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Research paper

Predicting puberty in partial androgen insensitivity syndrome: Use of clinical and functional androgen receptor indices

Ngee Lek [a,b,c,1], Rieko Tadokoro-Cuccaro [a,1], Jonathan B. Whitchurch [d], Bismoy Mazumder [d], Harriet Miles [a], Philippa Prentice [a], Trevor Bunch [a], Karolina Zielińska [a], Veronika Metzler [e], Nigel P. Mongan [e], David M. Heery [d], Ieuan A. Hughes [a,*]

[a] University of Cambridge, Department of Paediatrics, Cambridge, United Kingdom
[b] KK Women's and Children's Hospital, Department of Paediatrics, Singapore
[c] Duke-NUS Medical School, National University of Singapore, Singapore
[d] School of Pharmacy, University of Nottingham, United Kingdom
[e] School of Veterinary Medicine and Science, University of Nottingham, United Kingdom

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ABSTRACT

Background: PAIS exhibits a complex spectrum of phenotypes and pubertal outcomes. The paucity of reliable prognostic indicators can confound management decisions including sex-of-rearing. We assessed whether external masculinisation score (EMS) at birth or functional assays correlates with pubertal outcome in PAIS patients and whether the EMS is helpful in sex assignment.

Methods: We collected pubertal outcome data for 27 male-assigned PAIS patients, all with confirmed androgen receptor (*AR*) mutations, including two previously uncharacterized variants (I899F; Y916C). Patients were grouped as follows; EMS at birth <5 and ≥ 5 (EMS in normal males is 12; median EMS in PAIS is 4·7) and pubertal outcomes compared.

Findings: Only 6/9 patients (67%) with EMS <5 underwent spontaneous onset of puberty, *versus* all 18 patients with EMS ≥5 ($p = .03$). Only 1/6 patients (17%) with EMS <5 developed adult genitalia reaching Tanner stage 4 or 5, *versus* 11/13 (85%) with EMS ≥5 ($p = 0{\cdot}01$). There was no significant difference between the two groups of patients in being prescribed androgen replacement, who reached adult testicular volume ≥ 15 ml, pubic hair Tanner stage 4 or 5, above average adult height, had gynaecomastia, and mastectomy. No correlation was observed between EMS and *in vitro* AR function.

Interpretation: In PAIS with *AR* mutation, birth EMS is a simple predictor of spontaneous pubertal onset and satisfactory adult genitalia. This provides useful information when discussing the likely options for management at puberty.

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1. Introduction

Androgen insensitivity syndrome (AIS) is the most common cause of undermasculinisation in XY males with a disorder of sex development (DSD) when characterised by a phenotype consistent with androgen resistance [3–5]. The complete form (CAIS) is characterised by a female phenotype, while the partial form (PAIS) is expressed as a variable male phenotype. This includes hypospadias, micropenis, bifid scrotum and undescended testes at birth, or in later life, gynaecomastia and infertility as a result of oligospermia or azoospermia in a male with otherwise normal genitalia.

CAIS is invariably caused by a mutation in the androgen receptor (*AR*) gene, whereas a pathogenic *AR* mutation is found in only 22% of patients with a PAIS-like phenotype [6]. Consequently, it is important to exclude other conditions which can present with a similar XY DSD phenotype when undertaking studies in PAIS. Demonstrating an *AR* mutation which was confirmed to be pathogenic as a cause of the PAIS phenotype was a requirement for inclusion of cases for this present

Abbreviations: DSD, disorder of sex development; PAIS, partial androgen insensitivity syndrome; EMS, external masculinisation score; AR, androgen receptor; SDS, standard deviation score; DHT, dihydrotestosterone; NID, nuclear receptor interaction domain; LBD, ligand-binding domain; DBD, DNA-binding domain; WT, wild-type.

* Corresponding author at: Department of Paediatrics, University of Cambridge, Level 8, Box 116, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QQ, United Kingdom.
E-mail address: iah1000@cam.ac.uk (I.A. Hughes).

[1] Contributed equally.

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Research in context

Evidence before this study

There are limited data on pubertal outcome in patients with partial androgen insensitivity syndrome (PAIS) assigned male at birth. We searched PubMed using the search term "('androgen insensitivity' AND (partial NOT complete)) AND ('long term' OR 'follow up' OR pubert* OR outcome)" (last searched in June 2018). After review, we could only identify two relevant articles [1,2]. One study reported pubertal outcome in 14 PAIS patients, all of whom had an external masculinisation score (EMS) of 5 or more, thus excluding information on the more severely undermasculinised infants at birth [1]. A more recent study reported pubertal outcome in a larger group of 29 males with a proven *AR* mutation and a range of EMS at birth [2]. A specific feature of this study was the universal finding of pubertal gynaecomastia.

Added value of this study

The paucity of outcome data at puberty makes it difficult to predict what will happen at puberty in boys with PAIS particularly when there is severe undermasculinisation of the external genitalia at birth. This study analysed prospectively collected data on EMS at birth and function of *AR* mutations in 27 PAIS patients to determine whether useful predictors of puberty outcome could be identified. The cohort included two patients with *AR* mutations (I899F and Y916C) for which functional studies had not previously been undertaken. All 18 patients who had EMS ≥5 at birth had spontaneous onset of puberty, whereas three of nine patients whose EMS <5 at birth failed to start puberty spontaneously. In contrast to the clinical findings, there was no clear predictor of puberty outcome from the functional analysis of *AR* variants.

