69.6 ± 7.1	56.3 ± 2.2	79.1 ± 2.8	1.32 ± 1.14	139.5 ± 32.0
A870V	36.4 ± 5.4	91.1 ± 3.2	11.8 ± 2.7	78.6 ± 6.7	no data	no data	0.35 ± 0.08	164.7 ± 9.7

Table 7: Summary of functional data for AR mutations

Characterization of androgen memory

It is known that during embryogenesis, epigenetic mechanisms are involved in establishing and maintaining tissue specific gene-expression patterns. Epigenetics literarly means “upon genetics” and includes all modifications that alter gene expression without changing the DNA sequence itself. DNA methylation in CpG dinucleotides within gene promoters is among the most important epigenetic mechanisms contributing to tissue specificity. There is evidence that the androgen receptor regulates transcription by recruiting chromatin remodelling factors and histone modifiers that render the chromatin accessibility to the transcription machinery.

For the first time, we proved the existence of a long term epigenomic androgen memory in work package 04 by demonstrating different methylation signatures in genital fibroblasts comparing phenotypically male and female XY individuals having normal and mutated androgen receptors, respectively. 38 fibroblast strains were analysed on arrays (10 normal controls in duplicate each and 28 AIS cell strains, predominantly in duplicates each). In total, more than 100 – 250 sex – dimorphic methylation target genes have been identified, depending on filtering stringency and tissue analysed. Our most recent meticulous analyses show that patients with inactivating mutations of the androgen receptor gene have significant differences of the degree of methylation in 167 CpGs ($q < 0.04$). This corresponds to 162 different human genes. All methylation microarray data have already been posted on GEO and will be open to the public upon acceptance of our manuscript, which is currently prepared.

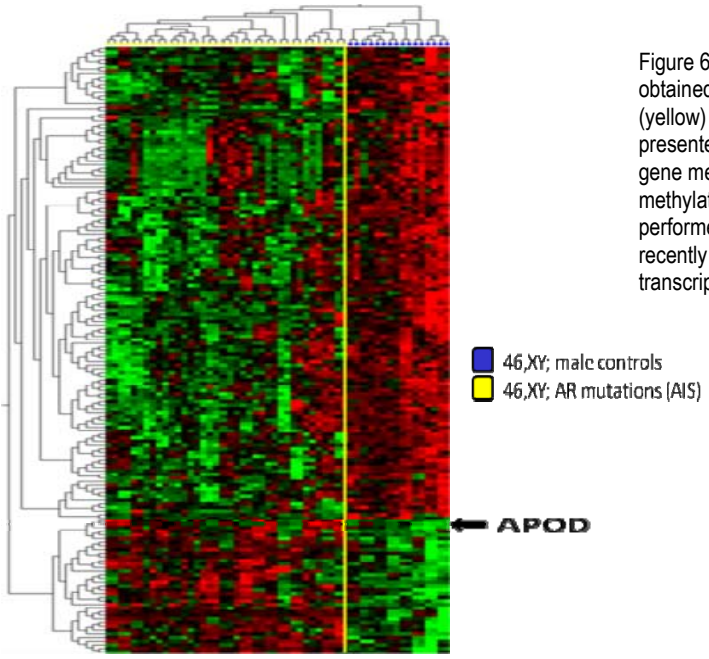
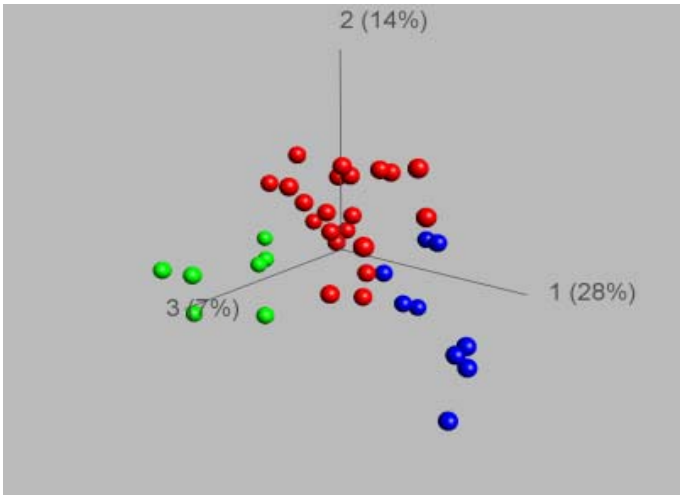


Figure 6: Supervised cluster analysis of DNA-methylation data obtained from genital fibroblasts separates individuals with AIS (yellow) from male controls (blue) (q<0.04). DNA methylation is presented on a relative scale. Increasing red means increasing % gene methylation, increasing green corresponds to decreasing gene methylation. To demonstrate reproducibility all hybridizations performed in duplicates are shown separately. APOD is a gene recently found by the researchers to be directly regulated in its transcription by androgen action

As written in the original grant proposal, epigenomics has undergone an extreme innovation of technology. Instead of the Affymetrix Tiling Arrays reported in the proposal, we used the HumanMethylation27 BeadChip (Illumina) containing 27,578 CpG sites corresponding to >14,000 human genes. This technological achievement went along with a significant drop of prices for methylation arrays. Therefore, recent technological developments did not support a reduced custom platform as in the original proposal, but clearly supported keeping with the large-scale HumanMethylation27 BeadChips for all samples investigated. This offered even more opportunities since all *EuroDSD* samples could be analysed on the large-scale platform without the restrictions of a custom platform initially proposed to save money when aiming at analysing large quantities of cell strains. An important advantage of this strategy was that informative methylation signatures of cell strains from patients with 17ß hydroxysteroid dehydrogenase deficiency and 5 alpha reductase type 2 deficiency did not necessarily restrict us to those genes identified as being different between CAIS and normal males but could unveil unique features of methylation (disease specific methylation signatures). In total, 46 additional microarrays with different phenotypes of AIS and also with mutations in genes coding for androgen biosynthesis (17ßHSDIII, SRD5A2) have been hybridized. Data analyses showed that different defects of androgen biosynthesis and action showed i*ndividual patterns different from AIS indicating disease specific signatures of DNA methylation reflecting the underlying DSD-diagnosis (Fig.4).* This is of high interest for European collaborative studies since it points to relevant and elegant diagnostic potential of epigenotyping in the future diagnostic workup of DSD

