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ORIGINAL ARTICLE

Hormonal evaluation in relation to phenotype and genotype in 286 patients with a disorder of sex development from Indonesia

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Summary

Objective The objective of this study was to determine the aetiological spectrum of disorders of sex development (DSD) in a large cohort of underprivileged and undiagnosed patients from Indonesia.

Methods A total of 286 patients with atypical external and/or internal genitalia were evaluated using clinical, hormonal, molecular genetic and histological parameters.

Results The age (years) at presentation was 0–0·5 in 41 (14·3%), >0·5–12 in 181 (63·3%) and >12 in 64 cases (22·4%). 46,XY DSD was most common (68·2%, $n = 195$), 46,XX DSD was found in 23·4% ($n = 67$) and sex chromosomal DSD in 8·4% ($n = 24$). In 61·2% of 46,XX DSD patients, 17·9% of 46,XY DSD patients and all sex chromosome DSD patients (29·4% in total), a final diagnosis was reached based on genetic or histological gonadal tissue evaluation. 17-hydroxyprogesterone and androstenedione levels were the most distinctive parameters in 46,XX DSD patients. In 46,XY DSD, diagnostic groups were identified based on the external masculinization score: androgen action disorder (AAD), unknown male undermasculinization (UMU), and gonadal dysgenesis (GD). LH, FSH and testosterone levels were most informative especially in the older age group. HCG tests were of no additional value as no patients with androgen synthesis disorders were found. Hormonal profiles of patients with sex chromosome DSD and a Y-chromosome sequence containing karyotype showed high levels of LH and FSH, and low levels of AMH, inhibin B and testosterone compared with the normal male range. Gene mutations were found in all patients with CAH, but in only 24·5% and 1·8% of patients with AAD and UMU. In 32% of 46,XY GD patients, copy number variants of different genes were found.

Conclusion A stepwise diagnostic approach led to a molecularly or histologically proven final diagnosis in 29·4% of the patients. The most informative parameters were serum levels of 17-hydroxyprogesterone and androstenedione in 46,XX DSD patients, and serum LH, FSH and testosterone levels in 46,XY DSD patients.

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Introduction

Sex determination and differentiation are regulated by a complex developmental network. Chromosomal sex determines gonadal sex, which in turn determines phenotypic sex.[1] Sex determination results from the expression of genes that cause the bipotential gonads to develop into either testes or ovaries.[2]

Testicular differentiation occurs after the onset of the expression of sex-determining region Y (SRY) in a subset of somatic cells. This leads to their subsequent differentiation into Sertoli

cells, which in turn produce anti-Müllerian hormone (AMH). AMH inhibits the differentiation of the Müllerian ducts into a uterus and other Müllerian structures. Furthermore, the developing Leydig cells start secreting testosterone, which causes the stabilization of the Wolffian ducts. In females, there is no obvious single dominant gene that determines the sex of somatic and germ cells in the ovary.[2]

Disorders of sex development (DSD) cover many different phenotypes of atypical sexual anatomy, which result from a breakdown of the underlying network that regulates gonadal development and differentiation. Investigations of hormone levels are the hallmark for the assessment of gonadal and adrenal function and for the identification of many potential pathogenic mechanisms underlying DSD.[3]

This study describes the various steps in the diagnostic approach in a large cohort of Indonesian patients with DSD. Due to the limited laboratory facilities in Indonesia, most of them had never received medical attention related to DSD, making it possible to include not only newborn, but also prepubertal and adult patients. The primary aim of our study was to determine the aetiological spectrum in this group of DSD patients. Furthermore, we wanted to analyse which clinical, hormonal and molecular genetic parameters proved most informative to reach a final diagnosis and to find a relevant strategy for further clinical management in these three age groups of patients.

