Eur J Pediatr (1997) 156 : 7–14 

ENDOCRINOLOGY

**G. H. G. Sinnecker · O. Hiort · E. M. Nitsche**
**P.-M. Holterhus · K. Kruse**
**German Collaborative Intersex Study Group**

# Functional assessment and clinical classification of androgen sensitivity in patients with mutations of the androgen receptor gene

Received: 13 February 1996 / Accepted: 4 June 1996

**Abstract** In the genetic male, mutations of the androgen receptor (AR) gene cause phenotypes ranging from female to subfertile male. Binding assays on genital skin fibroblasts and DNA analysis alone provide incomplete information about receptor function. We used the sex hormone-binding globulin (SHBG) response to stanozolol as a measure of AR function and correlated the results with phenotypes which were classified according to the degree of defective masculinization. Of the 34 patients investigated, 9 had complete, and 14 had partial androgen insensitivity syndrome (AIS) with predominantly female, ambiguous, or predominantly male phenotype. Eleven subjects served as controls. Mutations were characterized using polymerase chain reaction-single strand conformation polymorphism analysis and direct DNA sequencing. DNA analysis revealed two major deletions, two minor defects leading to premature stop codons in exon 1, and 19 point mutations in the DNA- and hormone-binding domains of the AR gene. After stanozolol, SHBG remained unchanged in patients with complete AIS (102.0 ± 3.8 [SE]%; range 92.4%–129% of the initial value). The SHBG decrease was diminished in partial AIS with predominantly female (83.8% ± 1.7%; range 81.3%–87.0%), ambiguous (80.4% ± 4.4%, range 68.4%–89.1%), and predominantly male (mean 65.9% ± 4.9%, range 48.6%–80.8%) phenotypes, and normal in controls (51.4% ± 2.1%, range 35.6%–62.1%). Differences between controls and each AIS group were statistically significant ($P < 0.05 - < 0.0001$). A close correlation was found between the degree of undermasculinization (AIS phenotype) and the SHBG response.

**Conclusions** The SHBG test provides functional information about the severity of the receptor defect in vivo and hence adds to the structural information provided by DNA analysis. It detects receptor defects due to mutations within the entire gene, including the DNA-binding domain, and is a rapid, simple, and cost effective procedure. It may provide useful information for the diagnosis and management of affected children.

**Key words** Sex hormone-binding globulin · Androgen receptor · Mutations · Androgen insensitivity syndrome · Pseudohermaphroditism

**Abbreviations** *AIS* androgen insensitivity syndrome · *AR* androgen receptor · *LH* luteinising hormone · *SHBG* sex hormone-binding globulin

## Introduction

Mutations of the androgen receptor (AR) gene cause a wide spectrum of phenotypes in patients who have a normal male (46,XY) karyotype and normal male gonads but defective masculinization due to reduced or absent AR function [9]. The phenotype may be either female in complete androgen insensitivity syndrome (AIS), predominantly female with slight signs of virilization, ambiguous, or predominantly male in partial AIS, or, if minimal androgen resistance is present, unequivocally male with impaired spermatogenesis and/or pubertal undervirilization [23]. In infants and children the diagnosis can be difficult, since phenotypes are variable, the hormonal profile is not diagnostic until after puberty, and ligand binding assays on genital skin fibroblasts are cumbersome and not infor-

Participants of the German Collaborative Intersex Study Group who referred patients to this study: I. Akkurt, Hamburg; N. Albers, Hannover; W. Blunck, Hamburg; J. Brämswig, Münster; H. G. Dörr, Erlangen; H. Guhde, Celle; W. Hecker, Stuttgart; P. Heidemann, Augsburg; V. Hesse, Berlin; K. Hinkel, Dresden; W. Hoepffner, Leipzig; M. Holder, Stuttgart; M. Klasen, Osnabrück; G. Krüger, Rostock; F. A. Leidenberger, Hamburg; M. Mix, Rostock; M. Morlot, Hannover; R. Mühlenberg, Krefeld; L. Pelz, Rostock; H. Reich, Vechta; D. Schnabel, Berlin; R. Sinnecker, Berlin; R. P. Willig, Hamburg

G. H. G. Sinnecker (✉) · O. Hiort · E. M. Nitsche
P.-M. Holterhus · K. Kruse
Department of Paediatrics, Medical University of Lübeck,
Kahlhorststrasse 31–35, D-23538 Lübeck, Germany
Tel. and Fax: +49-451-500-2191

mative in up to one third of cases [9, 23]. DNA analysis of the AR gene allows a definitive diagnosis but is costly and confined to specialized laboratories. Furthermore, it provides solely information on receptor structure but not on its function. Since phenotypes may vary even in patients who carry the same mutation, factors other than variations in the coding sequence of the AR gene may affect receptor function [3, 5, 8, 10, 19, 33].

In a previous study we have shown that the anabolic-androgenic steroid stanozolol induces a distinct decrease of serum sex hormone-binding globulin (SHBG) levels in normal individuals similar to that induced by exogenous testosterone [6, 30], whereas a virilizing effect on accessory sexual characteristics was not observed. This decrease has been shown to be diminished in patients with AIS [29]. The aim of the present study was to prove if the SHBG test can be used for: (1) assessment of AR function in vivo; (2) diagnosis of AIS; (3) detection of receptor defects due to mutations in all exons of the AR gene, including the DNA-binding domain; (4) differentiation of patients who will exclusively feminize at puberty from those who are at risk for pubertal virilization. Furthermore, we investigated if a correlation exists between the SHBG responses and phenotypes which were classified according to the degree of undermasculinization.

