Identification of an *AR*-mutation negative class of androgen insensitivity BY DETERMINING endogenous AR-ACTIVITY

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Context: Only about 85% of patients with clinical diagnosis complete androgen insensitivity syndrome (CAIS) and less than 30% with partial androgen insensitivity syndrome (PAIS) can be explained by inactivating mutations in the androgen receptor (*AR*) gene.

Objective: To clarify this discrepancy by in-vitro determination of AR transcriptional activity in individuals with disorders of sex development (DSD) and male controls.

Design: Quantification of dihydrotestosterone (DHT)-dependent transcriptional induction of the AR target gene apolipoprotein D (*APOD*) in cultured genital fibroblasts (GF) (APOD-assay) and next generation sequencing (NGS) of the complete coding - and non-coding *AR*-locus.

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received April 29, 2016. Accepted August 26, 2016.

Abbreviations:

Setting: University Hospital Endocrine research laboratory

Patients: GF from 169 individuals were studied encompassing control males (N=68), molecular defined DSD other than AIS (N=18), *AR*-mutation positive AIS (N=37) and previously undiagnosed DSD including patients with clinical suspicion of AIS (N=46).

Intervention(s): None.

Main Outcome Measure(s): DHT-dependent *APOD*-expression in cultured GF and *AR*-mutation status in 169 individuals.

Results: The APOD-assay clearly separated control individuals (healthy males and molecular defined DSD patients other than AIS) from genetically proven AIS (cutoff <2.3-fold *APOD*-induction; 100% sensitivity, 93.3% specificity, p<0.0001). Of 46 DSD-individuals with no *AR*-mutation, 17 (37%) fell below the cutoff indicating disrupted androgen signaling.

Conclusions: *AR*-mutation positive AIS can be reliably identified by the APOD-assay. Its combination with NGS of the *AR*-locus uncovered an *AR*-mutation negative, new class of androgen resistance which we propose to name AIS type II. Our data support the existence of cellular components outside the *AR* affecting androgen signaling during sexual differentiation with high clinical relevance.

Sexual development is a complex process involving three crucial steps: development of the gonads in the embryo, synthesis of sex hormones, and sex hormone action. Genetic errors in any of these processes can lead to a wide range of sexual phenotypes that can be broadly included under the umbrella term of disorders of sex development (DSD) (1–3). Androgen insensitivity syndrome (AIS) (OMIM#300068) is a DSD that is classically characterized as a disorder of hormone action due to a reduced or absent functionality of the androgen receptor (AR) protein encoded by the *AR* gene. AIS is often suspected to be a common cause of DSD in a 46,XY individual and may either be associated with complete feminization of the external genitalia due to a complete lack of AR transcriptional activity (complete AIS, CAIS) (4), a variable level of feminization/masculinization due to a partial lack of transcriptional activity (partial AIS, PAIS) or isolated male infertility (mild AIS, MAIS).

For optimal function, the AR is activated through its ligands, testosterone and the more potent dihydrotestosterone (DHT), following which it translocates into the nucleus and binds to its target genes whose expression entails the development of male internal and external genitalia. This process is tightly regulated through coactivators and corepressors of the AR (5, 6). Many AR target genes have been described in prostate cancer-derived cell lines, however, only a handful have been identified in healthy male genital tissue (7). Among these, apolipoprotein D (*APOD*) has been reported to exhibit the most significant induction upon DHT treatment. *APOD* is a direct transcriptional target of the AR (8, 9) and a DHT-dependent secretion of APOD has been observed in prostate cancer cells (10). APOD belongs to the lipocalin protein family (11) and is able to carry *E*-3-methyl-2-hexenoic acid (*E*-3M2H), the most abundant axillary odorant in males, to the skin surface ultimately used for pheromonal communication (12).

While the clinical diagnosis of CAIS is relatively easy and can be confirmed by identifying a genetic abnormality in the *AR* coding sequence (*AR*-CDS) in more than 85% of cases, the clinical diagnosis of PAIS is more difficult and, in addition, less than 30% of cases that are clinically suspected of PAIS are associated with a mutation in the *AR* (13). It is not known whether some of those with 46,XY DSD may, in fact, have a currently unidentified new class of androgen insensitivity despite the absence of an *AR*-CDS mutation, or whether some, or even all, rather have normal cellular AR function, thus excluding AIS.

To understand the possible coexistence of androgen resistance without any genetic evidence of a defect in the *AR*, we analyzed a cohort of 169 individuals including male controls, individuals with genetically proven AIS and individuals with a clinical suspicion but no molecular proof of AIS in whom genital fibroblasts were available. Combining *AR*-sequencing analysis with a functional assay for AR-activity by measuring the DHT-dependent transcriptional induction of the androgen-regulated *APOD*-gene in cultured genital fibroblasts (GF) (APOD-assay) enabled us to discover a new *AR*-mutation negative class of androgen resistance which we propose to name AIS type II.

Materials and Methods

The study was performed in agreement with the vote of the Ethical Committee of the Medical Faculty of the Christian-Al-

brechts-University, Kiel, Germany (AZ: D415/11) (File S1). GF received from collaborating partners were included in this study according to the recommendations of the local ethical committees. All GF included in this study were double encrypted and numbered from 1 to 169.