Subjects and methods

Patients

After exclusion of patients with diagnoses such as mild non-penoscrotal hypospadia and of those patients who did not consent to be included in the study, 286 consecutive patients with various DSD phenotypes were referred for chromosomal analysis by clinicians of the departments of Urology, Paediatrics, Internal Medicine and Obstetrics to the gender team of the Dr Kariadi Hospital, Semarang, Indonesia. Referral and data collection took place between 2004 and 2010 at the Center for Biomedical Research, Faculty of Medicine, Diponegoro University (FMDU), Semarang, Indonesia. Reasons for referral were the presence of ambiguous genitalia or atypical external or internal genitalia, including severe hypospadias, with or without palpable testes. Eighty-eight of the patients have been described previously.[4]

Methods

A detailed interview was performed at recruitment. Data concerning medical history, age of initial presentation, sex of rearing, family history (relatives with a genital disorder) and consanguinity were collected.

The patients were clinically evaluated; a detailed description of the external genitalia was obtained; the genitalia were staged according to Quigley,[5] and using the External Masculinization Score (EMS) [6] by a trained andrologist. The Prader stage was used to determine the degree of virilization in patients with 46, XX DSD.[7] Dysmorphic features were also recorded.

A blood sample was obtained for karyotyping, hormonal analysis and DNA extraction. Karyotypes were determined using a G-banding technique. In patients with 46,XY DSD or Y-chromosome containing aneuploid DSD, an additional blood sample was obtained 72 h after the intramuscular injection of 1500 IU human chorionic gonadotrophin (hCG; Pregnyl®, MSD, Oss, The Netherlands).

The medical ethics committee of Dr Kariadi Hospital/FMDU approved this study, and informed consent was obtained from all participants, as well as their parents or guardians prior to their participation in this study.

Diagnostic criteria

Patients were categorized according to the primary root of the classification based on karyotype: 46,XX DSD, 46,XY DSD or sex chromosome DSD.[8]

Serum hormone measurements

Measurements of inhibin B, AMH, LH and FSH in serum samples were taken as described previously.[9] Concentrations of testosterone and androstenedione were determined in serum collected before and after injection of hCG using the Coat-a-Count radioimmunoassay (Siemens, Los Angeles, CA) and the Immulite 2000 analyser (Siemens), respectively. Levels of 5α-dihydrotestosterone were measured using the enzyme-linked solid-phase immunoassay obtained from Diagnostics Biochem Canada (Dorchester, Ont, Canada) in post-hCG samples, provided sufficient material was available. Finally, 17-hydroxyprogesterone and 11-desoxycortisol levels were estimated using in-house methods.[10] Age- and sex-dependent hormone levels in the normal population are given in Tables S1 and S2.[9,11]

Genetic analysis

DNA was extracted from leucocytes of EDTA blood using the salting out method.[12] Based on the clinical and hormonal information, specific genes were analysed such as *CYP11B1* (11β-hydroxylase; P450c11β),[13] *CYP21A2* (21-hydroxylase; P450c21),[14] *LHCGR* (LH receptor) [15] and *NR3C1* (glucocorticoid receptor, GR) [16] using Sanger sequencing. The genes encoding the androgen receptor (AR),[17] *SRY* and *WNT4*, were analysed by Sanger sequencing of the coding exons and exon-flanking intronic regions. Primer sequences and locations are available upon request.

Patients' DNAs were also analysed for large genomic re-arrangements and copy number variations (CNVs) using genomewide SNP chip arrays (different versions of Illumina arrays) and/or Multiplex Ligation-dependent Probe Amplification (MLPA). Microarray analysis was performed as described previously[18]; MLPA was also used to confirm CNVs which were identified using arrays.[19]

Pathology

Histopathological assessments of gonadal tissue following gonadal excision or biopsy were performed using haematoxylin and eosin staining and immunohistochemistry for various germ cell markers, for example OCT3/4, TSPY, VASA, SCF (including double staining for OCT3/4-TSPY or VASA), as well as SOX9 and FOXL2 for supportive cells, that is Sertoli cells of the testis and granulosa cells of the ovary, respectively, as described earlier.[4,20]

Flow of the diagnostic process

For the first diagnostic evaluation, patients were grouped based on their karyotype, and further assessment for the second stage of diagnosis was conducted based on clinical data, notably EMS with or without ultrasonographic or magnetic resonance imaging. The analysis of candidate genes was carried out based on the results of these assessments and the results of the hormonal evaluation.