## Materials and methods

### Classification of phenotypes

For the calculation of correlations between the severity of the receptor defect in utero (as evidenced by the degree of undermasculinization) and receptor function in vivo (as measured by the SHBG response in the androgen sensitivity test), we adapted a classification system for male pseudohermaphroditism due to steroid 5α-reductase deficiency [32] equivalent to that introduced by Prader [21] for patients with female pseudohermaphroditism due to congenital adrenal hyperplasia. In contrast to congenital adrenal hyperplasia patients, however, not only the genital anatomy at birth (i.e. the severity of defective masculinization in utero) has to be taken into account, but also subtle androgen effects which are not detectable until after puberty (i.e. spermatogenesis, pubic and axillary hair growth). This classification is based, therefore, on the clinical categories: (1) minimal androgen resistance (AIS type 1); (2) partial AIS with predominantly male phenotype (AIS type 2, Reifenstein syndrome); (3) partial AIS with frankly ambiguous genitalia (AIS type 3); (4) partial AIS with predominantly female phenotype (AIS type 4); and (5) complete AIS (AIS type 5, testicular feminization syndrome). If detailed information about genital anatomy, pubertal development and/or spermatogenesis was available, an alphanumerical subclassification for each type of AIS was used with regards to subtle differences in the severity of the masculinization defect. For details see Table 1 and Fig. 1.

### Subjects

Twenty three patients with AIS and 11 patients with genital malformations not due to AIS were studied. The latter served as controls. All patients with AIS had a normal male karyotype (46,XY). Informed consent for participation in this study, which had been approved by the local ethical committee, was obtained from all patients. If the subject was less than 18 years old and assented, the parents were informed and consented.

Nine patients with complete androgen insensitivity syndrome (AIS type 5) had female external genitalia, a blind vaginal pouch, and no uterus. Six of them were postpubertal (patients 1, 2, 4, 6, 7, 8). At adolescence they developed female sexual characteristics and had well developed breasts, scanty or no pubic and/or axillary hair in spite of high serum testosterone levels. All these patients had gonads located in the inguinal region or in the labia majora. Histologically, they contained immature seminiferous tubules, few spermatogonia, no spermatogenesis, and hyperplastic Leydig cells (patients 6, 7, 8). In all but two patients (patients 7, 8) the SHBG androgen sensitivity test was performed before gonadectomy. In patient 2 one gonad was removed before the test. In patient 8, who received sequencial oestrogen/gestagen replacement therapy, the test was performed during the 2nd week of the cycle. In the adult patients the hormonal plasma profile was characteristic for AIS: high or increased testosterone (14.8–43.5 nmol/l), luteinising hormone (LH) (13.0–16.8 IU/l), LH × testosterone product (192–731), and oestradiol concentrations (63–205 pmol/l). Receptor analysis in cultured genital skin fibroblasts of patient 6 had revealed a quantitative and qualitative receptor defect [29]. Patients 7 and 8 were siblings, the others were unrelated.

**Table 1** Classification of AIS phenotypes (see also Fig. 1)

| Type | Phenotype | Subtype | Phenotype/Function |
|---|---|---|---|
| 1 | Male | 1a | Impaired spermatogenesis |
| | | 1b | 1a and/or impaired pubertal virilization |
| 2 | Predominantly male | 2a | Isolated hypospadias |
| | | 2b | Micropenis and severe hypospadias, bifid scrotum |
| 3 | Ambiguous | 3a | Microphallus with clitoris-like underdeveloped glans, labia majora like bifid scrotum, perineoscrotal hypospadias |
| | | 3b | As 3a but sinus urogenitalis with a short, blind ending vagina |
| 4 | Predominantly female | 4a | Clitoromegaly and labial fusion, sinus urogenitalis with a wide opening, short, blind-ending vagina |
| | | 4b | Slight signs of androgen effects: slight clitoromegaly or partial labial fusion, distinct urethral and vaginal opening |
| 5 | Female | 5a | Pubertal development of female secondary sex characteristics (breast and scanty pubic and/or axillary hair) without signs of virilization (i.e. no clitoromegaly, no deepening of the voice, no hirsutism) |
| | | 5b | As 5a but no development of any androgen dependent structures (i.e. no pubic and/or axillary hair) |

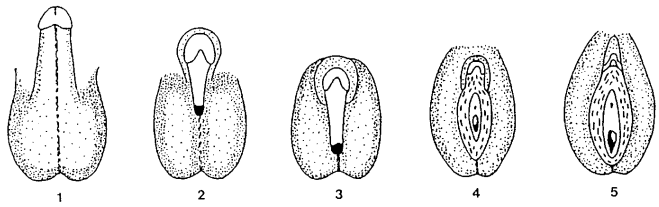


**Fig. 1** Classification of phenotypes in male pseudohermaphroditism due to AIS (adapted from Sinnecker et al. 1996 [32]). Type 1 is anatomically normal male, however, spermatogenesis and/or pubertal virilization are impaired. Type 2 is predominantly male with either isolated hypospadias, or micropenis, bifid scrotum and severe hypospadias. Type 3 is frankly ambiguous, the microphallus appears clitoris-like, the bifid scrotum appears like labia majora, the orifitium urethrae externum is either perineal or ends in a sinus urogenitalis, associated with a short, blind-ending vagina. Type 4 is predominantly female with slight signs of excess androgen effects: clitoromegaly, partial labial fusion with a sinus urogenitalis or distinct urethral and vaginal openings. Type 5 is anatomically female without signs of excess androgen effects (for details see Table 1)

Patient 1 had most extensive clinical signs of testicular feminization: female phenotype with almost invisible blonde vellus hair in the pubic region (i.e. no pubic hair), scanty lateral eyebrows, hypoplastic labia majora, good breast development, normal stature and normal intelligence (AIS type 5b). The hormonal plasma profile was also characteristic for AIS: LH and testosterone were increased to 15.7 IU/l and 30.0 nmol/l, respectively, the LH × testosterone product was consecutively increased to 471. The oestradiol concentration in serum was increased to 125 pmol/l.