Sample collection

The GF herein analyzed belonged to four major clinical groups:

The first group (group 1) was established in collaboration with local urologists and pediatric surgeons and includes scrotum-derived control GF from fertile adult patients with normal virilization of the external genitalia, who underwent vasectomy

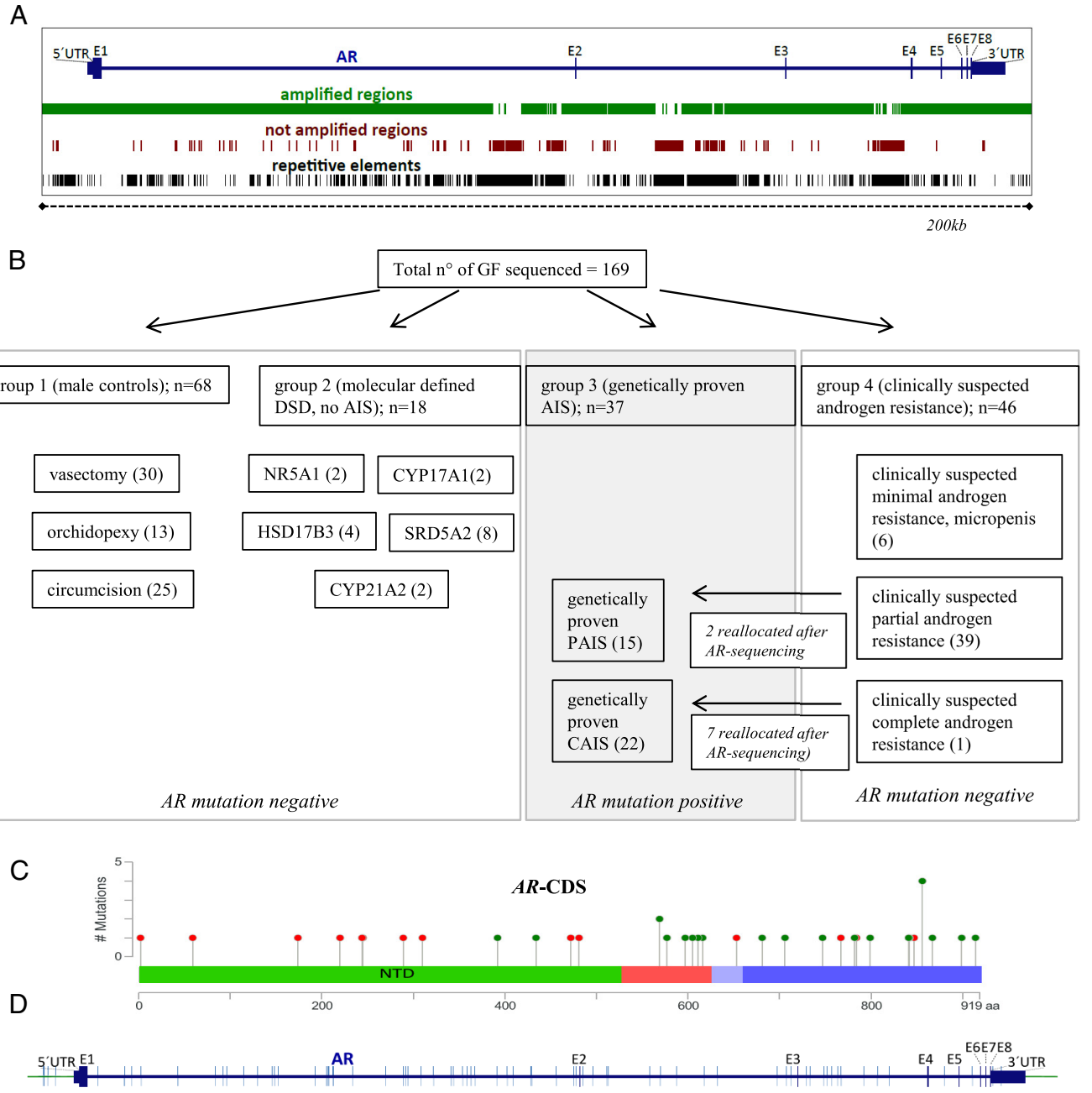

Figure 1. Next generation sequencing of the AR locus. a) Graphic representation of the AR locus and the regions amplified by the haloplex design (chrX:66,754,874–66 955 461 (hg19)) (shown in green). Highly repetitive sequences were excluded from the design (shown in brown). For comparison, repetitive elements present in this locus are shown in black. b) Division of the four patient groups from whom cultured GF were analyzed. When mutations were found in the AR-CDS of GF from group 4 (clinically suspected androgen resistance), they were reallocated to group 3 (genetically proven AIS). Therefore, two of the initially 41 samples from group 4 with clinically suspected PAIS and seven of the initially eight samples with clinically suspected CAIS were reallocated to group 3 resulting in 15 GF samples with genetically proven PAIS and 22 samples with genetically proven CAIS, respectively c) Distribution of mutations found in the CDS of the AR. Red dots represent nonsense mutations, green dots missense mutations. Synonymous mutations in the coding region were not considered as CDS mutations. The graph was designed using the Mutation Mapper software from cBioPortal d) Distribution of not annotated SNPs along the sequenced region (green bars).