Implications of all the available evidence

Our study indicates that in PAIS patients with a confirmed *AR* mutation, the EMS at birth is a simple predictor of spontaneous pubertal onset and adult genital development. Consistent with recent reports of gynaecomastia prevalence in PAIS [1,2], the majority of patients in this study developed the problem at puberty and irrespective of their EMS at birth. Consideration should be given to selectively starting an anti-estrogen or aromatase inhibitor in early puberty. Functional analysis of *AR* mutations *in vitro* provided detailed information to explain the PAIS phenotype but was not as predictive as clinical findings for puberty outcome. However, these assays together with *in silico* modelling of AR structure may prove beneficial in guiding optimal treatment in those patients requiring high dose androgen treatment.

study and knowledge of *AR* mutation status is important to guide management of PAIS in the longer term [2].

Sex assignment at birth for an infant with DSD is a central component of management undertaken jointly by health professionals and the family. In PAIS characterised by severe undermasculinisation, a key question raised is whether masculinisation is likely to occur at puberty. Data on such outcomes are limited [1,2]. Consistent findings appear to be a variable degree of spontaneous masculinisation at puberty, the need for androgen supplements to induce puberty in nearly half the cases, and the frequent occurrence of gynaecomastia.

There is a trend in recent decades towards male sex-of-rearing for all causes of XY DSD, including PAIS infants who are severely undermasculinised [7]. This poses a challenge of formulating predictive factors which inform puberty and subsequent development in early adulthood in males. We hypothesised that the degree of masculinisation at birth as assessed by a validated external masculinisation score (EMS) [8] and functional analysis of the cognate mutant *AR* would be clinically informative. Thus, clinical follow-up data and the results of *in vitro* functional studies for 19 unique *AR* mutations within the study cohort were analysed, including two previously uncharacterised *AR* mutants (I899F and Y916C) for which preliminary structure/function analysis is presented.

2. Patients and methods

2.1. Patients

The Cambridge DSD Database contains detailed information on each case based on a questionnaire completed by the referring clinician at the time of notification. Using this resource, 27 PAIS patients were identified with known *AR* mutations characterised functionally who were assigned male and were of pubertal or post-pubertal age at the last known clinical assessment.

2.2. Clinical data

Information on the external genitalia at birth was verified and further information on the status of pubertal development was obtained *via* a second questionnaire distributed to their current clinician following written informed consent from the patient and/or parents. The questionnaire was completed opportunistically during a routine clinic visit. The pubertal data were collected in binary format (yes/no): spontaneous onset of puberty; whether androgen replacement was given; testicular volume ≥ 15 ml (as assessed using Prader Orchidometer); Tanner stage 4 or 5 for pubic hair and genitalia (penile length was not measured consistently, with 'satisfactory penile development' often recorded); final adult height standard deviation score (SDS) ≥ 0 (*i.e.* taller than the average adult male in the UK using 1990 UK population reference); presence of gynaecomastia and whether mastectomy was performed. Data on testosterone and gonadotrophin concentrations were available in only 10 subjects, thereby an insufficient number for analysis in this study. The degree of virilisation at birth was quantified by the EMS [8]. The composite EMS ranges from a minimum of 0 (indicating complete lack of masculinisation) to a maximum of 12 (normal masculinisation).

The median EMS among all PAIS patients raised male in the Cambridge DSD Database ($n = 41$) is 4·7. The 27 patients in this study cohort were sub-divided into 2 groups based on an EMS at birth <5 (below median) and ≥ 5 (at or above median). The clinical data collated at puberty and beyond were compared between the two groups and analysed using Fisher's exact test for categorical variables as being the appropriate test for the relatively small sample size; statistical significance was taken to be $p < 0{\cdot}05$.

2.3. PAIS-associated AR mutations

Genomic DNA was isolated from peripheral leukocytes or genital skin fibroblasts using standard techniques. The coding exons and exon/intron boundaries of the *AR* gene were analysed by direct sequencing. Amino acid numbering for the human AR (1—920) is based on NM_000044.2 (NCBI). A total of 19 unique *AR* mutations were identified in the study cohort, which were assessed for their impact on AR-dependent reporter activation. Two previously uncharacterised variants were also assessed for dimerization and coactivator binding in yeast two-hybrid assays [9].

2.4. Mammalian expression plasmids

Wild-type (WT) human *AR* cDNA expression vector pSVAR0 was used to generate *AR* mutant expression vectors by QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies) [10]. All *AR* constructs were verified by sequencing. The luciferase reporter construct pGRE2-TATA-Luc has been described previously [11]. Renilla luciferase constructs pGL4-TK and phRG-TK (Promega) or pCH110 (β-galactosidase) were used as transfection controls.

2.5. Transient transfection and reporter assays

For AR transactivation studies, COS-1 or HeLa cells were seeded into 12-well tissue culture plates in Dulbecco's modified essential medium (DMEM) containing 2 mM glutamine and 10% charcoal-stripped serum. Cells were transfected with 250 ng pGRE2-TATA-Luc, 25 ng pSVAR0 and a control reporter 25 ng phRG-TK (Renilla Luciferase) or pCH110 (β-galactosidase) as indicated using standard transfection procedures. After 16 h incubation the cells were exposed to fresh medium containing 0–10 nmol dihydrotestosterone (DHT; Sigma) or the synthetic androgen mibolerone (Steraloids Inc) for a further 24 h. The cells were then lysed in 500 μl passive lysis buffer (Promega) and the ratio of firefly to renilla was determined using Nanolight technology Alternatively, Dual-light System (Applied Biosystems) was used to measure Luciferase and β-galactosidase activities. Reporter assays were quantified using a Microplate Luminometer LB 960 (Berthold).

2.6. Yeast two-hybrid assays

Yeast two-hybrid interaction studies were performed as described previously using the *S. cerevisiae* L40 reporter strain transformed with expression vectors for LexA-SRC1 NID 431–761 (nuclear receptor interaction domain) in combination with VP16-AR LBD (627–920) [9]. VP16-AR LBD mutants I899F and Y916C were generated by site-directed PCR mutagenesis, and constructs were validated by sequencing. For AR-LBD dimerization assays, LexA-AR-LBD was co-transformed with VP16-*AR* expression plasmids. Construct expression levels were determined by western blotting using antibodies specific for the LexA (06–719; Millipore) and VP16 (sc-7546; Santa Cruz) epitopes. Co-transformants were purified and grown in selective medium in the presence of 1 μM Mibolerone or vehicle. Reporter β-galactosidase activities are presented as the mean of three independent clones with error bars to indicate standard deviations.