Figure 7: Principal component analysis (PCA) of fibroblasts obtained from patients with mutations either in the AR- (red), SRD5A2- (green) or 17ßHSDIII- (blue) gene based on the CpG-loci showing the highest variance (/max=0.066). In essence, PCA uncovers the existence of epigenomic signatures specific for 17ßHSDIII-, SRD5A2-, and AR-gene defects.



However, this work package could not only demonstrate androgen memory at the epigenome level in genital fibroblasts but, moreover, it showed its existence also in peripheral blood mononuclear cells (PBMC)-derived genomic DNA. This finding corresponds well with our previous data at the transcriptome level (Holterhus et al. 2009,

BMC-Genomics). Due to the much better availability of blood samples compared with fibroblasts, the findings on PBMCs strengthen future clinical utility of epigenomics in diagnostic categorization of DSDs.

Steroid metabolomics

In work package 05, we employed steroid profiling by gas chromatography/mass spectromy (GC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) (task 1) as a discovery tool in patients with DSD to identify hitherto unknown steroidogenic disorders as underlying biochemical and genetic causes. During the course of the project around 150 plasma/serum samples and 150 urine samples were collected. The samples were obtained from patients with a broad range of underlying diagnoses of DSD including unknown causes of 46,XY DSD and 46,XX DSD; patient ages also varied from newborn to adulthood, which provided us with some possibilities for analysis of age differences in steroid secretion, with the aim to identify age-specific diagnostic parameters for the rapid identification of the underlying diagnosis.

Steroid data were entered into the local databases and linked via web-based communication to the virtual research environment VRE. We successfully implemented software for the automated transfer of steroid output data from the mass spectrometry equipment to the database software and also implemented tools for the automatic visualisation of steroid output data, illustrating both the entire profile and specific steroid/product substrate ratios reflective of distinct enzymatic activities. We also generated comprehensive report formats for reporting back to the referring clinician with the aim of developing tools for implementation in routine clinical practice. Data were firstly analysed by comprehensive visualisation, as illustrated by linear and logarithmic representation of steroid excretion data of the DSD patients in comparison to the sex- and age-matched normal reference cohorts. In a second step we generated reference ranges for defined conditions underlying DSD. Thirdly, we gathered extensive experience with the application of computational data analysis, comprising of heat map generation and also of machine learning techniques for advanced data analysis. This generated very promising first results and our novel approaches to steroid data visualisation and computational analysis have yielded several publications in leading journals in the field. It is predictable that further accumulation of biomaterial (plasma + urine) from patients with defined and unknown conditions associated with DSD will strengthen this diagnostic approach further. During the course of the project we already applied it successfully for the discovery of several prismatic cases of previously unknown DSD causes, with significant beneficial consequences for the management of the patients.

We succeeded in developing a comprehensive presentation of steroid output data. This was an important aim as steroid analysis results are often difficult to understand by the clinician and communicating the meaning of the results is an important means to achieve better understanding of the underlying conditions associated with DSD.

Firstly, we developed a colour coding system for the labelling of the 32 steroid metabolites we quantify by GC/MS analysis of urinary steroid excretion (Figure 8A). This clearly indicates whether the measured steroid is part of the androgen synthesis pathway, the glucocorticoid synthesis pathway or the mineralocorticoid synthesis pathway. We generated sex- and age-specific reference cohorts (Figure 8B shows tan adult reference range; similar reference cohorts were created for healthy 46,XX and 46,XY children and adolescents). We subsequently applied the same presentation mode and colour code to the plasma steroid results that were measured by LC/MS.