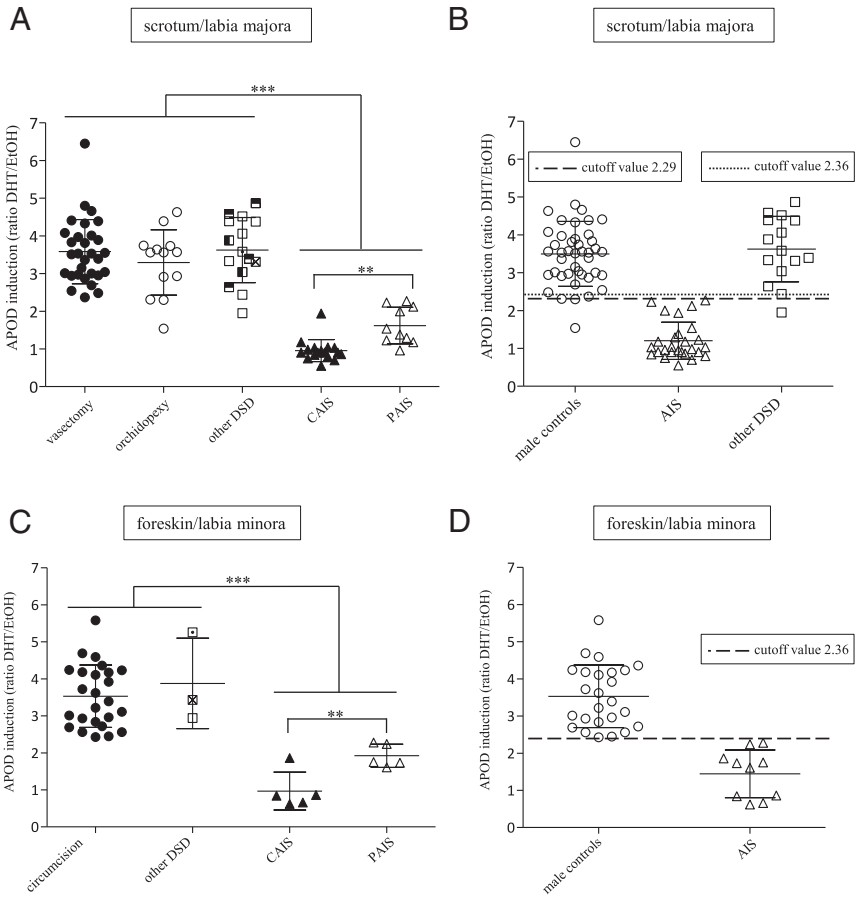

Figure 2. DHT-dependent AR induced APOD mRNA expression represented as ratio between ethanol (EtOH)- and DHT-treated GF. A) Scrotum derived male controls (vasectomy, orchidopexy (group 1)), labioscrotal derived molecular defined DSDs (other DSD (group 2)), and AR-CDS mutation positive AIS (CAIS, PAIS (group 3)) B) Depiction of cutoff values between male controls (vasectomy and orchidopexy) and AR-CDS mutation positive AIS (CAIS and PAIS) of 2.29 (100% sensitivity, 97,7% specificity, $P < .0001$) and between the same male controls and molecular defined DSDs (other DSD) of 2.36 (100% sensitivity, 93,3% specificity, $P < .0001$) C) Foreskin derived male controls (circumcision (group 1)), molecular defined DSDs (other DSD (group 2)), as well as AR-CDS mutation positive AIS (CAIS, PAIS (group 3)). D) Depiction of the cutoff value between male controls (circumcision) and AR-CDS mutation positive AIS (CAIS and PAIS) of 2.36 (100% sensitivity, 100% specificity, $P < .0001$). Means and standard deviations are included as error bars. p-values < 0.001 are denoted by three stars, those < 0.01 bei two stars. Among the DSD diagnoses other than AIS, empty squares represent SRD5A2-, horizontally half-filled squares HSD17B3-, vertically half-filled squares CYP17-, crossed squares CYP21A2- and dotted squares NR5A1-mutations.

(n = 30). We included scrotal biopsies of patients under the age of 18 who underwent orchidopexy due to maldescended testes (n = 13) with normal external genitalia, ie, no hypospadias. In addition, we used control foreskin fibroblasts from patients who underwent circumcision due to cultural reasons or phimosis (n = 25). Genomic DNA of all male control GF cultures was sequenced using our custom haloplex NGS (next generation sequencing) including up- and downstream sequences, untranslated regions and the introns (Figure 1a).

The second group consists of GF from previously characterized 46,XY DSD individuals with a defined molecular diagnosis other than AIS (group 2). In particular, these individuals carried mutations in the steroidogenic factor 1 (SF1) gene (*NR5A1*) (n = 2), the 17α-hydroxylase gene (*CYP17A1*) (n = 2), the 17β-hydroxysteroid-dehydrogenase type III gene (*HSD17B3*) (n = 4) and the 5α-reductase type II (5αRDII) gene (*SRD5A2*) (n = 8) in conjunction with ambiguous or female external genitalia. Biopsies were taken from either labioscrotal or foreskin/labia minora tissue. We added GF from female (46,XX) individuals with congenital adrenal hyperplasia (CAH) due to 21-hydroxylase deficiency (*CYP21A2*) (n = 2). Genomic DNA derived from GF cultures of the second group was sequenced via the custom haloplex NGS *AR*-panel.