2.7. Ethical approval

Ethical approval for this study was obtained from the local research ethics committee (09/H0308/158), and institutional approval was obtained from the Research and Development Committee at the Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK.

2.8. Role of the funding source

The funders had no role in study design, collection, analysis or interpretation of the data, writing the report, or the decision to submit the report for publication.

3. Results

3.1. EMS at birth and age of last clinical assessment

Table 1A, 1B shows EMS at birth and pubertal status at the last assessment for each of the 27 study participants in relation to their *AR* mutation type. The age range at last assessment was 14–44 years. Two of the participants (2-E and 2-O) were aged 14 years and both had entered puberty spontaneously but were not contactable at the time of the analysis. The remaining 25 participants had either recently completed puberty or were in young adulthood.

3.2. EMS and AR mutation type

Fig. 1 illustrates the distribution of mutations in the *AR* gene in this PAIS cohort. All were missense mutations and located in the DNA-binding (DBD) or ligand-binding (LBD) domains. There was no association between EMS at birth and the nature of the missense mutation. Indeed, some participants harbouring identical mutations displayed a widely variable EMS. For example, four participants (1-D, 1-E, 2-F and 2-G) with the same *AR* mutation (R841C) had an EMS of 1, 4, 5, and 9, respectively (see Table 1A, 1B).

Table 1A
Clinical pubertal data for the 9 patients with PAIS Group 1 (EMS at birth <5).

Study participant		1-A	1-B	1-C	1-D	1-E	1-F	1-G	1-H	1-I
AR mutation		R630W	S704G	F755 L	R841C	R841C	I899F #	R856H	A897E	A897E
Codon change		2247C > T	2469 A > G	2624C > G	2880C > T	2880C > T	3057 A > T	3016 G > A	3049C > A	3049C > A
Clinical features at birth										
EMS at birth (0−12)		3	2	1	1	4	1	3	3	3
	Scrotal fusion (0; 3)	0	0	0	0	0	0	0	0	0
	Microphallus (0; 3)	0	0	0	0	0	0	0	0	0
	Urethral meatus (0; 1; 2; 3)	0	0	0	0	1	0	0	0	0
	Right gonad (0; 0.5; 1.0; 1.5)	1.5	1	0.5	0.5	1.5	0.5	1.5	1.5	1.5
	Left gonad (0; 0.5; 1.0; 1.5)	1.5	1	0.5	0.5	1.5	0.5	1.5	1.5	1.5
Last known clinical features										
Age (years) at the last assessment		26	31	18	38	18	16	21	15	18
Spontaneous onset of puberty		Yes	No	No	Yes	No	Yes	Yes	Yes	Yes
Androgen replacement given		Yes	Yes	Yes	No	Yes	No	Yes	No	No
Adult testes ≥ 15 ml		No	No	Yes	–	Yes	Yes	No	–	Yes
Adult pubic hair PH4-PH5		Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes
Adult genitalia G4-G5		No	–	Yes	No	No	No	No	–	–
Adult height SDS > 0		No	No	Yes	No	Yes	Yes	No	Yes	Yes
Presence of gynaecomastia		Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Mastectomy done		Yes	Yes	Yes	Yes	Yes	Yes	No	No	No

Table 1B
Clinical pubertal data for the 18 patients with PAIS Group 2 (EMS at birth ≥ 5).

Study participant		2-A	2-B	2-C	2-D	2-E	2-F	2-G	2-H	2-I	2-J	2-K	2-L	2-M	2-N	2-O	2-P	2-Q	2-R
AR mutation		I665T	F674C	D691E	F755S	Y764C	R841C	R841C	R841H	I870M	I870M	A871V	M625 L	Y916C #	A597T	A871V	L713F &	L713F &	L713F &
Codon change		2353 T > C	2380 T > G	2432C > A	2623 T > C	2650 A > G	2880C > T	2880C > T	2881 G > A	2969 T > G	2969 T > G	2971C > T	2232 A > T	3106 A > G	2148 G > A	2971C > T	2496C > T	2496C > T	2496C > T
Clinical features at birth																			
EMS at birth (0–12)		6	6	5	8	8	5	9	5	6	6	10	7	5	6	6	8	8.5	9
	scrotal fusion (0; 3)	3	0	0	3	3	0	3	0	0	0	3	0	0	0	0	0	3	3
	microphallus (0; 3)	0	3	0	0	3	3	3	0	3	3	3	3	0	3	3	3	0	0
	urethral meatus (0; 1; 2; 3)	0	0	2	2	1	1	1	2	0	0	1	1	2	0	0	3	3	3
	right gonad (0; 0.5; 1.0; 1.5)	1.5	1.5	1.5	1.5	0.5	0.5	1	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1	1	1.5
	left gonad (0; 0.5; 1.0; 1.5)	1.5	1.5	1.5	1.5	0.5	0.5	1	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1	1.5	1.5
Last known clinical features																			
Age (years) at the last assessment		18	18	31	17	14	17	27	20	18	18	44	21	30	16	14	19	17	16
Spontaneous onset of puberty		Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Androgen replacement given		–	Yes	Yes	Yes	–	No	–	Yes	–	–	No	No	Yes	No	–	Yes	Yes	Yes
Adult testes ≥ 15 ml		Yes	Yes	Yes	No	–	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Yes	–	No	No	–
Adult pubic hair PH4-PH5		Yes	Yes	–	–	–	Yes	Yes	Yes	Yes	Yes	–	Yes	–	Yes	–	Yes	Yes	Yes
Adult genitalia G4-G5		Yes	Yes	No	–	–	Yes	No	Yes	Yes	Yes	–	Yes	–	Yes	–	Yes	Yes	Yes
Adult height SDS > 0		–	Yes	No	Yes	–	No	Yes	Yes	No	Yes	–	No	Yes	–	–	Yes	Yes	–
Presence of gynaecomastia		Yes	Yes	Yes	Yes	–	–	Yes	Yes	Yes	–	Yes	Yes	Yes	Yes	–	Yes	Yes	Yes
Mastectomy done		–	–	Yes	Yes	–	–	Yes	Yes	Yes	–	–	No	No	–	–	Yes	Yes	Yes