Fourteen patients had partial androgen insensitivity syndrome (AIS type 2–4), two of them were siblings (patients 21, 22), the others were unrelated. Three of these patients (patients 10, 11, 12) had a predominantly female phenotype (AIS type 4) with a short, blind-ending vagina, and mild signs of masculinization (clitoromegaly). The gonads were located in the inguinal region or in the labia majora. Patient 11 had no clitoromegaly, but rugation of the labia majora. On examination at the age of 4 years, patient 12 appeared completely female except for mild fusion of the labia minora and a consecutively small introitus vaginae. The binding affinity for dihydrotestosterone in cultured genital skin fibroblasts was reduced [11].

Four patients had frankly ambiguous genitalia at birth (AIS type 3). Patient 13 had a microphallus (< 1 cm), a smooth bifid scrotum which appeared like labia majora, and a perineal introitus sinus urogenitalis (AIS type 3b). She was initially assigned male. Since the effect of testosterone therapy (50 mg testosterone-enanthate i.m. monthly × 3) had been considered insufficient (phallic size increased to 1.7 cm) gender had been reassigned female. Patients 14, 15, and 16 had AIS type 3a (ambiguous genitalia, but no sinus urogenitalis). They are being reared as boys. Patient 16 had a clitoris-like micropenis of < 1 cm, a bifid scrotum with smooth skin which appeared like labia majora, and penile hypospadias.

Seven patients had partial androgen resistance with predominantly male phenotype (AIS type 2). Each of these patients had a variable degree of micropenis, bifid scrotum, and hypospadias, but no sinus urogenitalis, vagina, or uterus (for details see Table 2). All patients had surgical repairs of hypospadias. The older patients developed severe gynecomastia at puberty, which was treated by means of mastectomy in all of them (patients 18, 20, 21, 22). The hormonal profile in serum was characteristic of AIS in the postpubertal patients: high or increased testosterone (26.7–97.1 nmol/l), LH (9.9–12.4 IU/l), LH × testosterone product (264–1204), and oestradiol concentrations (136–327 pmol/l). Receptor analysis had revealed a qualitative receptor defect in patients 21, and 22 [11]. Patient 23 had only minor signs of defective masculinization (penoscrotal hypospadias, but otherwise normal male genitalia, AIS type 2a).

Eleven subjects had genital malformations due to other causes than AIS. Subject 24, 25, and 26 had micropenis and scrotal or perineal hypospadias due to 5α-reductase deficiency. Subjects 27–31 had incomplete masculinization due to gonadal dysgenesis or true hermaphroditism. Subject 27 and 28 are being reared as girls, subjects 29–31 have been assigned a male gender. Subject 32 had a female phenotype with clitoromegaly (2 cm) and slight posterior labial fusion due to Leydig cell hypoplasia. Subject 33 had a small penis (2 cm) without any other symptoms of defective masculinization. Subject 34 was a genetic (karyotype 46,XX) and gonadal female with a female phenotype, but agenesis of vagina and uterus (Mayer-Rokitansky-Küster-Hauser syndrome) (for details see Table 2).

## DNA-analysis

DNA analysis was performed as previously described [11–13]. Briefly, DNA was extracted from blood leucocytes. Exon 1–8 (except for a segment of exon 1, spanning nucleotides 1248 –1691) of the AR gene were amplified by polymerase chain reaction [11]. The amplification products were screened for variations by non-radioactive single strand conformation polymorphism analysis [13]. The samples showing aberrant migration on single strand conformation polymorphism analysis were directly sequenced in two separate reactions in sense and antisense direction. Primers were labelled with γ-[$^{33}$P]ATP and sequencing performed using the Sequenase sequencing kit (Amersham Buchler, Braunschweig, Germany) [11].

## SHBG assay

Serum SHBG was measured as previously described [29]. In brief, samples were incubated with [$^{125}$I]p-hydroxyphenylpropionyl-SHBG (10–20 × $10^3$ cpm), and rabbit anti-SHBG antiserum for 16–20 h at room temperature. Goat anti-rabbit γ-globulin containing 12.5 g/l normal rabbit serum was added and the tubes incubated for another 1.5–3 h at room temperature. The mixtures were centrifuged at 4° C for 30 min at 2000 *g*, the supernatants aspirated, and the radioactivity was measured in the pellets. Alternatively, a commercial SHBG-radioimmunoassay kit (Milab, Sweden) which employs the same tracer ([$^{125}$I]p-hydroxyphenylpropionyl-SHBG) was used according to the manufacturers instructions, except for the standard curve, which was always derived from a pool of sera from women in late pregnancy. This pool had been calibrated with a standard pregnancy plasma, which was a gift from Drs. Khan and Rosner, New York, USA. Using the same standard curves, the variance between results obtained with the above described assay and with the commercial kit was within the interassay variance determined for the above described assay (11.2% in ten repeat assays of three samples). The mean within assay coefficient of variation was 7.8% (ten measurements in each of three samples). Each sample and standard were analysed in quadruplicate at two different dilutions. Results were computer analysed by fitting standard curves to a four-parameter logistic function and interpolating unknowns from the resulting curve [25].