Statistics

Data were processed using IBM SPSS statistics 20 software. To calculate the differences between hormone concentrations for each diagnosis, we used ANOVA followed by post hoc Tukey analysis. Pearson's correlation coefficients were used to determine the relationship between EMS score and androgen sensitivity index. Mann–Whitney *U*-tests were used to determine the difference of various parameters among androgen action disorder with and without AR mutation.

Data in tables and figures are given as medians and interquartile ranges unless otherwise stated, data in text are given as means ± SEM. *P*-values < 0·05 were considered significant. Furthermore, sensitivity, specificity and positive and negative predictive values (PPV and NPV, respectively) of the endocrine tests used in the diagnostic procedures to discern congenital adrenal hyperplasia (CAH) patients from other 46,XX DSD patients and 46,XY gonadal dysgenesis patients form other groups of 46,XY patients were calculated using the algorithms at https://www.medcalc.org/diagnostic_test.php.

Results

46,XY DSD was most common, accounting for 68·2% (195/286) of patients. 46,XX DSD accounted for 23·4% (67/286) of cases, and 8·4% (24 cases) had a sex chromosome DSD (Table 1; Fig. 1). The age at presentation was 0–0·5 years in 41 cases (14·3%), >0·5–12 years in 181 cases (63·3%) and >12 years in

Table 1. Summary of the diagnoses and characteristics of the patients

Diagnosis		Total	Median (range) age at presentation (years)	Sex of rearing			Prader score	EMS score	Number of families
				Male	NA	Female			
46.XX DSD		67							
Disorders of gonadal development		2		2	0	0			2
Androgen excess									
Foetal (CAH)							3·7 (2–6)		
– Mutation CYP21A2		38	6·5 (0·01–33)	11 (6)	4	23 (28)		4 (1–7)	34
– Mutation CYP11B1		2	11 (3–12)	0	0	2		(4–7)	1
Unclassified androgen excess		3	0·1 (0.04–29)		2	1		1 (1–4)	3
Other									
Defect of mullerian development (MRKH)		12	21 (2–27)	0	0	12		1	12
Unclassified		10	3 (0·2–15)	2	0	8		1 (1–5)	10
46.XY DSD		195					Quigley stage		
Androgen action disorder (AAD)									
AR mutation +		24	9 (0·1–26)	20	0	4	3 (2–6)	6 (1–8·5)	21
AR mutation –		73	1·95 (0·02–23)	64	2	7	3 (2–6)	6 (2–8·5)	72
Disorders of gonadal development		31	14·5 (0·1–42)	15	2	14	3 (2–7)	5 (1–10)	29
Other									
Unknown male undermasculinization (UMU)		55	7 (0·1–29)	55	0	0	2 (2–4)	9 (9–11)	54
unclassified		12	1·2 (0·01–12)	10	1	1	4 (1–6)	6 (1–8)	12
Sex chromosome DSD		24	10 (0·1–39)	13	1	10		4·5 (1–10)	24
Turner syndrome variants	X/XX	4	20 (18–25)			4		1 (1–1)	4
Klinefelter syndrome variants	XY/XXY	3	7 (1·5–10)	3				9 (9–10)	3
Mixed gonadal dysgenesis	X/XY	8	9·5 (0·6–19)	5	1	2		6 (1–10)	8
Chimeric. ovotesticular DSD	XX/XY	6	2·75 (0·1–22)	5		1		4·5 (1–8·5)	5
Other		3	21 (20–39)			3		1 (1–4)	3
Total		286		192	11	83			

NA*: Not available.