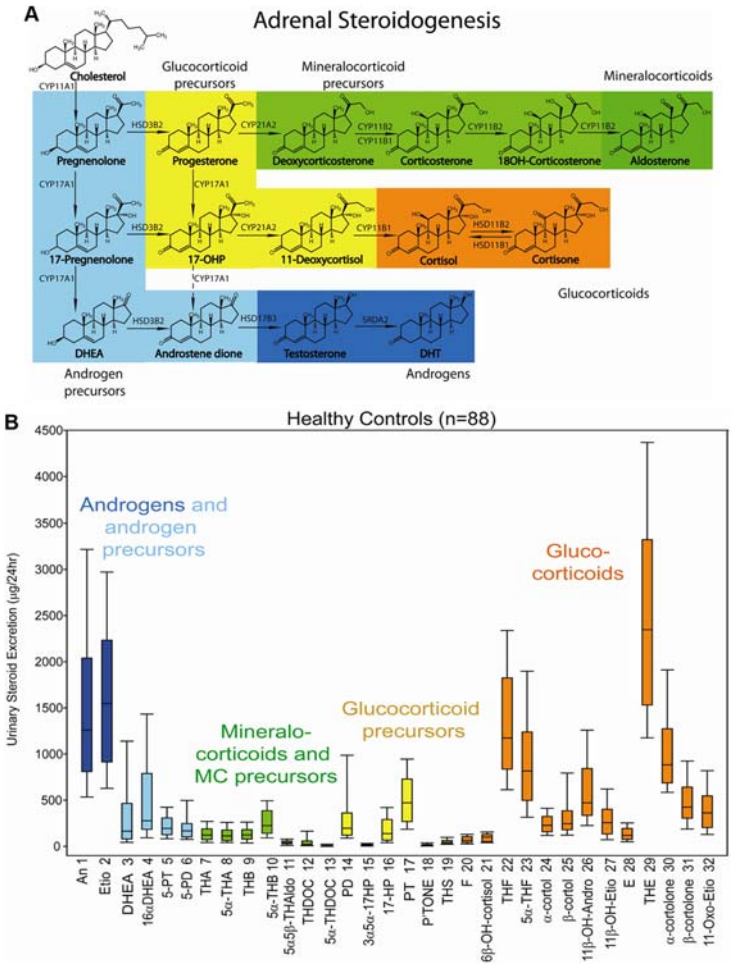


Figure 8:

A, Schematic representation of steroidogenesis depicting the major products of adrenocortical steroid synthesis, the mineralocorticoid aldosterone (*dark green*) and its precursors (*light green*), glucocorticoid precursors (*yellow*), the active glucocorticoid cortisol (*orange*) and its metabolite cortisone, and the adrenal androgens and their precursors (*light blue*). Synthesis of active androgens (*dark blue*) mainly takes place in the gonads.

B, The 24-h urinary steroid metabolite excretion in healthy controls (n = 88). Box plots represent median and interquartile ranges; the whiskers represent 5th and 95th percentile, respectively. Colour coding of steroid metabolites mirrors that used for depicting the major adrenal corticosteroid classes in A.

CYP, Cytochrome P450; HSD, hydroxysteroid dehydrogenase; DHT, 5α-dihydrotestosterone.

Importantly, for the LC/MS analysis, one initial task was the establishment of reference intervals for gonadal and adrenal steroids in children and adults, as in contrast to the GC/MS approach method-specific reference data with respect to age, sex and pubertal development were not yet available. This, however, is a prerequisite for a meaningful biological interpretation of the data. We achieved this successfully for the plasma androgens androstenedione, testosterone and dihydrotestosterone (Kulle et al. JCEM 2010, 95:2399-2409) as well as for adrenal pregnenolone, progesterone, deoxycorticosterone, corticosterone, aldosterone, 17-hydroxypregnenolone, 17-hydroxyprogesterone, deoxycortisol, cortisol, cortisone, and dehydroepiandrosterone (Kulle et al., Clin Chemistry, submitted) in 269 and 905 children and healthy adults, respectively.

For the urinary steroid excretion analysis, we also implemented the representation of steroid data in the form of diagnostic ratios, i.e. the ratio of the substrate(s) and product(s) of a specific enzymatic reaction, which therefore is indirectly reflective of the activity of distinct steroidogenic enzymes. This approach makes it relatively straightforward to diagnose circumscriptive conditions, as exemplified below for congenital adrenal hyperplasia due to 17-hydroxylase deficiency, which is a rare cause of 46,XY DSD associated with mineralocorticoid excess and glucocorticoid deficiency due to inactivating mutations in the CYp17A1 gene encoding for the 17-hydroxylase/17,20 lyase enzyme that is positioned at a crucial branchpoint of steroidogenesis (Figure 9)