## SHBG androgen sensitivity test

The androgen sensitivity test was performed according to the previously described protocol [29]: the anabolic-androgenic steroid stanozolol (17β-hydroxy-17α-methyl-5α-androstano-[3, 2-c]pyrazol) was administered orally for 3 consecutive days (0.2 mg/kg/day in a single evening dose). Blood samples were taken once before and 5, 6, 7, and 8 days after the beginning of the test. The initial SHBG concentration in serum (day 0) was compared to the lowest level obtained after application of stanozolol (days 5,6,7, and 8) and expressed as a percentage of the initial value. This percentage was used as the measure of response to stanozolol. Since the androgen-induced SHBG decrease may be superimposed

**Table 2** Age, phenotype, AR gene mutation, and SHBG values in the androgen sensitivity test in 23 patients with AIS

| Patient | Age (years; months) | AIS type | Mutation | Exon | Codon | Base change | Amino acid change | SHBG initial[a] (nmol/l) | SHBG nadir[b] (%) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 17;3 | 5b | Deletion | 1–8 | | | | 55.6 | 129.0 |
| 2 | 32;1 | 5b | Deletion | 3 | | | | 126.5 | 109.0 |
| 3 | 9;7 | 5 | Deletion | 1 | 140–148 | | Stop 154 | 86.4 | 105.6 |
| 4[c] | 18;6 | 5b | Insertion | 1 | 215 | GCT-CGCT | Stop 232 | 66.1 | 99.2 |
| 5[c] | 0;1 | 5 | Point | 5 | 765 | GCC-ACC | Ala-Thr | 182.0 | 92.4 |
| 6 | 24;4 | 5a | Point | 5 | 754 | TTC-GTC | Phe-Val | 118.0 | 96.0 |
| 7[c] | 23;0 | 5a | Point | 6 | 779 | CGG-TGG | Arg-Trp | 117.0 | 97.0 |
| 8[c] | 25;8 | 5a | Point | 6 | 779 | CGG-TGG | Arg-Trp | 185.1 | 93.0 |
| 9[c] | 0;4 | 5 | Point | 8 | 870 | GCG-GGG | Ala-Gly | 406.0 | 96.5 |
| 10[c] | 12;2 | 4b | Point | 2 | 582 | TTC-TCC | Phe-Ser | 69.1 | 83.0 |
| 11 | 1;10 | 4b | Point | 4 | 695 | GAC-AAC | Asp-Asn | 116.9 | 87.0 |
| 12[c] | 5;0 | 4b | Point | 7 | 840 | CGT-CAT | Arg-His | 96.6 | 81.3 |
| 13[c] | 0;2 | 3b | Point | 5 | 771 | AAT-CAT | Asn-His | 94.2 | 80.7 |
| 14[c] | 6;5 | 3a | Point | 3 | 604 | GAT-TAT | Asp-Tyr | 115.5 | 68.4 |
| 15 | 30;5 | 3a | Point | 5 | 746 | GTG-ATG | Val-Met | 111.6 | 83.4 |
| 16[c] | 7;9 | 3a | Point | 5 | 754 | TTC-TTA | Phe-Leu | 125.8 | 89.1 |
| 17 | 0;10 | 2b | Point | 4 | 686 | TGT-CGT | Cys-Arg | 101.5 | 78.8 |
| 18 | 24;11 | 2b | Point | 4 | 687 | GCT-GTT | Ala-Val | 49.0 | 80.8 |
| 19[c] | 1;7 | 2b | Point | 4 | 708 | GGA-GCA | Gly-Ala | 136.5 | 48.6 |
| 20 | 37;0 | 2b | Point | 4 | 712 | CTT-TTT | Leu-Phe | 98.7 | 67.0 |
| 21[c] | 15;4 | 2b | Point | 7 | 866 | GTG-TTG | Val-Leu | 72.1 | 65.6 |
| 22[c] | 11;6 | 2b | Point | 7 | 866 | GTG-TTG | Val-Leu | 51.0 | 71.2 |
| 23[c] | 2;1 | 2a | Point | 8 | 870 | GCG-GTG | Ala-Val | 119.7 | 49.3 |

[a] Serum SHBG concentration before stanozolol application
[b] Lowest SHBG concentration 5–8 days after initiation of the 3-day stanozolol administration period (0.2 mg/kg/day)
[c] Mutations that we have previously described [11–13]

Amino acid numeration is according to the nomenclature of Lubahn et al. [18]

by the physiological SHBG increase in neonates (from 43.7 ± 6.7 [SE] nmol/l at birth to 106.7 ± 3.2 nmol/lL after 1 month [28]), we usually perform the test after the neonatal period and prefer an age older than 3 months, if the initial SHBG value has not already reached a high childhood level, indicating that the neonatal SHBG surge has already been occurred. All samples from each subject were analysed in the same assay. The majority of these tests were performed on an outpatient basis by the participants of the German Intersex Study Group. The serum samples were stored at –20° C until completion of the test and then sent to our laboratory by first class mail.

### Reagents and hormone measurements

Stanozolol (Stromba) was obtained from Sterling Research Laboratories, U.K.; SHBG-Tracer ([$^{125}$I]p-hydroxyphenylpropionyl-SHBG; 34.8 Mbq/nmol) from Milab Malmö, Sweden; Hormone measurements were performed as previously described [29, 32].

### Statistics

The results are expressed as the mean ± SE. Statistical analysis was performed using the SPSS/PC+ (SSPS, Inc., Chicago, IL) statistical software. Kruskal-Wallis test was performed as appropriate. $P < 0.05$ was considered significant.

## Results

### Complete AIS (AIS type 5)

In the patients with AIS type 5, mutations were spread out all over the AR gene leading to deletions, insertions or non-conservative amino acid substitutions in the AR protein. SHBG did not decrease in the androgen sensitivity test (mean 102.0 ± 3.8 [SE]%; median 97%; range 92.4%–129% of the initial value) (see also Fig. 2, Table 2).

In patient 1 the whole gene (exons 1–8) was deleted. SHBG increased to 129.0% of the initial value. In patient 2 a deletion of exon 3, encoding the second zinc finger of the receptor protein, was found. SHBG increased slightly to 109.0% of the initial value. In patient 3 a deletion of 23 base pairs was found in exon 1 (affecting codons 140–148 according to the nomenclature of Lubahn et al. [18], leading to a stop codon in position 154) and in patient 4 an additional guanine was inserted in codon 215 initiating a stop in codon 232. The SHBG concentration remained unchanged after administration of stanozolol (Table 2).