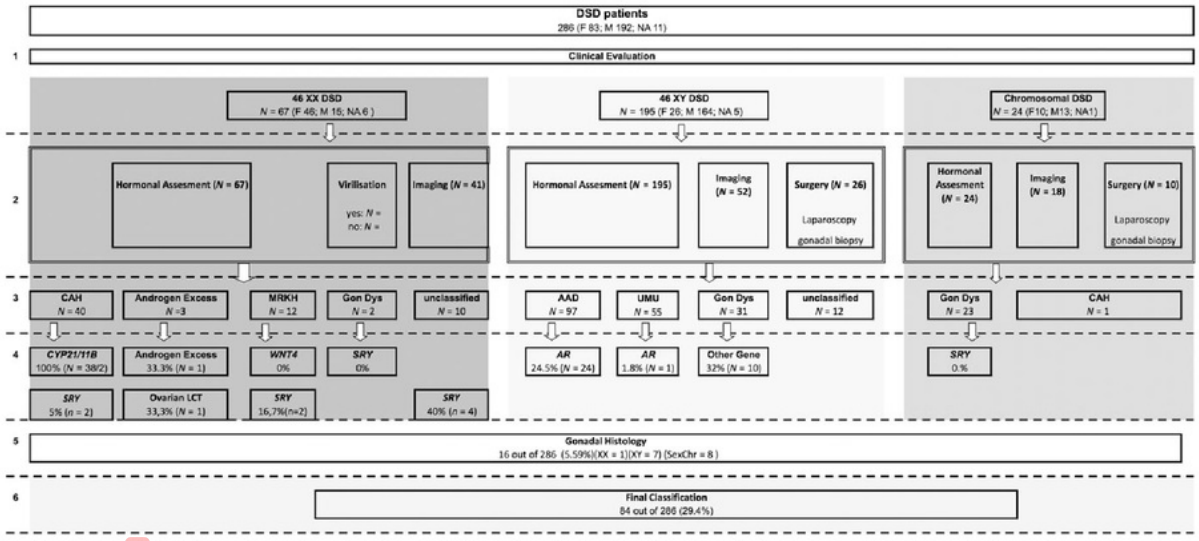

Fig. 1 Stepwise diagnostic evaluation in 286 patients with DSD in Semarang, Indonesia. CAH, congenital Adrenal Hyperplasia; GonDys, Gonadal Dysgenesis; AAD, Androgen Action Disorder; UMU, Unknown Male Undermasculinization; MRKH, Mayer-Rokitansky-Kuster-Hauser Syndrome; LCT, leydig cell tumour.

64 cases (22·4%). Familial DSD was reported in 11 cases from 5 families with 46,XX DSD and 13 cases from 6 families with 46, XY DSD. Consanguinity of parents was not reported for any of the patients. Clinical details of (post)pubertal patients are provided in Table S3.

46,XX DSD

Hormonal evaluation. Forty-three of 67 46,XX patients presented with varying degrees of virilization of the external genitalia. Seven of these patients were already treated with glucocorticoids on basis of the clinical picture, 33 of them showed hormonal levels characteristic for CAH, while 3 had unclassified hyperandrogenism. 21-hydroxylase deficiency (caused by a mutation in *CYP21A2*) was suspected in 38 patients based on their previously started treatment or increased levels of 17-hydroxyprogesterone, androstenedione and testosterone (Table 2), while in 2 cases 11β-hydroxylase deficiency (defect in *CYP11B1*) was suggested by a different pattern of adrenal steroids: increased levels of 11-desoxycortisol (890 and 977 nmol/l, respectively) in combination with undetectable levels of cortisol (Table 2). Clinically salt wasting was suspected in at least 11 of these cases based on intercurrent illnesses with hyponatraemic episodes, but no confirmation by laboratory tests was available. Five patients died during this study. One patient with the sex chromosome abnormality 46,XX (96%)/46,XY (4%) was also diagnosed with a 21-hydroxylase deficiency proven by gene mutation analysis. In all of these hyperandrogenized patients, LH, FSH, AMH and inhibin B levels were normal for age and levels of cortisol were low (Table 2). In all but one of the 33 untreated patients diagnosed with CAH by sequencing of *CYP21A2* or *CYP11B1*, serum 17-hydroxyprogesterone exceeded the upper limit of normal (10 nmol/l), leading to a sensitivity of 97%. However, in the group of 27 remaining 46,XX DSD patients, 2 newborn children who were suspected of glucocorticoid resistance on basis of very high levels of cortisol (see below) and one patient with transient virilization of unknown origin also showed 17-hydroxyprogesterone levels >10 nmol/l, leading to a specificity of 89% for this assay. This results in PPV and NPV of 91 and 96%, respectively (Table 3). In the patients with CAH, serum concentrations of androstenedione were above the cut-off levels of 5 nmol/l (below 12 years of age) or 10 nmol/l (over 12 years of age) in 29 of 33 patients; in four children with ages below 6 years, androstenedione levels below 5 nmol/l were measured. In the children suspected of glucocorticoid resistance, the patient with an ovarian Leydig cell tumour (see below) and one further patient without further endocrine abnormalities, androstenedione levels above the cut-off value were found. Resulting sensitivity, specificity, PPV and NPV for androstenedione are shown in Table 3.