In this cohort of 27 patients with PAIS, a total of 19 different mutations in the *AR* were found.
All the mutations except I899F and Y916C (the ones marked with #) have accompanying data from functional studies for comparison with the clinical pubertal data.
Three siblings with *AR* mutation L713F (the ones marked with &) were included in the study by Lucas-Herald et al. See reference 2.

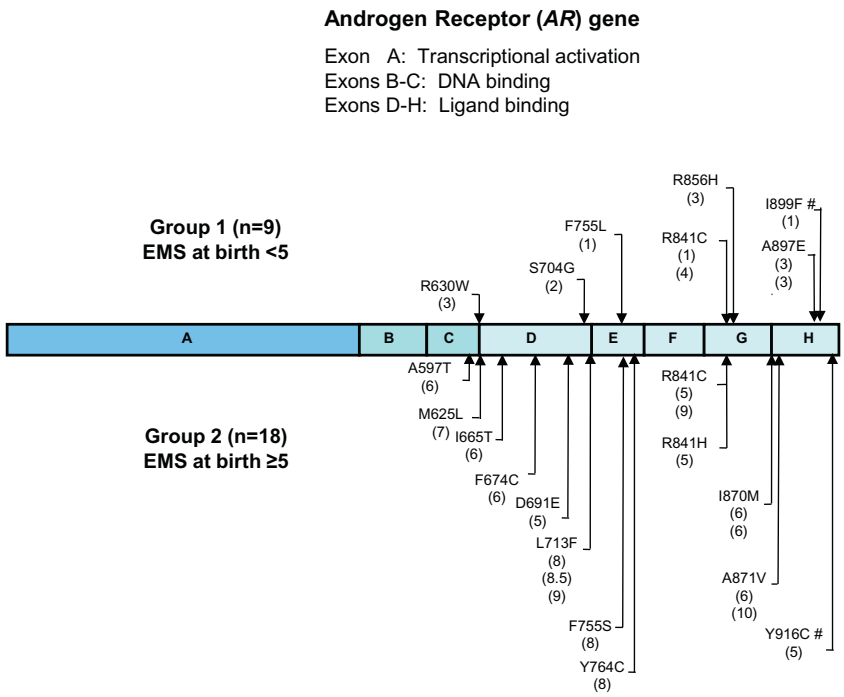

Fig. 1. Scheme of androgen receptor gene. Androgen receptor mutations and patients' EMS at birth (in parentheses) were illustrated. Upper; Group 1 (EMS < 5) and below; Group 2 (EMS ≥5). Mutations I899F and Y916C (marked with #) were characterised in Fig. 3.

3.3. EMS and pubertal outcome

Table 2 shows a quantitative summary of the study cohort (where data were available). For comparison of pubertal outcomes, the participants were sub-divided according to the EMS: Group 1, EMS <5 at birth ($n = 9$) and Group 2, EMS ≥5 at birth ($n = 18$). This cohort of 27 PAIS patients had a higher median (interquartile range) EMS of 6·0 (3·0 to 7·5), compared to an EMS of 4·8 (3·0 to 6·0) recorded in a previously published cohort of 36 male-assigned PAIS patients [6] and a median EMS of 4·7 in all 41 male-assigned PAIS patients recorded in the Cambridge DSD Database. In this study, there was no statistically significant difference between Groups 1 and 2 for age at last assessment. There was a trend towards less data on pubertal outcome in Group 2, and particularly on whether mastectomy had occurred ($p = 0·03$). Overall differences in data availability between the two groups did not reach statistical significance.

3.4. Spontaneous onset of puberty

24 of the total cohort of 27 participants (89%) entered puberty spontaneously, based primarily on evidence of an increase in testicular volume. There was a statistically significant difference in spontaneous *versus* non-spontaneous pubertal onset according to the EMS: 6/9

Table 2
Results of statistical analyses on the clinical pubertal data for the 27 patients with PAIS in the study cohort.

Study cohort [median (IQR)]	Group 1 (EMS at birth < 5), n = 9				Group 2 (EMS at birth ≥ 5), n = 18				*p*-value *	(Group 1 + Group 2), *n* = 27	
EMS at birth (0–12)	3.0 (1.0 to 3.0)				6.0 (6.0 to 8.0)				<0.001	6.0 (3.0 to 7.5)	
Age (years) at last assessment	18.0 (18.0 to 26.0)				18.0 (17.0 to 20.8)				0.53	18.0 (17.0 to 23.5)	
	Group 1 (EMS at birth < 5), *n* = 9				Group 2 (EMS at birth ≥ 5), n = 18					(Group 1 + Group 2), n = 27	
Pubertal outcomes [binary]	Yes	No	Total data, n [%] ^		Yes	No	Total data, n [%] ^		p-value **	Total available data, n [%] ^	
Spontaneous onset of puberty	6	3	9	[100]	18	0	18	[100]	0.03	27	[100]
Androgen replacement given	5	4	9	[100]	8	4	12	[67]	0.67	21	[78]
Adult Testes ≥ 15 ml	4	3	7	[78]	10	5	15	[83]	1.00	22	[81]
Adult Pubic Hair PH4-PH5	7	2	9	[100]	12	0	12	[67]	0.17	21	[78]
Adult Genitalia G4-G5	1	5	6	[67]	11	2	13	[72]	0.01	19	[70]
Adult Height SDS > 0	5	4	9	[100]	8	4	12	[67]	0.67	21	[78]
Presence of gynaecomastia	7	2	9	[100]	14	0	14	[78]	0.14	23	[79]
Mastectomy done	6	3	9	[100]	8	2	10	[56]	0.63	19	[70]