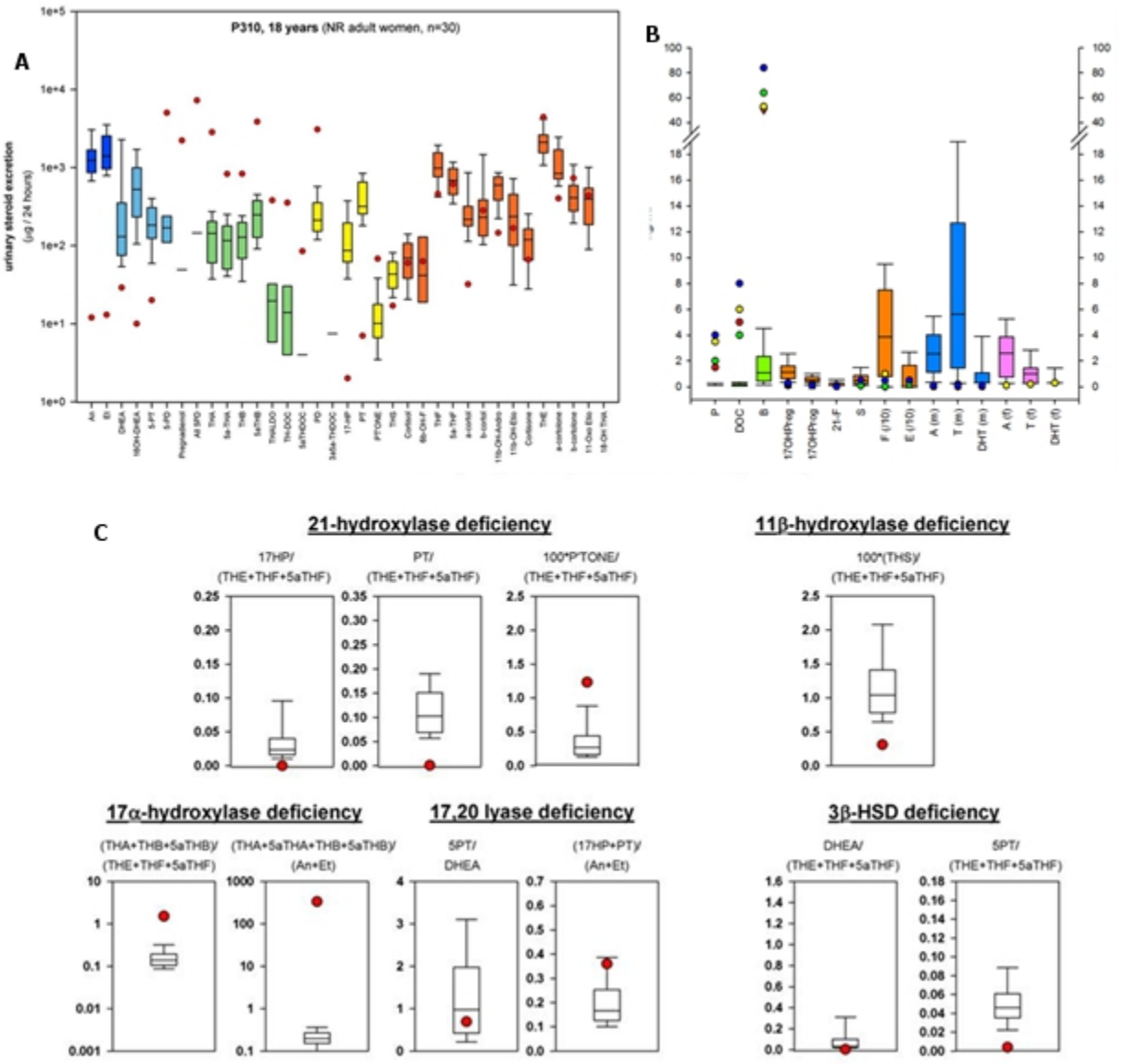


Figure 9

A, *GC/MS* 24-h urinary steroid excretion profile in an 18-year-old patient with 46,XY DSD (female gender presentation); note the logarithmic scale y axis that allows for comprehensive visualisation of all results including steroids that are present only at a low concentration. Colour coding of the normal reference range (n=30, young women) as in Fig. 8, the measurement result of the patient is represented as a red closed circle.

B, LC/MS plasma multi-steroid profile in four patients with genetically confirmed 17-hdyrxoylase deficiency projected on the age- and sex-specific reference range (in this case 0-12 months of life)

C, GC/MS results visualised as diagnostic steroid ratios indicative of distinct enzymatic activities which allows for the diagnosis of six distinct steroidogenic disorders (not shown: P450 oxidoreductase (POR) deficiency; note the significantly elevated ratios indicative of 17-hydroxylase deficiency).

Applying both GC/MS and LC/MS analysis we were able to confirm and to diagnose a broad variety of conditions in the patients with *EuroDSD* biomaterial collections, including several conditions manifesting with 46,XX DSD (21-hydroxylase-deficiency, 11-hydroxylase-deficiency, POR deficiency) as well as 46,XY DSD forms (17ß-hydroxysteroiddehydrogenase type 3 deficiency, 5-alpha-reductase type 2 deficiency, 17-hydroxylase deficiency, POR deficiency, Cytochrome b5 deficiency). Steroid data in complete and partial androgen insensitivity syndrome as well as complete and partial gonadal dysgenesis were collected, but did not allow for differential diagnosis most likely because these diseases are not primarily steroidogenic and because of a degree of heterogeneity in that data set (absence or presence of gonads, broad age range, concurrent treatment).

In the prospective setting, we were able to collect and analyze by LC/MS repetitive samples in DSD patients of different age. We were able to show that androgen levels (A, T, DHT) at birth do not correlate with the genital phenotype. Furthermore we were able to diagnose several cases of 46,XY DSD and followed these during the next

three years including e.g. the treatment with DHT. Steroid determination and clinical work up led to the diagnosis of StAR deficiency in two patients with 46,XY DSD with sex reversal and adrenal failure. The mutational analysis performed in these families revealed homozygous mutations in the STAR gene without residual activity therefore pathogenetic for both cases (Bens et al. JCEM 2010;95:1301-1308) (Fig. 5). Follow up data on 14 patients with partial androgen insensitivity and 23 patients with complete androgen insensitivity were collected and the natural cause of gonadal and adrenal steroids in this DSD form will be reported in the near future.

DSD e-learning web portal

Within *EuroDSD* we aimed to develop an e-learning portal in work package 06 that took the newest information of the project to health care providers to enhance understanding and knowledge of DSD. As the Council of the European Society for Paediatric Endocrinology (ESPE) had pledged its financial support of the development of the basic requirements of an e-learning web portal on DSD, most of the technical foundation of the web portal had been laid by February 2008 prior to the project start. In the *EuroDSD* project we aimed to extend the pre-existing CME functionalities of the portal and construct a reviewing, reporting and content management functionality which enables multiple authors and editors to interact in the creation of the content. Subsequently, new medical content could be developed for different target groups at post-doc level: residents, fellows, specialists, and researchers. Furthermore, we constructed forum functionality. This forum functionality enables the users to post comments and remarks and discuss certain topics. The forum can be used for specific discussions on a case or study results or knowledge sharing. Several levels of accessibility are foreseen. Finally, a Mediclopedia was constructed offering the user the possibility to search for specific subjects in an alphabetically ordered list with subjects. The programme will continue under the auspices of the ESPE and is meant to sustain among other endocrine e-learning programmes.