In patients 5–9, point mutations leading to non-conservative amino acid substitutions in the hormone-binding domain of the AR gene were found. SHBG did not decrease significantly after administration of stanozolol (see also Fig. 2, Table 2). The differences between SHBG responses of these patients and all other groups were statistically significant ($P < 0.05 – < 0.0001$).

### Partial AIS with predominantly female phenotype (AIS type 4)

Point mutations in exons 2–8 of the AR gene which cause non-conservative amino acid substitutions in the AR protein were found in all patients with partial AIS. In the

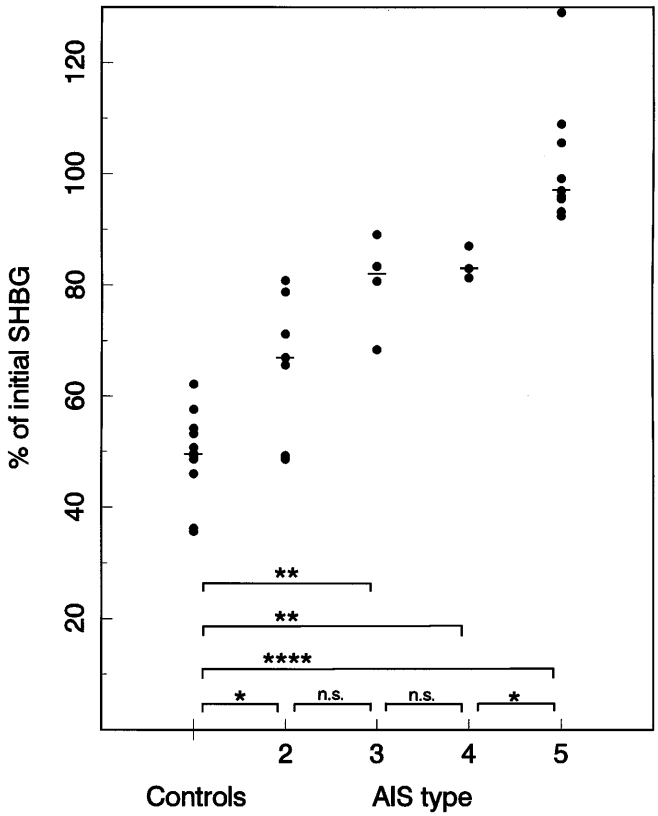


**Fig. 2** Decrease in serum SHBG concentration after 3 days of stanozolol administration (0.2 mg/kg), expressed as the percentage change from initial values in patients with AIS (phenotypes 2–5) and in control patients with genital malformations not due to androgen resistance. The *circles* represent the patients, the *lines* represent the median for each group. The *asterisks* indicate the significance levels of differences between the adjacent groups (n.s.=not significant; * = < 0.05; ** = < 0.01; *** = < 0.001; **** = < 0.0001)

three patients with AIS type 4, who were reared as females, SHBG decreased slightly to 83.8% ± 1.7% of the initial value (median 83.0%; range 81.3%–87.0%) in the androgen sensitivity test, which was significantly lower than in AIS type 5 ($P < 0.05$), but higher than in the controls ($P < 0.01$) (see also Fig. 2, Table 2).

### Partial AIS with ambiguous genitalia (AIS type 3)

In the four patients with AIS type 3 SHBG decreased also slightly after administration of stanozolol (mean 80.4% ± 4.7%, median 82.1%, range 68.4%–89.1%), which was statistically no different from AIS type 2 and 4, but lower than AIS type 5 ($P < 0.01$), and higher than in the controls ($P < 0.01$) (see also Fig. 2, Table 2).

### Partial AIS with predominantly male phenotype (AIS type 2)

In the seven patients with AIS type 2 (predominantly male phenotype) who were reared as males, a variable, but usually more pronounced SHBG decrease was observed in the androgen sensitivity test: mean 65.9% ± 4.9%; median 65.6%; range 48.6%–80.8% of the initial value. This decrease was statistically not different from AIS type 3, but differed significantly from the controls ($P < 0.05$) (see also Fig. 2, Table 2).

### Controls

In the 11 control patients (patients 24–34), in whom a cause other than AIS could be found to explain genital maldevelopment, no nucleotide variations were found in the AR gene. SHBG decreased in these patients to a mean of 51.4% ± 2.1% (median 49.6%, range 35.6%–62.1%) of the initial value, which was within the normal range (i.e. < 63.4% [28]) (for details see Table 3).

A close correlation was found between the degree of undermasculinization (AIS phenotype) and the SHBG response in the androgen sensitivity test (see Fig. 2).

## Discussion

In male pseudohermaphroditism, management regarding gender assignment, genital surgery, and gonadectomy should be based on accurate diagnosis and prognosis for pubertal development. However, due to the variability of clinical phenotypes and the lack of a diagnostic hormonal profile of AIS in infancy, diagnosis and prognosis can be difficult. Until recently, the evaluation of androgen binding in fibroblasts derived from genital skin biopsies has been used for the diagnosis of AIS [23]. However, androgen binding may be normal in up to one third of patients with AIS, in particular in those who carry mutations in the DNA-binding domain [1, 15, 16, 23]. Therefore, receptor binding studies are of limited practical value for clinical purposes.

The characterization of mutations in the AR gene can serve as a reliable tool for the diagnosis and molecular subclassification of AIS [2, 3, 11, 13, 26]. In contrast to biochemical studies, which require genital skin biopsies, molecular genetic analysis can be achieved as a minimally invasive procedure from blood leucocyte DNA. This has lead to an increasing number of patients diagnosed with AIS. However, molecular genetic studies provide information about AR structure, but not about AR function.