Among the three patients with unclassified androgen excess, one patient was later diagnosed with an ovarian Leydig cell tumour, which was confirmed by histopathological analysis.[21] In two children, glucocorticoid resistance was suspected based on the combination of the clinical phenotype of ambiguity in an 46,XX individual and markedly elevated serum levels of cortisol, 17-hydroxyprogesterone and adrenal androgens (Table 2).

Gonadal dysgenesis was presumed in two patients presenting with ambiguous genitalia. In one patient, the level of FSH was high and AMH was low for age, whereas the other patient showed a so-far-unexplained high level of AMH.

Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome was suspected in 12 phenotypic female patients with normal gonadal function with hypoplasia of the vagina and absence of the uterus. Eight of 10 patients in the "46,XX DSD other,

Table 2. Hormone concentrations in 46,XX DSD patients on basis of diagnosis and age group

Diagnosis age (yrs)	N	LH (IU/l)	FSH (IU/l)	Cortisol (nmol/l)	Androstenedione (nmol/l)	17-OHP (nmol/l)	Testosterone (nmol/l)	AMH (µg/l)	Inhibin B (ng/l)
CYP21A2*									
0–0.5	8	0·29 (0·1–1·0)	1·7 (0·6–3·6)	123 (33–143)	37 (8·5–53)	178 (62–299)	3·6 (1·3–7·4)	0·8 (0·4–2·5)	26 (17–37)
>0·5–12	18	0·19 (0·1–0·29)	1·9 (0·3–3·4)	119 (77–160)	46 (16–75)	401 (191–549)	7·7 (2·7–14)	3·6 (2·3–6·2)	21 (13–38)
>12	5	1·4 (0·48–2·8)	3·3 (3·2–3·5)	122 (67–136)	67 (50–131)	583 (237–771)	13 (11–20)	5·1 (3·8–6·5)	42 (33–188)
CYP11B1									
>0·5–12	2	<0·1–<0·1	0·3–<0·1	<35–<35	131–136	9·7–20	8·0–16	1·6–2·8	18–33
Ovarian tumour									
>12	1	12	8·1	191	18	9·9	60	2·8	NA
Suspected cortisol resistance									
0–0·5	2	0·14–0·26	0·79–6·1	1515–1172	47–80	15–14	2·7–5·4	0·05–0·44	73–3
Transient virilization									
>0·5–12	2	0·14–0·05	2·7–5·0	392–66	NA	NA–15·2	0·1–0·1	10·1–2·4	36–68
Suspected gonadal dysgenesis									
0–0·5	1	0·69	18	77	<1·05	0·7	0·1	0·8	62
>0·5–12	1	0·18	2·6	387	<1·05	4·5	0·3	60	47
Cloacal Malformation									
0–0·5	1	1·2	25	83	3·7	0·9	0·2	0·38	NA
>0·5–12	7	0·2 (0·1–2·8)	5·7 (1·7–11)	151 (95–643)	1·0 (1·0–4·0)	0·8 (0·1–3·1)	0·1 (0·05–0·8)	2·9 (0·2–9·8)	45 (37–180)
MRKH									
>0·5–12	2	0·14–0·16	2·5–2·5	160–214	<1·0–<1·0	<0·1–0·5	0·1–0·1	1·9–6·5	8–127
>12	10	6·2 (1·2–17)	4·2 (2·1–9·4)	294 (28–480)	9·3 (6·6–10)	4·6 (0·6–7·4)	1·2 (0·5–2·7)	8·9 (4·2–19)	86 (35–190)

Data are provided as medians and interquartile ranges. If $n = 1$ or 2, data of individual patients are shown.
17-OHP: 17-hydroxyprogesterone.
NA: not available.
*Glucocorticoid-treated patients have been excluded.