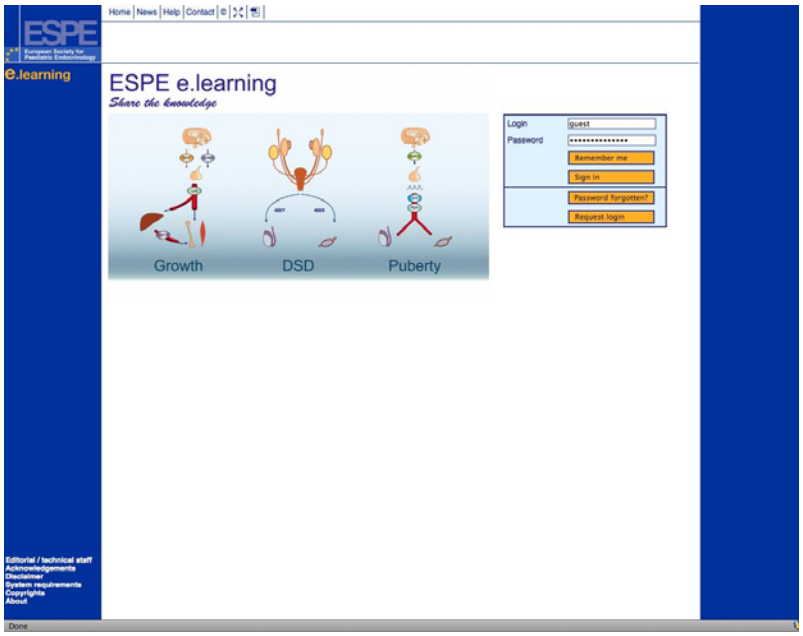


Figure 10: The homepage of the e-learning web portal: www.espe-elearning.org

We developed new medical content for an extended target group. The editorial board of DSD consists of: Faisal Ahmed, Glasgow, UK; Silvano Bertelloni, Pisa, I; Olaf Hiort, Lübeck, D; Garry Warne, Melbourne, Aus; Stenvert Drop, Rotterdam, NL; thus mainly members of the *EuroDSD* consortium, but also experts from outside. A variety of authors representing experts in DSD of the *EuroDSD* consortium as well as outside have contributed. Current content of chapters and cases is given below:

TITLE	AUTHOR(S)
The Androgen Receptor	Albert Brinkman (NL)
Management team of DSD	Olaf Hiort (D)
Occurrence of gonadal tumors in DSD patients	Martine Cools (B), Leendert Looijenga (NL)
Psychological aspects of DSD	Arianne Dessens, Peggy Cohen-Kettenis (NL)

Psychological management of DSD	Arianne Dessens, Peggy Cohen-Kettenis (NL)
Sex assignment in the newborn	Amy Wisniewski, Sowmya Krishnan, Jeanie Tryggestad (USA)
Sex development	John Achermann (GB)
Steroid hormone biosynthesis and its disorders	Wiebke Arlt, Nils Krone (GB)
Surgical management of DSD, fem. genitoplasty	Justine Schober (USA)
Surgical management of DSD, masc. genitoplasty	Justine Schober (USA)
The Initial Endocrine Approach To A Suspected Disorder of Sex Development	Faisal Ahmed, Martina Rodie (USA)
The missing link in DSD care	Ellie Margritte (GB)

Table 8: chapters of the e-learning programme and the responsible authors.

Figure 11 gives an example for the illustrative character of the e-learning programme, relying on painted images more than on photographs. Several images are also animated to demonstrate the variability of phenotype.

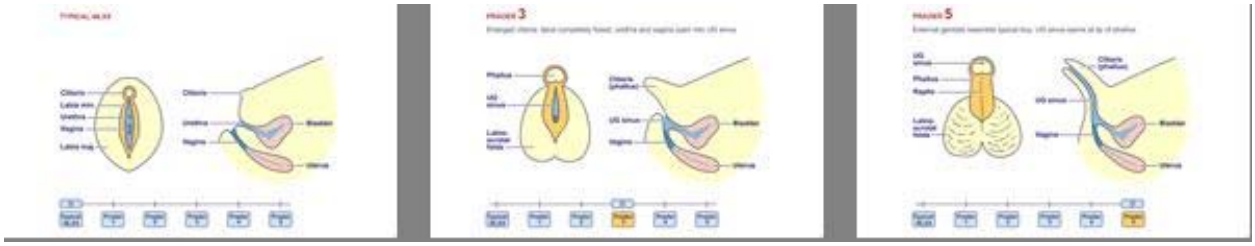


Figure 11: Prader stages in the chapter: “Surgical management of DSD; feminizing genitoplasty”

Forum functionality was constructed and further development will be influenced by feed-back functionality. It has been tested on the basis of a number of chapters and cases submitted. Based on comments and suggestions of users several adjustments were made resulting in a functional web portal module. Global access is provided through an automated login procedure via password protection.

A glossary-module has been constructed. The glossary selector is a list definitions and explanations. From every page in any chapter or case a link can be made to a definition in this glossary, as seen in figure 12.