Statistical analyses
There was no statistically significant difference in the age at last clinical assessment between patients in the two groups.
All the 18 patients with EMS at birth ≥ 5 (Group 2) had spontaneous onset of pubertal development, compared to 6 of 9 patients with EMS at birth <5 (Group 1).
Only 1 of 6 patients with EMS at birth <5 (Group 1) attained Genitalia Tanner Stage G4 or G5 in adulthood, compared to 11 of 13 patients with EMS at birth ≥ 5 (Group 2).
^ Data availability:
In this cohort of 27 patients with PAIS, information on evidence of spermatogenesis was available in six patients (22%).
There was fewer availability of pubertal outcome data in Group 2 (EMS ≥ 5) for whether mastectomy was done (Fisher's exact test; $p = .03$).
There was a trend towards fewer availability of pubertal outcome data in Group 2 (EMS ≥ 5) for whether androgen replacement was given,
Whether Pubic Hair Tanner Stage PH4 or PH5 in adulthood was attained, and whether Height SDS >0 in adulthood was attained (Fisher's exact test; all $p = .07$).
Differences in data availability between the two groups for the other pubertal outcomes did not reach statistical significance (Fisher's exact test; all $p > .10$).
* Mann-Whitney *U* test was used to test for differences in EMS at birth and age at last clinical assessment of puberty between patients in the two groups.
** Fisher's exact test was used to test for differences in pubertal outcome (binary) parameters between patients in the two groups.

participants (67%) with EMS < 5 had spontaneous onset of puberty, compared to all 18 participants with EMS ≥5 ($p = 0{\cdot}03$).

3.5. Tanner stage 4 or 5 in adult genitalia

Data on this outcome parameter were available in 19/27 participants (70%). Only 1/6 participants (17%) with EMS <5 had adult genitalia reaching Tanner stage 4 or 5, compared to 11/13 participants (85%) with EMS ≥5 ($p = 0{\cdot}01$).

For all the other pubertal outcomes, there was no statistically significant difference between Groups 1 and 2 for androgen replacement ($n = 21$; $p = 0{\cdot}67$), achieving adult testicular volume ≥ 15 ml ($n = 22$; $p = 1{\cdot}00$), pubic hair Tanner stage 4 or 5 (n = 21; p = 0·17), above average adult male height (n = 21; p = 0·67), development of gynaecomastia ($n = 23$; p = 0·14), or mastectomy surgery ($n = 19$; p = 0·63). Importantly, among the participants in whom data were available (n = 23), 21 (91%) developed gynaecomastia irrespective of the EMS at birth or the type of *AR* mutation. Two siblings (1-H and 1-I) with *AR* mutation A897E showed no gynaecomastia with EMS at birth of 3 in both.

3.6. EMS and functional analysis of AR mutants

PAIS–associated mutations in *AR* may potentially impact on different AR functions including expression of the *AR* gene, stability of the mRNA or AR protein, or protein functions such as DNA or ligand binding, dimerization and cofactor binding, nuclear localisation, and transcriptional activity. As *AR* transcriptional activity is a readout for most of these functions, we assessed androgen-dependent reporter gene activation by wild-type and mutant AR proteins in well-established reporter assays using transiently transfected cells. Fig. 2 depicts reporter gene activation by wild-type and mutant AR proteins in response to increasing DHT/mibolerone concentrations. Transcriptional activity of mutant *AR*s was significantly impaired except for R630W, A597T (Fig. 2A), and for A897E (Fig. 2B). Although these three mutant *AR*s show a normal transcriptional response, the associated EMS in the cognate participants were reduced to 3 (1-A, 1-H and 1-I) and 6 (2-N). Furthermore, the phenotype at birth as defined by the EMS was extremely variable (including the four participants with the R841C mutation) and did not correlate with the results of *in vitro* transcriptional activation studies. The poor correlation between the specific pathologic mutation in the *AR* sequence and the clinical presentation at birth suggests other factors impact on phenotype.

3.7. Mutant AR activity and spontaneous onset of puberty

The activity of 17 different mutant *AR*s as based on transactivation assays *in vitro* was analysed in relation to whether puberty occurred spontaneously or not. The data are summarised in Fig. 2 and Table 1A, 1B. Increasing concentrations of DHT or mibolerone were used in the assay and the transcriptional response of a reporter gene was compared with the wild-type *AR*. There was no consistent relationship between the degree of transcriptional *AR* deficit and puberty outcome. Mutants R841H and F674C in participants 2-H and 2-B, respectively, had low