Deletions and an unusual insertion mutation of the AR gene were found in patients 1, 2, 3, and 4. These nonsense mutations constitute the ultimate form of complete AIS (type 5b), as evidenced by absence of any androgen dependent sex characteristics (i.e. pubic and axillary hair in the adult patients) and complete absence of any stanozolol-induced SHBG decrease. SHBG even tends to increase in these patients, which may be due to the completely unopposed oestrogen effects, which may become visible if the androgen effect is totally abolished. However, the difference between the values of the patients

**Table 3** Age, phenotype, karyotype, diagnosis, and SHBG values in the androgen sensitivity test in 11 patients with genital malformations not due to AIS

| Patient | Age (years; month) | Genital malformation | Karyotype | Diagnosis | SHBG initial[a] (nmol/l) | SHBG nadir[b] (%) |
|---|---|---|---|---|---|---|
| 24 | 3;10 | Micropenis, perineal hypospadias | 46,XY | 5α-reductase deficiency | 184.3 | 62.1 |
| 25 | 7;10 | Micropenis scrotal hypospadias | 46,XY | 5α-reductase deficiency | 89.1 | 50.7 |
| 26 | 4;1 | Small penis | 46,XY | 5α-reductase deficiency | 98.6 | 57.8 |
| 27 | 6;3 | PHM type 2 | 45,X/46,XY | Gonadal dysgenesis | 90.8 | 49.4 |
| 28 | 15;11 | PHM type 4 | 46,XY | Gonadal dysgenesis | 21.9 | 57.6 |
| 29 | 10;4 | PHM type 2 | 46,XY | Gonadal dysgenesis | 19.5 | 49.6 |
| 30 | 1;3 | Micropenis, scrotal hypospadias | 45,X/46,XY | Gonadal dysgenesis | 80.3 | 48.6 |
| 31 | 4;6 | Cryptochidism, chordee | 46,XX/46,XY | True hermaphroditism | 121.4 | 35.6 |
| 32 | 8;10 | PHM type 4 | 46,XY | Leydig cell hypoplasia | 145.7 | 54.2 |
| 33 | 10;8 | Micropenis | 46,XY | Isolated micropenis | 117.7 | 46.0 |
| 34 | 20;10 | No uterus and vagina | 46,XX | Agenesis of uterus/vagina | 56.0 | 53.2 |

*PHM type*, classification of phenotypes in male pseudohermaphroditism, estimating the severity of defective masculinization according to the classification used in this study for AIS (see Fig. 1), except for the alphanumerical subgroups, which apply only for AIS

[a] Serum SHBG concentration before stanozolol application

[b] Lowest SHBG concentration 5–8 days after initiation of the 3-day stanozolol administration period (0.2 mg/kg/day)

with AIS type 5b and those with AIS type 5a, who carry missense mutations, is statistically not significant.

The other patients with complete AIS (patients 6–9) carry missense mutations within the hormone-binding domain of the AR gene (see Table 2). In accordance with the completely female phenotype SHBG remained unchanged in all of them after the application of stanozolol, indicating total loss of AR function in these patients, no matter whether they develop some pubic and/or axillary hair at puberty. Since they all do not virilize at puberty in spite of high endogenous testosterone levels, they constitute one clinical group of patients, AIS type 5 according to the classification used in this study.

In the patients with partial AIS and predominantly female phenotype (type 4) and in those with ambiguous genitalia (AIS type 3) missense mutations within the DNA- and hormone-binding regions of the AR gene were found (see Table 2). A moderate SHBG decrease could be observed in the androgen sensitivity test, which was significantly more compared to the patients with AIS type 5, and significantly less than in controls. Our patient 12 appeared almost completely female except for mild labial fusion. The SHBG decrease to 81% clearly indicates partial androgen activity. The mutation she carries, 840Arg-His, has been described several times in association with partial AIS [23]. The moderate SHBG decrease (i.e. severely inhibited SHBG decrease) serves as an indicator of severely disturbed AR function. The fact that SHBG decreases at all, however, indicates that some residual AR function is left. Thus, the SHBG test can be used for distinguishing patients with AIS type 5, who will exclusively feminize at puberty, from those, who are at risk for pubertal virilization due to remaining clinically relevant AR function (AIS type 4 or less), in whom it is prudent to remove the testes before puberty to avoid virilization.

In the patients with partial AIS and predominantly male phenotype (AIS type 2) point mutations were found within the steroid-binding domain of the AR gene (see Table 2). The mean SHBG decrease was more pronounced compared to the more severely affected patients (types 3–5). However, the individual SHBG response varied from values within the normal range (patients 19, 23) to values as usually seen in patients with ambiguous genitalia (AIS type 3, patient 18).

The sensitivity of this biological test may not be high enough to detect minor functional defects of the AR in all instances. For the clinical management, however, it may be a valuable information that the functional AR defect is of minor severity and hence, spontaneous virilization at puberty or at least response to high dose testosterone therapy may be expected. On the other hand, in patients 15 and 18, in whom SHBG decreased only moderately as usually seen in patients with AIS type 4, no response to high dose testosterone treatment could be observed, indicating a more severe functional AR defect than to be expected from the phenotype alone (unpublished observations).

The substitution of valine by leucine at position 866 has exclusively been reported to be associated with partial AIS [11, 17, 26]. Extensive characterization of this mutation in genital skin fibroblasts revealed that in the presence of high dihydrotestosterone concentrations, transcriptional activation by the mutant receptor approached that of the normal receptor [17], indicating dependency of receptor function on the actual androgen concentration. In keeping with this observation, SHBG decreased significantly, but less than normal in our patients 21 and 22 and they had substantial penile growth and pubertal virilization in response to treatment with high dose testosterone enanthate [31]. The androgen induced SHBG decrease may reflect androgen receptor function in vivo, comparable to the measurement of androgen induced transcriptional activity of mutant ARs in genital skin fibroblasts in vitro.