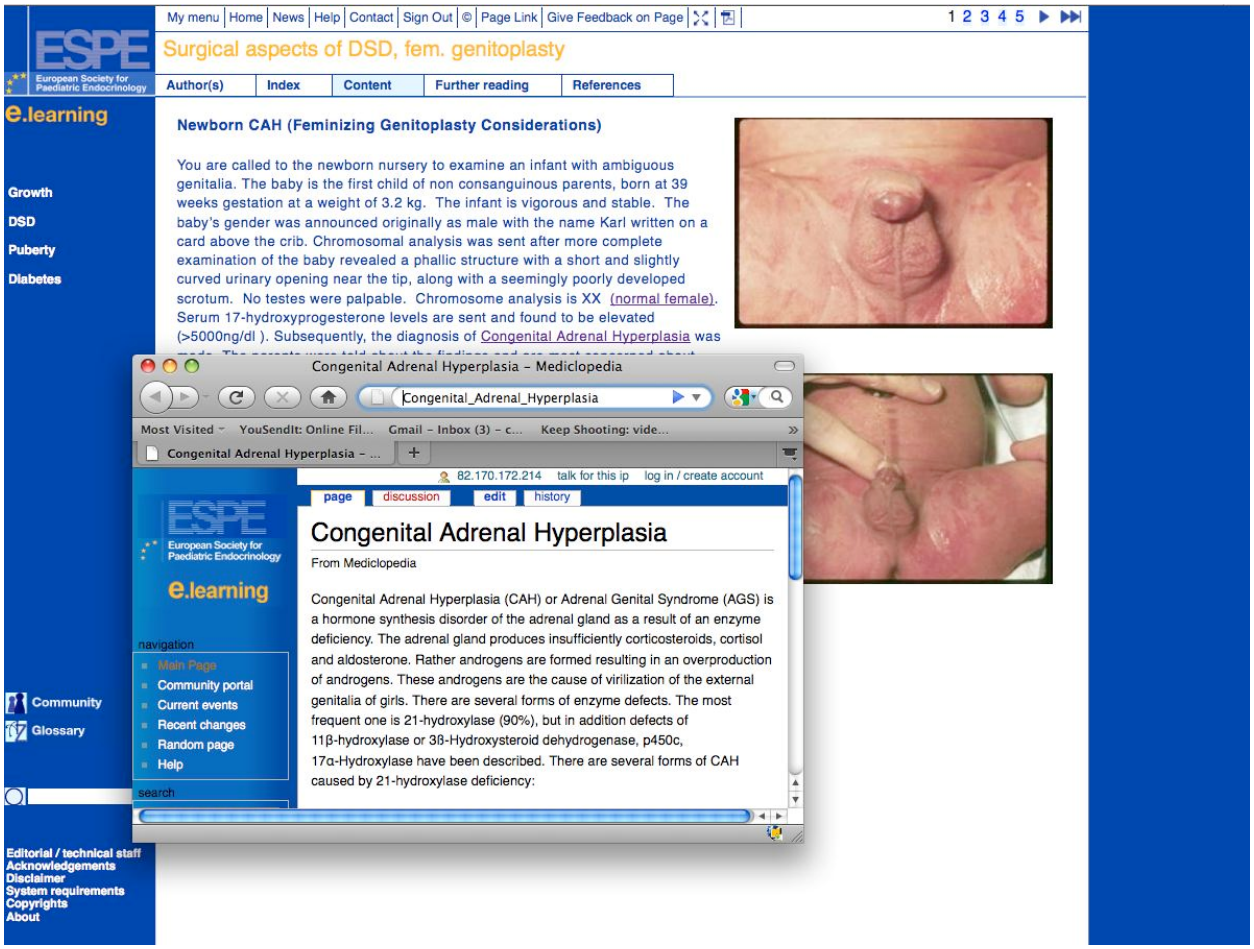


Figure 12: A pop-up window appears for further explanation after clicking on a linked definition.

In the first pilot study a selection of 3 chapters and 3 cases was studied by experts (n=9), fellows (n=12), residents (n=10) and students (n=6). There was an overall enthusiasm about the e-learning portal; the content was felt to be interesting and level of difficulty was appropriate; the average time spent per case/chapter was 30-60 min. .The number and level of questions were overall satisfactory; the quality of the questions was variable and constructive comments were given. Interaction with other uses was felt to be very useful.

Therefore a second pilot was performed evaluating personalized feedback to open questions: fellows (n=8) and residents (n=3) were asked to study two cases and to answer feedback questions; the given answers were sent to a senior expert, who provided personalised feedback in return. Both fellows and experts judged the way of interaction very positive. For the student/fellow it is a nice safe environment of learning. One expert indicated: "I think it's excellent. The students are anonymous, so they can feel free to say what they really think". In conclusion students, residents, fellows and experts consider e-learning very effective compared to other learning methods like literature and textbooks. Interaction with other users is felt to be useful, however takes time.

1.4 The potential impact

Socio-economic impact and the wider societal implications of the project

Contribution to Community and social objectives

DSD constitutes a defined group of rare disorders, some of them following Mendelian genetic traits, whilst the genetic basis of others is not elucidated. Rare disorders as a whole produce an enormous economic impact to society, because affected persons often do not find adequate diagnosis and managed care, thereby inducing enormous negative impact on them. Only the understanding of the pathophysiology of disease, development of standards for management and care, and bringing knowledge to health care providers as well as to affected individuals meets the goal of preventing chronic disabling disease and meeting the optimal prerequisites for uninhibited quality of life. DSD constitutes a very special group of rare disorders, because on the one hand, they are mostly not life threatening, nor should they constitute an obvious physical inhibition in most daily activities. However, DSD leads to an enormous disturbance both in health care providers as well as in the affected and their families and therefore have a great impact on quality of life.

A European data collection of these very rare disorders is needed to assess the natural course of the disease and to define aspects which modulate this course. The implementation of a European data collection as performed in work package 01 has brought together a critical mass to enable the extraction of relevant and highly informative data for the research applied for in the project. It is mandatory to increase the knowledge of the pathways of normal and abnormal sex development to enhance the diagnostic and prognostic possibilities of these disorders. This was investigated in work packages 02, 04, and 05, which dealt with novel approaches to the diagnosis of DSD employing advanced biochemical and molecular genomics technology. Moreover, in work package 03, unique insight into the functional properties of androgen action was obtained, devising novel cell models and delineating the role of co-regulators in the determination of sex phenotype. This enhances knowledge on the natural course of DSD in general. Furthermore, awareness and increasing information through structured learning programs for health care professionals, as carried out in work package 06, will lead to better management and care.

The implementation and achievements of *EuroDSD* have increased the public awareness of DSD and their acceptance of these conditions and thereby will help to alleviate the negative impact of DSD to patients and families.