The third group contains labioscrotal and foreskin/labia minora-derived GF with a genetic proof of AIS, in whom mutations in the *AR*-CDS were either found previously via Sanger sequencing or in this paper through the haloplex NGS *AR*-panel (n = 37) (group 3). All GF that revealed an *AR*-CDS mutation via the custom haloplex NGS *AR*-panel were validated by Sanger sequencing.

The fourth group was compiled from a collection of labioscrotal and foreskin/labia minora-derived GF samples from 46,XY DSD individuals without an established molecular diagnosis (group 4). It includes individuals with apparently unaffected androgen biosynthesis based on available hormone data supporting a clinical suspicion of androgen resistance (n = 46). When available, data on the external genital appearance, basal and stimulated testosterone levels (HCG-test) and measurements on AR-ligand binding (Bmax and Kd) were collected (suppl. Table 1 and 2). This group may also contain individuals with a so far undiagnosed form of DSD other than AIS. All GF of group four were sequenced through our custom haloplex NGS *AR*-panel. If an *AR*-CDS mutation was detected by NGS, Sanger sequencing was used for confirmation and the GF were subsequently reallocated to group 3.

Supplemental Table 3 lists all GF included in this study according to their location of biopsy together with the median age at biopsy.

Primary culturing of genital skin biopsies, the APOD-assay, next generation sequencing library preparation and sequencing and further methods are described in the supplemental material.

Results

Separation of GF into *AR* coding sequence mutation positive and negative entities

In the group of male controls (group 1) no mutations were detected in the CDS and the intron-exon boundaries of the *AR*. In group 2 (molecular-defined DSD diagnoses other than AIS) there were also no *AR*-CDS or intron-exon

boundary mutations. In all classical AIS individuals (genetically proven AIS, group 3) previously identified by Sanger sequencing, mutations in the *AR*-CDS could be validated by our custom NGS *AR*-panel, underlining the validity of the NGS approach. All those individuals in group 4 with previously undiagnosed forms of DSD in whom we identified a mutation in the *AR*-CDS by NGS (n = 9) were reallocated to group 3. In the remaining GF samples of group 4, including DSD samples where AIS was suspected (n = 46) neither mutations in the *AR*-CDS nor in the intron exon boundaries could be detected. A schematic representation of all four groups is shown in Figure 1b. The distribution of detected *AR*-CDS mutations within group 3 is schematically shown in Figure 1c and their exact position is listed in suppl. Table 4. Eight *AR-CDS* mutations are not currently listed in the AR mutation database (14) and, to our knowledge, have not been described in the literature. These unreported mutations are frameshift-mutations (n = 5), stop-mutations (n = 1) and missense mutations (n = 2) (suppl. Table 4). Outside the coding region, numerous nonannotated SNPs were found in all four groups. A distribution of those SNPs is shown in Figure 1d.

Calculation of a cutoff for the functional classification of male controls (group 1), molecular-defined DSD other than AIS (group 2) and AR-CDS mutation positive AIS individuals (group 3) using the APOD-assay

We now functionally characterized all 169 sequenced GF by measuring the DHT-triggered ability of the AR to induce transcription of its target gene *APOD* (APOD-assay). Male control scrotum-derived GF from group 1 (vasectomy, orchidopexy) showed a mean DHT-mediated *APOD* induction of 3.5 fold (SD 0.85), defining the normal range of transcriptional function of the AR in this group (Figure 2a). Scrotum-derived GF from group 2 (molecular-defined DSD other than AIS) showed the same degree of *APOD* up-regulation, confirming uncompromised functionality of the AR in these cells (Figure 2a). Only one orchidopexy-derived control GF cell line from an individual in group 1 showed an unexpectedly low induction of *APOD*. In the light of the complete dataset provided in this manuscript and since the final steps of testicular descent are androgen dependent, we retrospectively have to conclude that this individual has some degree of androgen resistance (15). In contrast to groups 1 and 2, *APOD* induction was significantly lower in scrotum-derived GF from classical AIS individuals (group 3) ($P < .001$). CAIS-derived GF showed on average no induction (0.96) while PAIS-derived GF demonstrated an average induction of 1.62 (Figure 2a). This confirms androgen resistance at the functional molecular level in these cells and underlines the validity of the APOD-assay. We now calculated the cutoff between scrotal-derived control GF from group 1 (adults and children together) and corresponding labioscrotal-derived GF from AIS individuals in group 3 (CAIS and PAIS together). Consequently, an *APOD* induction below 2.28 represents a form of androgen resistance with a sensitivity of 100% and a specificity of 97.67%, and indicates that the two groups are separable with high confidence (Figure 2a and b).

From the clinical perspective, it is of much greater relevance to distinguish AIS from other forms of DSD rather than from clinically unsuspicious male controls. We calculated a cutoff between labioscrotal-derived GF from group 2 (molecular-defined DSD other than AIS) and group 3 (*AR*-CDS mutation positive AIS) of 2.36-fold *APOD* induction. Hence, a DHT-mediated APOD induction under 2.35 distinguishes genetically proven AIS from other molecular-defined DSDs with a sensitivity of 100% and a specificity of 93.33% (Figure 2a and b).