The mean SHBG decrease in the control patients approached 50% of the initial value following the administration of stanozolol, which is significantly below the values obtained in the patients with AIS. All individual results were within or below the normal range (i.e. 51.6% ±

11.8% [± 2SD]), which was established previously by investigation of healthy volunteers [29].

Phenotype is an expression of multiple variables, of which AR binding characteristics, transactivation, type and concentration of the ligand are known factors, some others, however, are presumably unknown. The phenotypic classification system used in this study serves for the estimation of the severity of the receptor defect, expressed in terms of different types of defective masculinization (AIS types 1–5).

Figure 2 illustrates that the functional defect in AIS, expressed in terms of SHBG decrease in the androgen sensitivity test, comprises a continuum, ranging from completely abolished AR function over severely and less severely altered AR function in different types of AIS to the normal range found in the controls. The differences between controls and each AIS group and between AIS type 4 and 5 were statistically significant and a close correlation was found between the degree of undermasculinization (AIS phenotype) and the SHBG response in the androgen sensitivity test. However, due to the variability of SHBG responses within the groups with partial AIS (types 2–4), there is considerable overlap between these groups. For clinical purposes, the interpretation of these results should therefore always include the thorough comparison with the phenotype to control for plausability. This is true, of cause, for the interpretation of any laboratory result.

Another approach for the study of AR function is the analysis of transcriptional activity of mutant receptors by cotransfection assays [4, 7, 17, 22, 24] or by adenovirus-mediated delivery of a reporter gene in genital skin fibroblast cultures [20] in vitro and to correlate the results with the degree of defective masculinization in vivo. However, these methods are invasive, as a genital skin biopsy is required for fibroblast harvesting, they are cumbersome and restricted to spezialized laboratories. In contrast, the SHBG androgen sensitivity test is less invasive and can be performed easily on an outpatient basis independent from the location of the laboratory. Since SHBG in serum and plasma is stable for 7–8 weeks at room temperature, and even for 3 days at 37° C, it can easily be shipped within 1–2 days without loss of immunoreactivity [27].

The SHBG androgen sensitivity test detects receptor defects due to mutations in all exons of the AR gene, including the DNA-binding domain. It clearly helps to distinguish AIS from other causes of genital malformation in the genetic male and in particular, it helps to distinguish patients who will exclusively feminize at puberty from those who are at risk for pubertal virilization. Furthermore, it provides functional information about the severity of the receptor defect in vivo and hence, it adds to the structural information provided by molecular genetic analysis of the AR gene. The quantification of residual AR function may provide useful information for the diagnosis and management of the intersex child with AIS.

**Acknowledgements** The authors thank Dagmar Struve, Andrea Lehners, and Anke Zöllner for excellent technical assistance, and Dr. Dibbelt, Institut for Biochemical Endocrinology, Medical University of Lübeck, for measurement of LH, testosterone, and oestradiol. This work was supported by the German Research Foundation (DFG grants Si 323/2–2, Hi 497/3–1), and the Federal Ministry for Education and Research (BMBF grant 01KY93011 to GHGS and OH)

## References

1. Amrhein JA, Meyer III WJ, Jones Jr HW, Migeon CJ (1976) Androgen insensitivity in man: evidence for genetic heterogeneity. Proc Natl Acad Sci USA 73: 891–894
2. Batch JA, Williams DM, Davies HR, Brown BD, Evans BAJ, Hughes IA, Patterson MN (1992) Androgen receptor gene mutations identified by SSCP in fourteen subjects with androgen insensitivity syndrome. Hum Molec Genet 1(7): 497–503
3. Batch JA, Davies HR, Evans BAJ, Hughes IA, Patterson MN (1993) Phenotypic variation and detection of carrier status in the partial androgen insensitivity syndrome. Arch Dis Child 68: 453–457
4. Beitel LK, Kazemi-Esfarjani P, Kaufman M, Lumbroso R, DiGeorge AM, Killinger DW, Trifiro MA, Pinsky L (1994) Substitution of arginine-839 by cysteine or histidine in the androgen receptor causes different receptor phenotypes in cultured cells and coordinate degrees of clinical androgen resistance. J Clin Invest 94: 546–554
5. Brown TR, Maes M, Rothwell SW, Migeon CJ (1982) Human complete androgen insensitivity with normal dihydrotestosterone receptor binding capacity in cultured genital skin fibroblasts: evidence for a qualitative abnormality of the receptor. J Clin Endocrinol Metab 55: 61–69
6. Ciaccio M, Rivarola M, Belgorosky A (1989) Decrease of sex hormone-binding globulin as a marker of androgen sensitivity. Correlation with clinical response. Acta Endocrinol (Copenh) 120: 540–544
7. De Bellis A, Quigley CA, Marschke KB, El-Awady MK, Lane MV, Smith EP, Sar M, Wilson EM, French FS (1994) Characterization of mutant androgen receptors causing partial androgen insensitivity syndrome. J Clin Endocrinol Metab 78: 513–522
8. Forest MG, Mollard P, David M, Morel Y, Bertrand J (1990) Syndrome d`insensibilité incomplète aux androgènes. Difficultes du diagnostic et de la conduit a tenir. Arch Fr Pediatr 47: 107–113
9. Griffin JE (1992) Androgen resistance – the clinical and molecular spectrum. N Engl J Med 326: 611–618
10. Grino PB, Isidro-Gutierrez RF, Griffin JE, Wilson JD (1989) Androgen resistance associated with a qualitative abnormality of the androgen receptor and responsive to high dose androgen therapy. J Clin Endocrinol Metab 68: 578–584
11. Hiort O, Huang Q, Sinnecker GHG, Sadeghi-Nejad A, Kruse K, Wolfe HJ, Yandell DW (1993) Single strand conformation polymorphism analysis of androgen receptor gene mutations in patients with androgen insensitivity syndromes: application for diagnosis, genetic counseling, and therapy. J Clin Endocrinol Metab 77: 262–266
12. Hiort O, Klauber G, Cendron M, Sinnecker GHG, Keim L, Schwinger E, Wolfe HJ, Yandell DW (1994) Molecular characterization of the androgen receptor gene in boys with hypospadias. Eur J Pediatr 153: 317–321
13. Hiort O, Wodtke A, Struve D, Zöllner A, Sinnecker GHG (1994) Detection of point mutations in the androgen receptor gene using non-isotopic single strand conformation polymorphism analysis. Hum Molec Genet 3 (7): 1163–1166
14. Hughes IA, Evans BAJ (1986) Complete androgen insensitivity syndrome characterized by increased concentration of a normal androgen receptor in genital skin fibroblasts. J Clin Endocrinol Metab 63: 309–315