The topic of medical care of DSD is currently discussed extensively in the public. As children with DSD may be born with ambiguous genitalia, the decision-making of sex assignment has been perceived as extremely challenging for families and also to health care professionals. Often, multiple surgical interventions are performed for genital reconstruction to a male or female appearance. The gonads are often removed to avoid malignant development. Hormone replacement therapy is not studied well and usually follows common patterns of replacement used in other patient populations. Patients and their advocacy groups oppose the current use and management of surgical correction to adhere to morphological appearance with sex assignment and also demand specialized hormone therapy. The *EuroDSD* partners have informed the public through lay publications and public broadcasting. In 2010, a special feature on DSD was presented on the German-French TV programme ARTE with the participation of several *EuroDSD* partners. Lay publications included the German Apotheken Umschau, a biweekly information brochure by the German pharmacies with an expected readership of approximately 19 million people. Just recently, a "debate" was recorded by the German weekly newspaper "Die ZEIT" with arguments by a representative of the German XY-womens group and the coordinator of *EuroDSD*, which is soon to be published. Furthermore, the associate to the *EuroDSD* registry from Khartoum, Sudan has presented the challenges of DSD in light of the discussion on genital mutilation to a Sudanese TV audience in November 2011.

Main dissemination activities and exploitation of results

In May 2011, the *EuroDSD* consortium organized a public symposium "3rd Symposium on Disorders of Sex Development" in Lübeck to present the results of the *EuroDSD* project to a professional audience. A total of 144 active participants from all around the world discussed the topic vigorously and opted for further research in the field. Since May 2011, the German Ethical Council, which reports directly to the German parliament, is working on a recommendation for health care to DSD patients. A public hearing on the topic with experts of the *EuroDSD* consortium was followed by an internet-based survey of patients with DSD about their personal experiences and opinions towards past and current research and health care in DSD. The final recommendation of the German Ethical Council is still pending, but a debate in the German parliament on 24 November 2011 uniformly presented the

opinion that patients with DSD represent a very special group of patients with rare diseases, who need more attention by the public, require respect and tolerance, but also very specialized health care from trained experts. Also the members of parliament agreed that more interdisciplinary research is needed to prevent the devastating medical treatments of the past in current patients with DSD. Thus, the *EuroDSD* consortium has contributed enormously to a very timely and pressing public debate on research and health care of patients with rare diseases affecting the genito-urinary tract. This will have effects on the implementation of research and health care standards for patients and will hopefully lead to the creation of Centres of Reference for these disorders throughout Europe.

The *EuroDSD* project was approved by the respective national ethical committees, but additionally, we implemented an expert ethical advisory board, consisting of Prof. Claudia Wiesemann, Germany, a medical ethicist, Prof. Gabriele Gillessen-Kaesbach, Human Geneticist, Germany, and a member of the Dutch XY-women's support group, who also had a medical background. This has set the standards for implementing security in data bases in this very sensitive issue, but also was helpful in the discussion about translational research regarding DSD.

The work of the *EuroDSD* consortium has provided specific results and tools that will have impact on research and health care in patients with DSD. A virtual research environment (VRE) has been iteratively designed and rolled out to the wider DSD research community. This has resulted in several versions of the VRE – each having increased functionality as required by the wider community. This functionality now covers clinical information and associated characterisation of DSD as well as specific modules for following up patients and the genetic screening they may have undertaken (and genetic alterations that may have been found). To support the security requirements of the project, fine-grained access control is supported where researchers can restrict access to and use of their data sets based on a variety of reasons. For example, a clinician might insist that the data is: only available to researchers at their own site/hospital; only available to researchers in their given country, e.g. due to ethic recommendations; available to all *EuroDSD* researchers, or indeed to recognised DSD researchers around the globe. All of this is completely transparent to the clinicians and biomedical researchers themselves. The *EuroDSD* virtual research environment and data base now represents a globally unique resource for research into DSD. As well as supporting the portfolio of clinical and biomedical researchers associated with the *EuroDSD* project, a key result of the VRE work is that this resource is now used by a far greater range of researchers around the world. For example, researchers from Argentina, Australia, Brazil, the Czech Republic, Estonia, Jordan, Kuwait, Morocco, Poland, Portugal, Spain, Sudan, Turkey, UK and the USA now have access to the *EuroDSD* VRE and many are using it for storing of their own clinical data sets. As well as numerous clinical/biomedical research publications, the establishment and support of the VRE has also resulted in a collection of software engineering related publications. These results have been presented at international conferences and published in respected journals. The results of the work are also shaping many other efforts involving the VRE development team. For example, major new EU funded projects based upon the *EuroDSD* security-oriented access and usage model pioneered in *EuroDSD* are now starting. These include projects in the area of adrenal tumours and in other rare diseases.

The consortium aimed to identity new genetic changes that may affect the development of the urogenital system. These alterations may be in genes we already know to be involved in the development of the urogenital system or they may be new genes. To identify these causes we used two approaches. The first is a new sequencing technology that allowed us to rapidly, and at low cost, simultaneously sequence 36 genes known to be involved in sexual development. We developed a microarray (or chip) that contains all the sequence information of the 36 genes. Using this chip, patient DNA would be compared against this array for changes in DNA sequence. This might greatly improve the speed and efficiency of the genetic diagnosis in the near future. The second approach is to study DNA from patients for small rearrangements in their genomes. In this approach, called aCGH (array comparative genomic hybridization) we looked for deletions or duplications that may be either variants in the general population or they may be associated with an aberration of urogenital development. The aCGH approach to detect new deletions or duplications that may cause DSD has proven to be very informative. Our results suggest that in patients where the testis failed to form, small deletions or duplications can be found in 25% of cases where other organ systems are affected and in 5.6% of cases where only testis development was affected. Therefore, this aCGH approach is a very useful diagnostic tool in the management of DSD patients and should be included in the diagnostic work-up in the future.