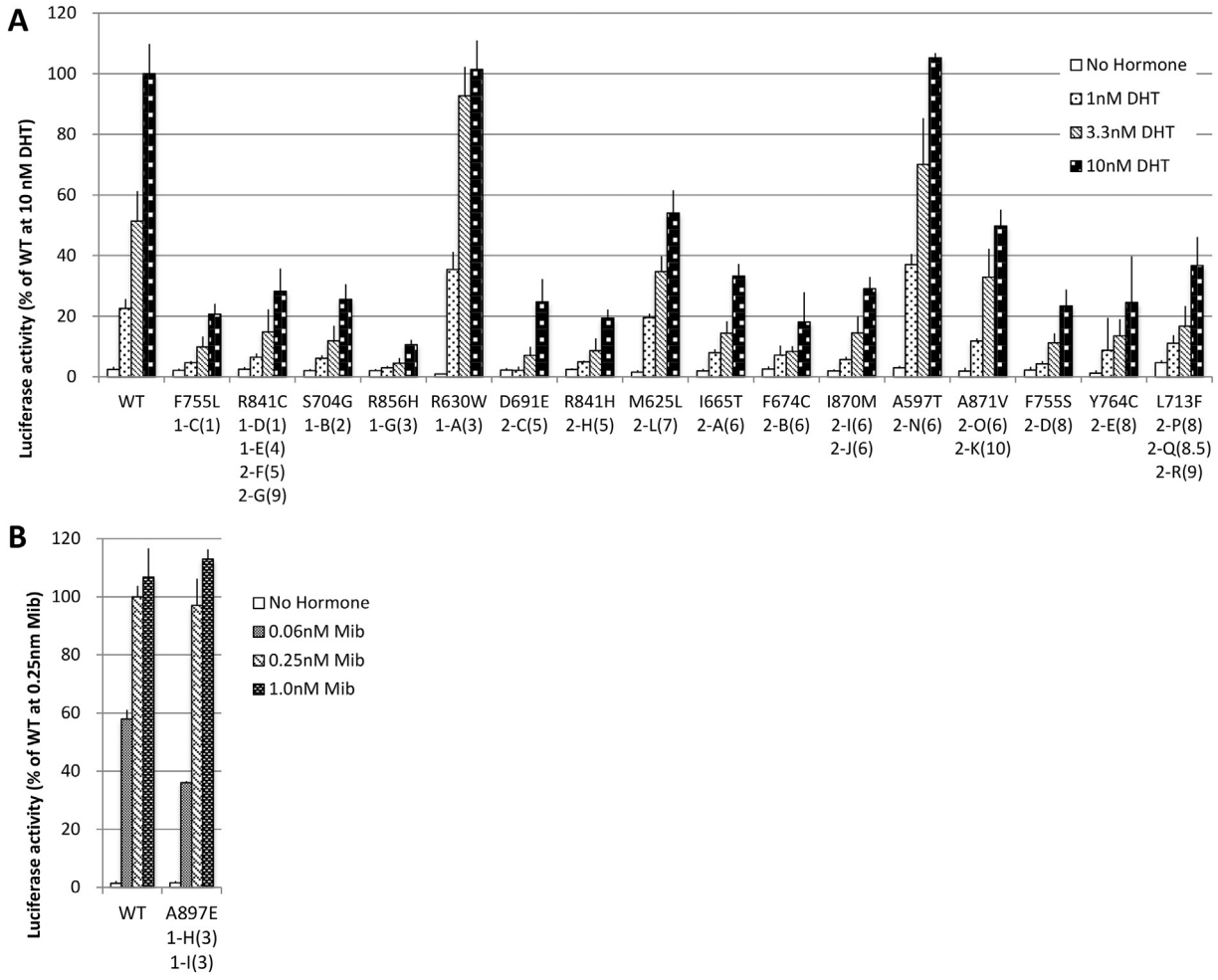

Fig. 2. A: Reporter assays using extracts of transiently transfected COS-1 cells showing dose-dependent activation of an androgen-responsive luciferase reporter gene by wild-type (WT) or variant AR proteins in response to dihydrotestosterone (DHT). B: Reporter assay for A897E was performed separately using COS-1 cells and mibolerone (Mib).

transcriptional activity relative to wild-type *AR* and yet in both cases, puberty occurred spontaneously. In contrast, mutant S704G identified in participant 1-B was similarly transcriptionally inactive and in this instance, puberty did not occur spontaneously and response to androgen treatment was not satisfactory. Nevertheless, although *AR* transcriptional activity in reporter assays was invariably reduced in most of the cohort, only three boys did not enter puberty spontaneously. Mutant R841C characterised by low transcriptional activity was identified in four separate participants of whom three entered puberty spontaneously and one did not. The widespread inconsistency between this phenotypic marker and the results of *AR* function assessed *in vitro* indicates that the *AR* functional assays employed here may not be a reliable predictor of pubertal outcome.

3.8. Structure and function analysis of I899F and Y916C AR variants

Two *AR* mutations were identified during our molecular investigation of this cohort of patients with PAIS who were raised male, namely I899F (identified in participant 1-G, Group 1) and Y916C (identified in participant 2-M, Group 2) where the variants were previously uncharacterised. A preliminary analysis of structure and function was undertaken as part of this study.

Examination of the crystal structures of agonist-bound AR LBD monomers (PDB: 3L3X, 4OEY) or the AR LBD homodimer (PDB: 5JJM) in complex with cofactor peptides revealed that the I899 sidechain forms part of the cofactor binding site whereas the Y916 side chain lies exposed on an outer surface of the AR LBD, distant from ligand binding, dimerisation or cofactor binding sites (Supplementary Figs. S1 and S2). Consistent with this, both *AR* mutants (I899F and Y916C) displayed reduced ability to activate the reporter, compared to wild-type *AR*, especially at the low concentration (0·1 nM) of exogenous androgen tested (Fig. 3A). This effect on activity was more pronounced with *AR* mutant I899F. To assess whether these mutations impact on the function of the AR LBD, we performed yeast two-hybrid studies as described previously [12]. As shown in Fig. 3B, wild-type AR LBD showed a strong ligand–dependent interaction with the nuclear receptor interaction domain (NID) of the Steroid Receptor coactivator (SRC1), a known cofactor for AR containing three LXXLL motifs. This interaction was significantly compromised by the I899F substitution (Fig. 3B and C) consistent with the proximity of the residue to the surface required for both cofactor binding and N/C domain interactions (Supplementary Fig. S2). In contrast the I899F mutation had no significant impact on the ability of the AR LBD to form homodimers in these assays in response to ligand (Fig. 3D), indicating that other major LBD functions were not affected.