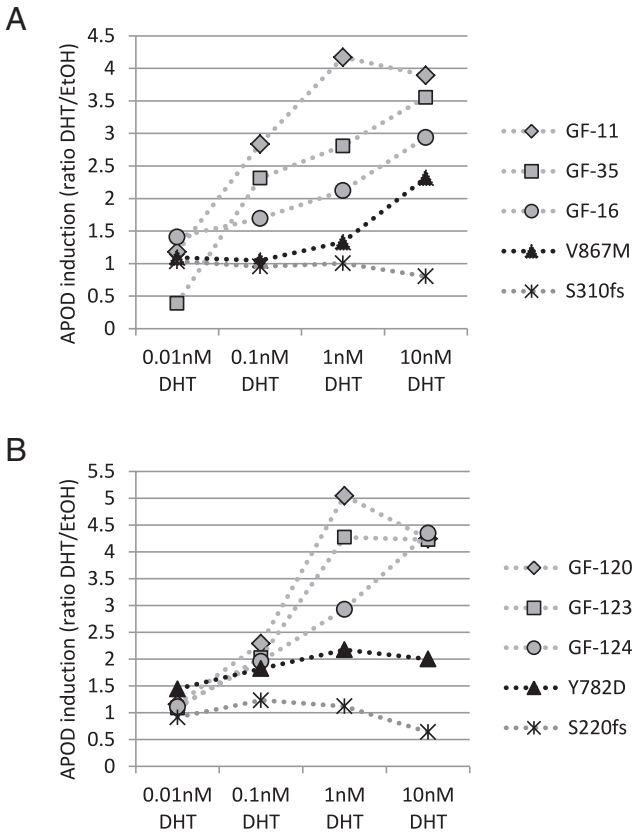

Figure 3. DHT-dependent AR-induced APOD induction in response to different DHT concentrations in the culture media. a) GF-11, GF-16 and GF-35 are scrotum-derived male control GF (suppl. Table 1). GF derived from a CAIS patient carrying the p.Ser310fs mutation served as negative control. The p.Val867Met mutation is shown in black. b) GF-120, GF-123 and GF-124 are foreskin derived male control GF (suppl. Table 2). GF derived from a CAIS patient carrying the p.Ser220fs mutation served as negative control. The p.Tyr782Asp mutation is shown in black.

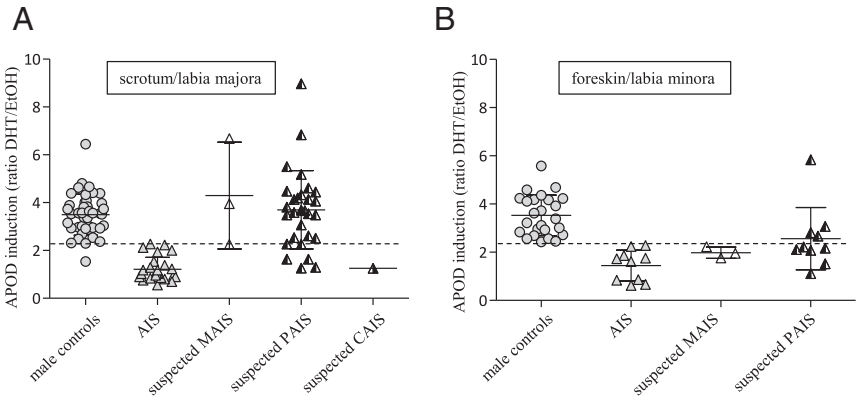

Figure 4. DHT-dependent AR-induced APOD induction in AR-CDS negative individuals with clinically suspected androgen resistance (group 4) derived from a) scrotum/labia majora GF b) foreskin/labia minora. Suspected androgen resistant GF of group 4 are divided into minimal androgen resistance (MAIS, micropenis), partial androgen resistance (PAIS, ambiguous external genitalia) and complete androgen resistance (CAIS, completely female external genitalia). Included are means and standard deviations. For comparison, the tissue specific controls and AR-CDS mutation positive GF are shown as well. The calculated cutoffs are drawn as dotted lines.

When analyzing foreskin/labia minora-derived GF, the APOD-assay could again reliably separate male control fibroblasts (group 1) and GF from AIS individuals harboring an *AR*-CDS mutation (group 3) (Figure 2c). A cutoff of 2.36-fold *APOD* induction was determined for foreskin/labia minora-derived tissue with 100% sensitivity and specificity (Figure 2c and d). The average DHT-mediated *APOD* induction in CAIS was 0.97 (= no APOD induction) and 1.92 in PAIS (Figure 2b). Although GF from individuals of group 2 showed an as high *APOD* induction as male controls (group 1) (Figure 2c), no cutoff could be calculated as there were not enough corresponding foreskin-derived GF strains available in our DSD-GF-biobank. When testing two GF strains derived from CAH individuals carrying *CYP21A2* mutations and having a 46,XX karyotype their *APOD* response to DHT was comparable to that of male controls, confirming that the AR can be activated by DHT in GF independently of the chromosomal sex (Figure 2a and c).