Male genital development is dependent on the action of androgens targeting a key receptor during a small developmental time window. The androgen receptor (AR) is the key player mediating the hormone signal by protein-protein interactions in responsive cells. We studied the effects on human development when the AR function is inhibited. Several androgen receptor mutations have been characterised and their function compared with the normal receptor. Importantly, we have been able to gather information on patients who have these mutations by studying

how they develop at puberty. It might now be possible to predict what will happen in later life when a child is born with a mutation in the androgen receptor and calculate the amount of androgen hormone that may need to be given at puberty for male development. We have also searched for known and so far unknown proteins that interact with the AR in this short but important time slot and started analysing their impact on genital formation. We have found several new candidate genes for AR binding partners. Candidate genes that play a role in proliferation, cell cycle control and differentiation were further analyzed by biochemical and cellular assays. We could identify new proteins that interact with AR and verify a regulatory function in AR-dependent gene transcription. By further experiments we aimed to elucidate the exact molecular function of the identified proteins. This will give further insight into the genotype-phenotype correlation in patients with androgen insensitivity syndrome and will also shed light on the overall variability of androgen action in male sex development.

Our research has revealed that the testosterone-induced difference between the male and the female external genitalia is paralleled by programming life-long sex specific programs of genome activity in the cells of the genital. In other words, these cells appear to have a life-long molecular memory reflecting the early action of testosterone. Further clinical and molecular research by us and by others supports the idea that androgen programming is not restricted to the external genitalia but occurs in other organs primarily seen as non-sex-specific as well, e.g., in the blood, in the brain. Since the production and the action of testosterone may be defective (either increased or decreased) in different forms of disorders of sex development, long term effects of androgen may influence the long term outcome in DSD patients. We investigated the hypothesis whether androgen programming occurs at the level of the methylome. „Methylome“ means the introduction of methyl-groups into specific regions of the genes at a genome wide level thus controlling their activity and eventually the specific development of organs and their functions. New gene chip technology enabled us to investigate many different sites where methyl groups are expected in our genes (about 15.000 genes). Indeed, our results support the idea that androgen-memory has an epigenomic background. Our research analysed this so called „epigenomic signature“ in normal males, females and in different forms of DSD to understand clearer their mechanisms and biological function in sex specific development of males, females and patients having DSD. It was demonstrated that the epigenomic signature correlates with the underlying diagnosis in individuals with DSD, therefore analysis of the methylation of target genes may be an additional tool to identify the specific underlying diagnosis in DSD. This, however, has to be proved by further research in the future.

Sex steroid hormones are of great importance for male or female development and phenotype. Patients affected DSD often suffer from disordered sex steroid production. We have employed mass spectrometry methods for steroid analysis. Liquid chromatography /mass spectrometry was used for plasma sample determinations, while gas chromatography /mass spectrometry was utilized for the analysis of steroid profiles in spontaneous and collected urine samples. Reference intervals for age and sex were determined for the LC/MS/MS method. Furthermore, we have discovered new causes of DSD due to errors in steroid metabolism previously unknown and have delineated profiles for several disease entities. This enabled us to define new diagnoses in DSD and to correlate the biochemical findings with molecular genetic aberrations. Therefore, we provide the basis for steroid metabolic profiling for the diagnosis of rare diseases, which may be used as diagnostic tools in the near future, and lead to targeted, cost-effective, and fast diagnosis in DSD.

The educational aspects of this project and its dissemination to the public were of high priority. We have cooperated with the European Society for Paediatric Endocrinology (ESPE) to provide the basis for education to professionals in the field. ESPE has implemented a broad portfolio of e-learning programmes for various endocrine disorders. Within the *EuroDSD* project, an e-learning programme on DSD was developed, proved by forum functionality, and assessed by professionals of different educational levels. This e-learning programme will continue under the auspices of the ESPE and it will be open to all those who register appropriately for its use. As the e-learning programme incorporated the results of the research of *EuroDSD* to the most appropriate standards, we believe this will have a long-lasting and sustainable effect on the knowledge and health care provision on DSD.

EuroDSD partners have published their work in several highly ranked scientific journals already and evaluation of certain aspects is still on its way, so more scientific publications will be submitted in the near future. Thus, dissemination of research results was highly successful and will lead to sustainable knowledge in the scientific community. *EuroDSD* was meant as a European Consortium to work on Disorders of Sex Development. It closely related to the working group on DSD of the European Society for Paediatric Endocrinology (ESPE). A regular presentation panel of this working group was initiated, preceding the yearly meeting of the society. This platform was used for dissemination of the results of the *EuroDSD* consortium to the professional public. Within the working group on DSD in ESPE is was also decided to invite other subspecialties working on DSD to the meetings and for further exchange, especially from paediatric surgery and paediatric urology, currently not part of the *EuroDSD* consortium.

The paediatric surgeons decided to plan a strategic meeting in 2012 to take the results of the *EuroDSD* project into their own future research agenda and management of patients with DSD.

The coordinator of *EuroDSD* was appointed to the European Union Committee of Experts on Rare Diseases (EUCERD) and provided information on the special needs of patients with DSD especially in the light of the most sensitive issue of sexual development and the possible misuse of these patients which has been reported in the past. As a member of EUCERD the coordinator also participated in the two initiating meetings of the International Rare Diseases Research Committee (IRDiRC), to provide the basis for a truly international research community in rare diseases. Further spin-offs of the *EuroDSD* project included a first clinical trial study financed by the German Ministry for Research and Education (BMBF), which elucidates the effects of testosterone versus estradiol in patients with complete androgen insensitivity in a double-blind, double-dummy over cross fashion.