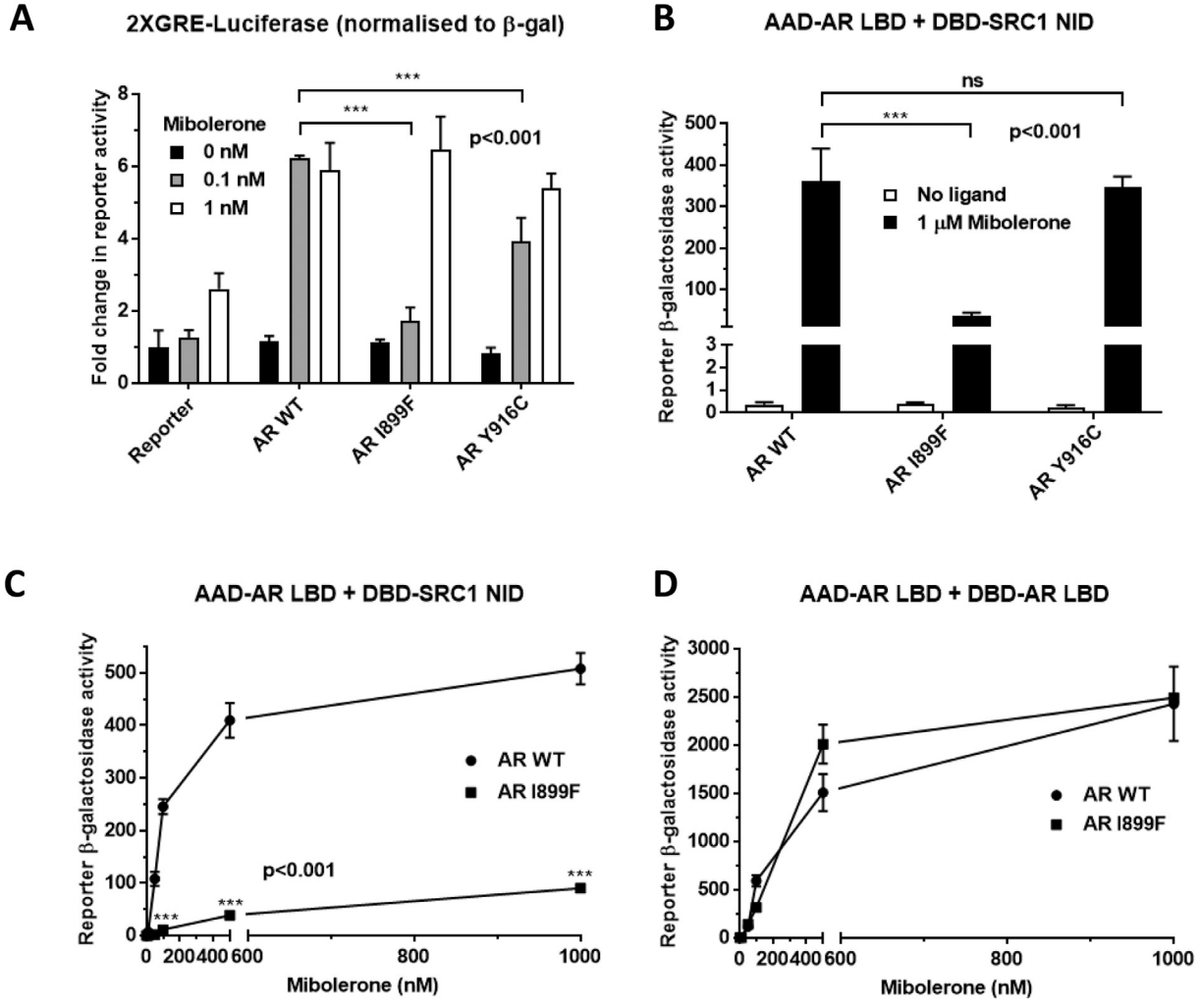

Fig. 3. A: Reporter assays using extracts of transiently transfected HeLa cells showing dose-dependent activation of an androgen-responsive luciferase reporter gene by wild-type (WT) or variant AR proteins in response to mibolerone. B: Yeast two-hybrid assays to assess cofactor binding by AR proteins. Shown is reporter activity due to interaction of AAD-AR LBD WT, I899F or Y916C constructs with the nuclear receptor interaction domain (NID) of steroid receptor coactivator 1 protein (DBD-SRC1 NID) in the presence of vehicle or mibolerone (1 μM) as indicated. C: Dose response curve in yeast two-hybrid assay comparing interaction of AAD-AR LBD WT and I899F proteins with DBD-SRC1 NID. D: Yeast two-hybrid assay assessing homo-dimerization capabilities of AR LBD WT or AR-LBD I899F constructs. The data shown represent the mean of triplicates and the error bars indicate standard deviations. *** = $p <$ .001 by 2-way ANOVA.

Western blots confirmed similar expression levels of the wild-type and mutant AR and SRC1 two-hybrid proteins (Supplementary Fig. S3),

4. Discussion

We report the largest cohort of PAIS patients to date whose puberty has been systematically characterised and quantitatively analysed in relation to the nature of their *AR* mutation. All 18 patients with median-or-higher EMS at birth achieved spontaneous onset of puberty, whereas three of the nine patients with lower-than-median EMS needed androgen treatment to induce puberty. This confirms the utility of assessing the degree of virilisation at birth to predict likely spontaneous pubertal development. The finding has practical importance in assisting health professionals and families when discussing the likely options for management at puberty, particularly in light of the recent trend for male assignment in PAIS [7].

Indicators of final pubertal progression were also informative. There was failure to reach Tanner stage 4 or 5 in more of the PAIS patients with low EMS. There were no significant differences between the two groups of PAIS patients with high or low EMS in terms of adult testicular volume, Tanner staging of pubic hair, adult height and incidence of gynaecomastia. Sperm analysis had been undertaken in 5 patients all of whom had oligo/azoospermia (1-A, 1-D, 2-C, 2-G, 2-L). A further patient was reported to have two children but no additional details were available (2-K). Overall, the results of this study suggest that the higher the EMS is at birth the more likely that puberty will occur spontaneously with satisfactory genital development.