We than examined whether GF derived from genetically proven CAIS and PAIS individuals within group 3 could be distinguished from each other by the APOD-assay. When comparing DHT-mediated *APOD* induction, we found a significant difference between PAIS and CAIS in both labioscrotal and labia minora/foreskin-derived tissues ($P < .01$). However, there was some overlap due to a few GF cultures (Figure 2a and c). One GF cell line carrying a p.Val867Met mutation in the ligand binding domain of the AR derived from a CAIS individual still showed residual *APOD* induction. Interestingly, when using lower DHT concentrations, *APOD* induction was abolished (Figure 3a). Labia minora-derived GF from another CAIS individual bearing the mutation p.Tyr782Asp again located in the AR-ligand binding domain also showed residual APOD induction. However, this partial activity was even present at lower DHT concentrations (Figure 3b). A third GF cell line was derived from an individual with predominantly female external genitalia, and therefore PAIS, carrying a p.Leu174stop mosaic. The latter was present in 94% of the cultured GF according to the NGS reads, which is most likely the cause for complete abolishment of DHT-mediated *APOD* induction in spite of the PAIS phenotype.

We also compared *APOD* induction between GF harboring nonsense or missense mutations in the AR protein. Nonsense mutations (stop- or frame shift mutations) never showed any *APOD* induction and always belonged to the CAIS group (apart from the p.Leu174stop mosaic), while missense mutations had a variable *APOD* induction and were present in both PAIS and CAIS-derived GF (see suppl. Table 1 and 2).

In conclusion, the APOD-assay does distinguish CAIS patients from PAIS individuals, albeit with slightly lower sensitivity (88.2%) and specificity (90%) (suppl. Figure 8c).

AR-activity in *AR*-CDS mutation negative GF from individuals with suspected diagnosis of AIS (group 4)

We then analyzed the large group of *AR*-CDS mutation negative GF derived from individuals with no previously established DSD diagnosis (group 4) using the APOD-assay and applied the above calculated cutoffs. Looking at labioscrotal-derived GF, 24% (n = 8) of fibroblast cultures from group 4 fell below the cutoff of 2.28 and therefore have to be defined as functionally androgen resistant (Figure 4a, suppl. Table 1). One of the GF cultures was from an individual with the suspected clinical diagnosis of CAIS and showed strongly reduced *APOD* induction. In contrast, the remaining 76% (n = 25) GF cell lines showed an *APOD* induction above the cutoff and had an AR-activity comparable to that of control groups 1 and 2 (Figure 4a). Therefore, these GF cell lines have to be defined as normally androgen responsive. Analyzing the foreskin/labia minora-derived GF in group 4, the majority (69%, n = 9) fell below the cutoff of 2.35. Again, these cultures have to be defined as androgen insensitive on a functional basis (Figure 4b, suppl. Table 2). The remaining 31% of foreskin/labia minora GF cultures (n = 4) behaved like male

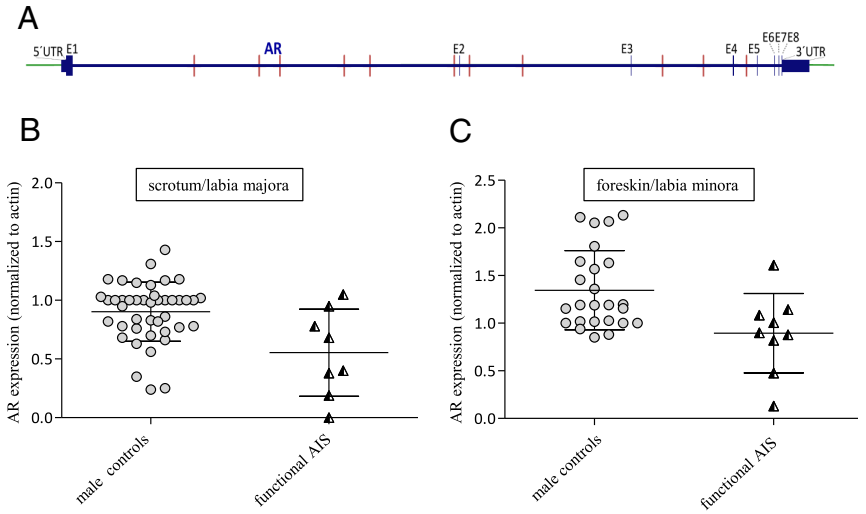

Figure 5. Analysis of AR-CDS mutation-negative but functionally androgen insensitive GF. a) Distribution of potentially damaging mutations in the sequenced region outside the AR-CDS b) AR protein expression in male scrotum-derived controls and AR-CDS mutation negative but functionally androgen insensitive labioscrotal GF c) AR protein expression in male foreskin-derived controls and AR-CDS mutation negative but functionally androgen insensitive GF. Included are means and standard deviations.

foreskin controls (group 1) in terms of *APOD* induction and hence androgen insensitivity can be ruled out. In summary, 17 of the 46 GF from group 4 have functionally proven androgen resistance based on androgen-induced *APOD* transcription in spite of the absence of an *AR*-CDS mutation.