Table 3. Sensitivity, specificity and positive and negative predictive values (%) of results of hormone estimations in congenital adrenal hyperplasia (CAH) and gonadal dysgenesis (GD) patients in comparison with other disorders of sexual differentiation

Group	Diagnosis	Parameter	Age group	Cut-off value	Sensitivity	Specificity	PPV	NPV
46,XX	CAH	17-OHP	all	>10 nmol/l	97	89	92	97
	CAH	Androstenedione	<12 and >12 years	>5 and >10 nmol/l	88	85	88	85
46,XY	GD	FSH	0·5–12 years	>1 IU/l	67	52	9	95
	GD	FSH	>12 years	>15 IU/l	90	90	90	90
	GD	LH	0·5–12 years	>0.5 IU/l	44	87	20	95
	GD	LH	>12 years	>10 IU/l	70	63	67	67
	GD	AMH	0·5–12 years	<100 µg/l	89	68	18	99
	GD	AMH	>12 years	<25 µg/l	100	63	73	100
	GD	Inhibin B	0·5–12 years	<100 ng/l	78	63	14	97
	GD	Inhibin B	>12 years	<120 ng/l	95	42	63	89

unclassified" group had cloacal malformations with hormonal concentrations within the normal range and two patients showed clinically transient hypervirilization. A follow-up examination revealed normal female genitalia and hormone levels (see Table 2).

Molecular Genetic evaluation. The diagnosis of CAH was confirmed in all patients by *CYP11B1* (n = 2) or *CYP21A2* (n = 38) gene mutation analysis. In the patients with 11β-hydroxylase deficiency, two sisters, the compound heterozygous mutations p.R374Q/R448C (NG_007954.1) were found. As reported previously,[14] gene sequencing of *CYP21A2* revealed p.R356W (NM_000500.7: c.1069C>T) and p.I172N (NM_000500.7: c.518T>A) as the most common mutations. In 2 CAH patients (aged 1 and 7 years), an additional SRY translocation was found; there was no hormonal evidence of gonadal dysgenesis. In the two patients with suspected glucocorticoid resistance, no *GR* mutations were detected.

In another six patients with 46,XX DSD, subsequent SRY PCR analysis showed the presence of SRY, probably due to a translocation. Two of these patients (25 and 18 years of age) showed clinical and sonographic characteristics of MRKH. One patient was classified as 46,XX gonadal dysgenesis and the remaining three were classified as other (cloacal malformations).

46,XY DSD

We observed a preference of male sex of rearing: of 130 patients with a severe degree of ambiguity (Quigley stage 3–5), 101 were assigned to the male sex (Fig. 2).

Hormonal evaluation. Ninety-seven patients were classified as suspected of androgen action disorder (AAD) based on an EMS score <9. Fifty-five patients with an EMS score ≥9 were classified as unknown male undermasculinization (UMU).[22] Comparison between hormone levels in the AAD and UMU groups showed similar concentrations for LH and FSH, testosterone before and after hCG, and for AMH and inhibin B in all age groups, with the exception of increased levels of LH and basal testosterone in postpubertal AAD patients (Fig. 3). In none of the AAD or

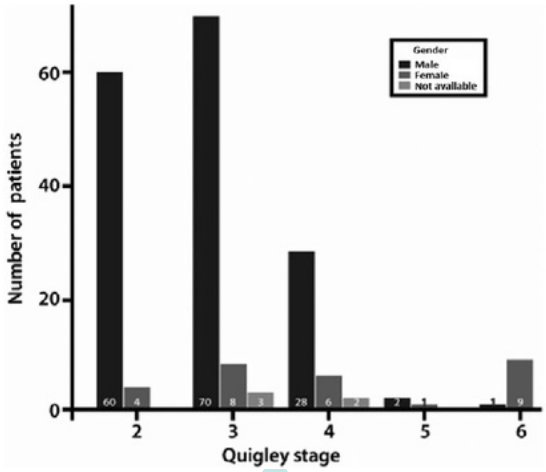

Fig. 2 Quigley stage and gender of patients with 46,XY DSD.