A previous study from Denmark reported gynaecomastia in 13 of 14 PAIS patients in their cohort [1]. The study group was unusual since eight patients had a normal EMS at birth and first presented in puberty with gynaecomastia as a sign of PAIS. The authors estimated that their small cohort accounted for 74% of PAIS patients in Denmark, suggesting the more severely undermasculinised patients presenting at birth were not included. A larger study of boys with PAIS through the International Disorders of Sex Development Registry reported universal development of gynaecomastia at puberty [2]. This also seemed to be the case in our study apart from two siblings who showed no gynaecomastia. Both had an EMS of 3 at birth and entered puberty spontaneously. That pubertal gynaecomastia is generally common in PAIS raises the possibility of prevention with the use of anti-estrogens and aromatase inhibitors to avoid surgical reduction mammoplasty [13].

A specific aspect of the current study was inclusion of patients in whom not only was the diagnosis confirmed by sequencing the *AR* gene, but the pathogenicity of the mutation was also assessed. The results show that all mutations located in the AR LBD displayed reduced transcriptional activity as measured in a reporter gene activation assay. However, this *in vitro* profile was not sufficiently specific in relation to clinical parameters such as the EMS and whether puberty started spontaneously. Two mutations, A597T and R630W, were located outside the LBD and both demonstrated wild-type activity *in vitro*. A597T occurs in the DNA-binding domain and was originally reported in two families with Reifenstein syndrome, a previously used eponymous term to describe the severe form of PAIS [14]. Subsequent studies showed this mutation disrupts AR dimerization and hence DNA binding [14,15]. The R630 residue lies at the DBD-hinge domain boundary and its role is poorly understood. A mutation in this residue (R629Q using the former numbering system) was identified in a prostate cancer patient with hormone refractory/androgen independent disease and was revealed to enhance *AR* transcriptional activation [16]. This suggests that the substitution of arginine by glutamine, an amino acid of similar volume, promotes AR function whereas the presence of a bulkier aromatic tryptophan side chain cannot be accommodated and thereby disrupts androgen signalling.

A combination of reporter assays, yeast two-hybrid assays and *in silico* modelling was used for functional characterisation of Y916C and I899F, two novel *AR* mutations identified during the course of this study. The results show that I899F compromises the recruitment of the coactivator, SRC1 and consequently is likely to impair AR-regulated transcription [17]. Structural data for the LBD in complex with LXXLL or FXXLF motifs support this conclusion. I899 makes important stabilising contacts with the first conserved leucine or phenylalanine in these motifs that would be sterically disrupted by a phenylalanine side chain, consistent with the yeast two-hybrid data (Figs. 3B-3D).

The Y916 residue does not participate in the cofactor binding site, nor at the recently characterised AR dimerization interface [18]. However, it may be involved in interactions with other proteins due to its exposed location on the AR LBD surface in both the monomeric and dimeric structures (Supplementary Fig. S1). A previous study has shown that Y916 is phosphorylated by Src kinase and that this event is important for recruitment of AR to chromatin [19]. Disruption of such function is unlikely to be detected in a reporter gene assay. Future studies should address the developmental regulation of AR phosphorylation as its physiological significance may have relevance to the timing of pubertal onset and subsequent progression [20].

Since the phenotype associated with PAIS is so variable and pleiotropic in its causation, it is generally accepted that a diagnosis of PAIS should be confirmed by identifying a mutation in the *AR* gene [3]. Furthermore, additional studies of the mutant receptor are needed, particularly when the mutation is novel. While the additional use of yeast two-hybrid assays provides mechanistic insights into the nature of the functional defect in AR signalling, it is evident that the use of *in vitro* assays of androgen action used in this study is not sufficiently informative to predict how puberty will develop in PAIS associated with a particular *AR* mutation. This may be due to variable responses according to different promoters used in reporter gene assays under the conditions employed [21,22]. The complexity of nuclear receptor coregulator dynamics is now well established, where the activity of the receptor itself is influenced by the DNA elements bound, together with coregulator expression and recruitment. Current *in vitro* methods are likely to be sensitive enough only to detect the most extreme of functional defects. There are also examples of mosaic expression of *AR* mutations in PAIS which can explain variable phenotypes [23–25]. The question of mosaicism was not systematically examined in this study. It is likely to be infrequent as in most cases, genital skin fibroblast lines had been established for androgen binding assays and furthermore, many of the cases were familial.

The strength of the present study is a relatively large cohort of patients with PAIS demonstrating a range of EMS values which reliably indicated the likelihood of spontaneous onset of puberty. The outcome parameters of puberty were generally complete, apart from systematic evidence of spermatogenesis. Furthermore, the clinical dataset was coupled with detailed information about the *AR* mutation identified in each patient, including the use of *in silico* modelling. The propensity for infants with PAIS now to be more likely assigned male at birth requires a management policy that addresses the clinical challenges which may be faced at puberty and beyond. The majority of patients with PAIS assigned male appear to enter puberty spontaneously. Thereafter, androgen supplementation may be required for which it may be possible to utilise data on AR structure and function as a guide for the type and dose of androgen preparation.

Contributors

NL, RTC, PP, HM, and IAH designed the study, collected, analysed, and interpreted the data and wrote the report. TB, KZ, and JW performed reporter assays. DMH designed the Y2H experiments, analysed data and co-wrote the manuscript. JW and BM generated Y2H constructs and performed Y2H reporter assays, and western blots. NPM, VM analysed data and edited the manuscript.

Declaration of interests

We declare no competing interests.

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Appendix A. Supplementary data

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