Molecular characterization of the *AR*-CDS mutation negative but functionally androgen resistant GF

Finally we wanted to know if mutations detected within the *AR* locus but outside the *AR*-CDS in individuals of group 4 could potentially have influenced AR activity. Out of the 17 *AR*-CDS-mutation negative androgen resistant GF, nine had one or more not annotated SNPs outside the *AR*-CDS while eight had only previously annotated and clinically unsuspicious SNPs in the region covered by our NGS approach. The distribution of the not annotated SNPs along the analyzed *AR* locus is shown in Figure 5a. We speculated that a low *APOD* induction could be due to a reduced AR protein expression or stability in these GF. We checked AR protein levels in all the 17 GF cultures and compared them to their appropriate control groups (scrotum and foreskin) (Figure 5b and c, Table 1). A lower AR protein expression was seen in four GF of the *AR*-mutation negative androgen resistant group, indicating that AR protein expression was impaired in these cases. Two of these four individuals had not annotated SNPs outside the CDS (Table 1). In conclusion, we show that reduced AR protein expression or stability can explain a reduced *APOD* induction in about one fourth of the analyzed cases.

Discussion

Functional assays for AR activity in GF have been described before (16). Lacking a target gene for the AR, however, they were dependent on the transfection of exogenous reporter constructs in order to monitor endogenous AR-activity. We here provide the APOD-assay as a tool for the functional characterization of cellular AR function in GF derived from DSD patients. We validate its diagnostic suitability in a very large cohort using male controls, various molecular well-defined DSD patients other than AIS, as well as several genetically proven classical AIS individuals. The resulting diagnostic cutoffs not only helped to exclude the diagnosis of AIS in many cases but also lead to the identification of an androgen resistant but *AR*-CDS-negative new class of AIS which we suggest to call AIS type II. This is not only a significant addition to the current classification of 46,XY-DSDs but also a starting point for a better understanding of AR signaling, including the identification of new AR cofactors in future clinical and molecular DSD studies.

While the APOD-assay separates classical AIS from male controls with high sensitivity and specificity, the separation was slightly less specific when comparing classical AIS from defined DSD diagnoses other than AIS. In fact, one individual with documented 5αRD-deficiency (group2) showed reduced AR activity in the APOD-assay. A possible explanation could be that this individual has a defect both in DHT synthesis and in androgen action. An additional 5αRD-deficiency may also be responsible for the CAIS phenotype of an individual carrying a p.Tyr781Asp mutation in the ligand binding domain of the AR, as biochemical data indicate reduced 5αRD-activity in GF of this patient ((17); ID:C31). Both the residual *APOD* induction shown in this paper and previously published DHT binding and dissociation studies (17) indicate only an incomplete loss of AR-function in this individual in spite of a complete female phenotype.

Also, the APOD-assay did not distinguish between PAIS and CAIS in all cases. This overlap may be affected by specific functional and molecular conditions in some individual GF cultures. In GF from a CAIS individual carrying a p.Val867Met mutation in the AR-ligand binding domain we observed residual *APOD* induction with 10 nM DHT despite a clinical CAIS. Interestingly, no *APOD*

Table 1. List of AR-CDS mutation negative but functionally androgen insensitive GF

GF	origin of biopsy	SNP (chromosome reference>alternate)	APOD induction	AR protein expression	Encode regulation (hg19)
GF-89	S	nothing	1.26	0.00	
GF-107	S	chrX:66 912 572 G>A	2.27	0.19	strong enhancer in HSMM
GF-104	S	nothing	1.30	0.38	
GF-105	S	nothing	1.64	0.40	
GF-90	S	chrX:66 839 548 G>A	2.26	0.68	strong enhancer in HUVEC
GF-109	S	chrX:66 811 878 T>C	1.64	0.78	polycomb repressed in GM12878, K562, H1-hESC, HELA, HUVEC, HepG2
GF-86	S	nothing	2.25	0.95	
GF-118	S	chrX:66 922 786 A>G	1.25	1.05	/
GF-164	F	chrX:66 877 648 G>A	1.52	0.13	polycomb repressed in GM12878, K562, H1-hESC, HELA, HUVEC, HepG2
GF-158	F	nothing	1.76	0.48	
GF-162	F	chrX:66 864 354 A>G; chrX: 66 860 551 C>A	2.08	0.82	polycomb repressed in GM12878, K562, H1-hESC, HELA, HUVEC, HepG2
GF-160	F	chrX:66 795 584 A>G; chrX: 66 817 032 3bp del	2.11	0.88	polycomb repressed in GM12878, K562, H1-hESC, HELA, HUVEC, HepG2; Pol2 associated transcription in H1-hESC
GF-159	F	chrX:66 933 579 G>C	1.95	0.90	transcription in HUVEC
GF-168	F	nothing	2.17	1.00	
GF-157	F	nothing	2.23	1.09	
GF-163	F	chrX:66 833 033 A>G	2.22	1.14	strong enhancer in HUVEC and HSMM
GF-166	F	nothing	1.11	1.61	

HSMM (Human Skeletal Muscle Cells and Myoblasts); GM12878 (B-lymphocyte); K562 (leukemia cell line); H1-hESC (H1 human embryonic stem cells); HELA (cervical carcinoma cells); HUVEC (Human Umbilical Vein Endothelial Cells); HepG2 (liver hepatocellular carcinoma cells).