UMU cases, the measured concentrations of gonadal and adrenal steroids before and after hCG administration as well as the testosterone/dihydrotestosterone ratios after hCG revealed the underlying diagnosis of an androgen synthesis defect or 5α-reductase deficiency (data not shown).

Gonadal dysgenesis was presumed in 31 patients on the basis of clinical features. In this group of patients, external genitalia were often ambiguous depending on the degree of impairment of gonadal function. The correlation between EMS score and testosterone levels in the postpubertal group of 20 patients with gonadal dysgenesis showed a correlation coefficient of 0·544 ($P < 0·01$). Serum levels of LH and FSH were elevated compared with reference values, whereas AMH and inhibin B levels remained low for age in all age groups (Fig. 3). FSH levels were significantly higher in the group of gonadal dysgenesis compared with the groups of AAD and UMU patients until puberty. However, due to the wide range of the results, sensitivity and specificity of increased FSH (>1 IU/l) in the age group between 0·5 and 12 years were only relatively low (Table 3). In the patients with ages over 12 years, sensitivity, specificity, PPV and NPV of FSH concentrations >15 IU/l were higher. Data on sensitivity, specificity, PPV and NPV for LH were

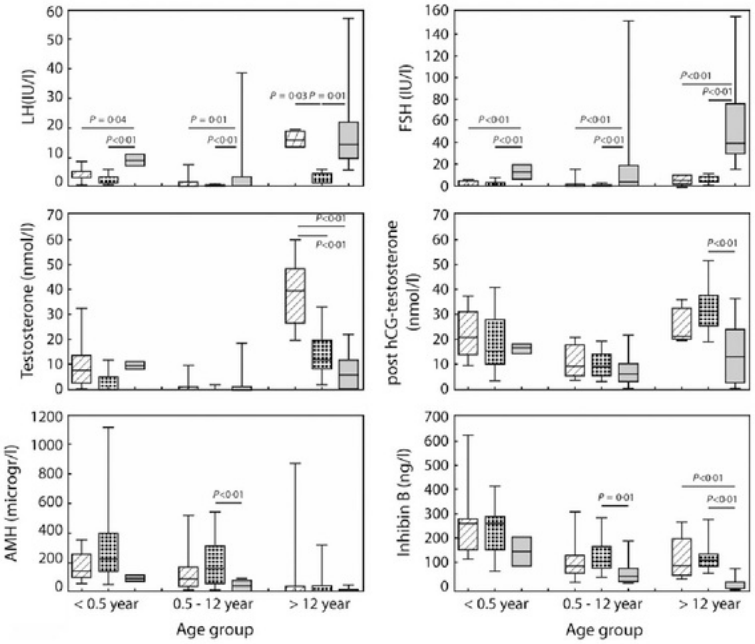

Fig. 3 Serum hormone levels in 46,XY patients based on diagnosis and age: patients with androgen action disorders (AAD, hatched bars), unknown male undervirilization (UMU, stippled bars) and gonadal dysgenesis (shaded bars). Significance of differences has been indicated.

less satisfying than for FSH (Table 3), especially in the age group over 12 years, where LH levels were equally increased in AAD and gonadal dysgenesis patients.

AMH levels in patients with gonadal dysgenesis were decreased compared with the AAD and UMU groups, but significant differences were only shown in the age groups >0·5–12 and >12 years (Fig. 3). Inhibin B levels were significantly lower in the gonadal dysgenesis group, compared with the AAD group, except in newborn patients (Fig. 3). In the postpubertal age group (>12 years), inhibin B levels were significantly different between the UMU and gonadal dysgenesis patients. Data on the sensitivity, specificity, PPV and NPV using the indicated cut-off values for AMH and inhibin B in the diagnosis of GD in 46,XY DSD patients are summarized in Table 3. As for FSH, higher values for specificity and PPV were found in the postpubertal group when compared with levels in the prepubertal group.