15. Hughes IA, Evans BAJ (1987) Androgen insensitivity in forty-nine patients: classification based on clinical and androgen receptor phenotypes. Horm Res 28: 25–29
16. Kaufman M, Pinsky L, Baird PA, McGillivray BC (1979) Complete androgen insensitivity with a normal amount of 5α-dihydrotestosterone-binding activity in labium majus skin fibroblasts. Am J Med Genet 4: 401–411
17. Kazemi-Esfarjani P, Beitel LK, Trifiro M, Kaufman M, Rennie P, Sheppard P, Matusik R, Pinsky L (1993) Substitution of valine-865 by methionine or leucine in the human androgen receptor causes complete or partial androgen insensitivity, respectively with distinct androgen receptor phenotypes. Mol Endocrinol 7: 37–46
18. Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS (1989) Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. Proc Natl Acad Sci USA 86: 9534–9538
19. McPhaul MJ, Marcelli M, Zoppi S, Wilson CM, Griffin JE, Wilson JD (1992) Mutations in the ligand-binding domain of the androgen receptor gene cluster in two regions of the gene. J Clin Invest 90: 2097–2101
20. McPhaul MJ, Deslypere J-P, Allman DR, Gerard RD (1993) The adenovirus-mediated delivery of a reporter gene permits the assessment of androgen receptor function in genital skin fibroblast cultures. J Biol Chem 268: 26063–26066
21. Prader A (1954) Der Genitalbefund beim Pseudohermaphroditismus femininus des kongenitalen adrenogenitalen Syndroms. Helv Paediatr Acta 9: 231–248
22. Quigley CA, Evans BAJ, Simental JA, Marschke KB, Sar M, Lubahn DB, Davies P, Hughes IA, Wilson EM, French FS (1992) Complete androgen insensitivity due to deletion of exon C of the androgen receptor gene highlights the functional importance of the second zinc finger of the androgen receptor in vivo. Mol Endocrinol 6: 1103–1112
23. Quigley CA, De Bellis A, Marschke KB, El-Awady MK, Wilson EM, French FS (1995) Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr Rev 16 (3): 271–321
24. Ris-Stalpers C, Trifiro MA, Kuiper GGJM, Jenster G, Romalo G, Sai T, Rooij HCJ van, Kaufman M, Rosenfield RL, Liao S, Schweikert H-U, Trapman J, Pinsky L, Brinkmann AO (1991) Substitution of aspartic acid-686 by histidine or asparagine in the human androgen receptor leads to a functionally inactive protein with altered hormone-binding characteristics. Mol Endocrinol 5: 1562–1569
25. Rodbard D, Hutt DM (1974) Statistical analysis of radioimmunoassays and immunoradiometric (labeled antibody) assays: a generalized, weighted, iterative, least-squares method for logistic curve fitting. Symposium on radioimmunoassay and related procedures in medicine. Int Atomic Energy Agency, Vienna. Unipub, New York, pp 165–192
26. Saunders PTK, Padayachi T, Tincello DG, Shalet SM, Wu FCW (1992) Point mutations detected in the androgen receptor gene of three men with partial androgen insensitivity syndrome. Clin Endocrinol (Oxf) 37: 214–220
27. Sinnecker G (1989) Stability of sex-hormone-binding globulin in serum and plasma. Clin Chem 35: 1253
28. Sinnecker GHG (1993) Sexualhormon bindendes Globulin. Physiologische Bedeutung im Wirkungsmechanismus der Steroidhormone und klinische Bedeutung für die Diagnostik endokriner Erkrankungen. Thieme, Stuttgart, pp 77–78
29. Sinnecker G, Köhler S (1989) Sex hormone-binding globulin response to the anabolic steroid stanozolol: evidence for its suitability as a biological androgen sensitivity test. J Clin Endocrinol Metab 68: 1195–200
30. Sinnecker G, Lork S, Willig RP (1986) Gonadal and adrenal steroids and their binding globulins SHBG and CBG in patients treated with ethinylestradiol and testosterone oenanthate. Acta Endocrinol (Copenh) 111 [Suppl 274]: 18–20
31. Sinnecker GHG, Hiort O, Brack C, Nitsche E, Kruse K (1993) Partial androgen insensitivity due to val→leu substitution in codon 866 of the androgen receptor can be treated with high doses of testosterone. Pediatr Res 33 [Suppl]: 17
32. Sinnecker GHG, Hiort O, Dibbelt L, Albers N, Dörr HG, Hauß H, Heinrich U, Hemminghaus M, Hoepffner W, Holder M, Schnabel D, Kruse K (1996) Phenotypic classification of male pseudohermaphroditism due to steroid 5α-reductase deficiency. Am J Med Genet 63: 223–230
33. Wilson JD, Harrod MJ, Goldstein JL, Hemsell DL, MacDonald PC (1974) Familial incomplete male pseudohermaphroditism type 1. Evidence for androgen resistance and variable clinical manifestations in a family with the Reifenstein syndrome. N Engl J Med 290: 1097–1103