induction was measured when using 1 nM DHT, suggesting that the CAIS phenotype might have originated from low local genital DHT concentrations during embryogenesis. This observation is supported by the literature associating this mutation with different AIS phenotypes, ranging from CAIS through PAIS to MAIS (14). Another phenomenon with functional relevance for the APOD-assay may be the presence of somatic mosaicism which is an apparently frequent condition in AIS due to the high new mutation rate (18, 19). In the PAIS subgroup within group 3 of our study one cell line contained a p.Leu174stop mosaic (20) present in 94% of the cultured GF according to the NGS reads. No *APOD* induction could be detected, well in line with the high percentage of the mutation in this PAIS cell culture. Hence, while the APOD-assay correctly identified AIS in this situation, somatic mosaicism may influence the detected level of functional impairment which may be in contrast to the clinical phenotype. We have previously described this phenomenon of discrepancy between molecular studies and the clinical phenotype in mosaic AIS, using other functional approaches (19, 21). Ultimately, due to limited information regarding the exact AIS-grades of the genital phenotypes in our DSD-GF-biobank (eg, according to Quigley et al (22) or to Ahmed et al (23)) we cannot provide a meaningful AIS-grade/APOD-assay correlation to date. The APOD-assay is therefore currently not a statistically proven tool for assessing the quantitative extent of androgen resistance in a given individual with DSD.

By analyzing sequencing data of the *AR* locus outside the *AR*-CDS we could detect so far not annotated SNPs within potentially regulatory regions. Some of these SNPs are potential candidates for influencing AR activity as they are paralleled by reduced AR protein expression in the corresponding GF cultures which could explain the lower AR activity in the AIS type II individuals. Interestingly, we previously detected a mutation in the 5′UTR of the *AR* in an individual having CAIS and experimentally showed that this mutation is sufficient to strongly reduce AR protein levels and AR activity (24). This underlines the importance of detection of potential mutations outside the *AR*-CDS. Another promising group of factors outside the *AR* gene region that might contribute to AIS type II are AR-cofactors which are needed for proper AR activity (5, 6). Numerous cofactors of the AR have been described in prostate cancer (25) but a coregulator that exclusively reg-

ulates the AR has not been described so far. Since the *AR* gene was cloned in 1988 (26, 27), only one single case of disrupted AR activation through a coactivator defect has been reported in a CAIS individual (28), but this coactivator has never been identified. No further case has since been described.

We do not yet know whether the AIS type II cohort identified in this study has a monogenic origin or whether multiple aberrant genes may contribute to this entity. Exome sequencing of the *AR*-CDS-negative AIS type II cohort in comparison with the other three cohorts of this study is one of the next important experimental steps planned. Furthermore, we cannot exclude that mild functional AIS type II may play a role as secondary modifier contributing to a DSD phenotype, even in certain molecular-defined DSDs and in unknown DSDs. This is supported by previous reports documenting the existence of more than one compromised molecular factor in the same DSD individual (29–31). According to Cox K. et al (32) associated conditions occur in about a quarter of analyzed DSD cases. Looking specifically at cases with suspected androgen insensitivity syndrome in 11% anomalies were reported. In our AR-CDS-negative AIS type II cohort we found documented minor syndromic signs in four out of 46 cases, hence 9%. In addition, prenatal conditions leading to low birth weight (LBW) may have programming effects on androgen responsiveness of genital cells, since a correlation of a LBW and a ´PAIS-like´ phenotype in individuals without an *AR*-gene mutation has been described before (33).

Currently, our data are based on retrospective analyses of fibroblasts obtained from our DSD-biobank, but they can, nevertheless, be of potential value for the clinical endocrinologist. Apart from being an explanation for the phenotypic development of a DSD individual, reduced *APOD* induction may be associated with a reduced future AR sensitivity during puberty and may influence clinical response to androgen treatment. Prospective data are needed to correlate *APOD* expression with clinical outcome parameters in affected individuals. Given the high significance of the data provided in this manuscript, the scientific community in DSD research should revisit the clinical indication of a diagnostic genital skin biopsy in specific unclear DSD cases.

Acknowledgments

We would like to thank Brigitte Karwelies, Tanja Stampe and Gila Hohmann for their excellent laboratory support. We are grateful to Rieko Tadokoro-Cuccaro for providing GF. The study has been funded by the Medical Faculty of the Christian-Albrechts-University, CAU, Kiel, Germany (Forschungsförderung 2015 – Anschub to NH) and the German Research Council (Deutsche Forschungsgemeinschaft, DFG) (Ho 2073/7–1/7–3 to PMH and Am 343/2–1/2–3 to OA). We thank the KinderKrebsInitiative Buchholz/Holm-Seppensen for providing infrastructure. S.F. Ahmed is supported by a UK Medical Research Council partnership award G1100236. I.A.Hughes was supported by the NIHR Cambridge Biomedical Research Centre.

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This work was supported by Grants or fellowships supporting the writing of the paper: The study has been funded by the Medical Faculty of the Christian-Albrechts-University, CAU, Kiel, Germany (Forschungsförderung 2015 – Anschub to NH) and the German Research Council (Deutsche Forschungsgemeinschaft, DFG) (Ho 2073/7–1/7–3 to PMH and Am 343/2–1/2–3 to OA). The KinderKrebsInitiative Buchholz/Holm-Seppensen provided infrastructure. S.F. Ahmed is supported by a UK Medical Research Council partnership award G1100236. I.A-.Hughes was supported by the NIHR Cambridge Biomedical Research Centre.

Disclosure Statement: The authors have nothing to disclose

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