Basal testosterone levels in prepubertal boys (>0·5–12 years) with gonadal dysgenesis were higher compared with those in UMU but not different from levels in AAD boys. These levels increased after puberty but remained substantially lower than those in the other two groups. Post-hCG testosterone levels showed significantly different levels only in the age group of over 12 years and showed insufficient response to hCG in the group of gonadal dysgenesis (Fig. 3). The ratio of FSH over inhibin B was significantly higher in patients with gonadal dysgenesis (2·14 ± 0·57 IU/ng) compared with UMU (0·02 ± 0·004) and AAD (0·03 ± 0·009); $P < 0·001$ for both comparisons. This was already apparent in the age group of >0·5–12 years and became more pronounced in patients over 12 years of age.

Statistical analysis of the data on the androgen sensitivity index (ASI, the product of the serum concentrations of LH and testosterone)[23] for postpubertal patients with AAD and UMU revealed a Pearson correlation between ASI and EMS score of $r = -0·615$ ($P < 0·01$). In our study, mean ASI in the postpubertal AAD group was 516 ± 92 IU × nmol/l^2 and 44 ± 12 IU × nmol/l^2 in the postpubertal group of UMU ($P < 0·001$). No such differences were found in the younger age groups. This indicates that the distinction between AAD and UMU can only be made on the basis of the ASI in the postpubertal patients.

Evaluation of the 12 unclassified cases revealed hormonal values within the normal range for the following cases: aphallia ($n = 2$), cloacal anomalies ($n = 2$), double penis ($n = 1$) and severe hypospadias with multiple malformations ($n = 2$). In the remaining five cases, results were compatible with hypogonadotropic hypogonadism ($n = 2$, low levels of LH, FSH and testosterone), Leydig cell hypoplasia (LCH, $n = 2$, low testosterone after hCG test) and CYP11A1 deficiency ($n = 1$, high gonadotrophins and low levels of testosterone and adrenal steroids) (see Table S4).

Molecular genetic evaluation. The AR gene was sequenced in all patients with AAD and UMU. We detected AR sequence variants in 24 of 97 patients (24·5%) with AAD (23 pathogenic mutations and 1 unclassified variant). The phenotype was partial androgen insensitivity in 22 patients and complete androgen insensitivity in two. In one of 55 patients with UMU, a pathogenic AR mutation was detected. We identified a total of 19 different AR mutations, none of which was found to be most prevalent. Four mutations had been unclassified so far, but we showed three of these to be pathogenic.[24] *SRY* sequencing revealed no variants in these patients. As a final step, DNA of AR mutation-negative patients with AAD or UMU ($n = 73$ and

54, respectively), or with gonadal dysgenesis ($n = 31$), was subjected to further analysis using MLPA. In the group with AR mutation-negative AAD and UMU, no potentially causative CNVs were found. However, in the group of 31 gonadal dysgenesis cases the following CNVs were detected: *DAX1* duplication ($n = 2$), deletions of *DMRT1* ($n = 1$), of *WT1* ($n = 1$), of *FHIT* ($n = 1$) and of *WWOX* ($n = 3$). In 2 cases, a 3-bp deletion in the *NR5A1* gene (also known as steroidogenic factor-1) was found by exome sequencing.[25]

AR mutation positive vs *AR mutation-negative patients.* Multiple variables were analysed to find out whether AAD patients having an AR mutation ($n = 24$) differed from patients without detected AR mutations ($n = 73$) (referred to as AAD(+) and AAD(−), respectively). With regard to the clinical appearance such as EMS, micropenis, location of the urethral opening and Quigley stage, there were no significant differences between AAD (+) and AAD(−) patients except for the scrotal fusion score ($1{\cdot}50 \pm 0{\cdot}31$ *vs* $0{\cdot}78 \pm 0{\cdot}18$, $P < 0{\cdot}05$, respectively). There were hardly any differences in hormonal values between the AAD(+) and AAD(−) patients except for the inhibin B standard deviation score ($-0{\cdot}55 \pm 0{\cdot}35$ *vs* $0{\cdot}19 \pm 0{\cdot}18$, $P < 0{\cdot}001$) and the increase in testosterone after administration of hCG ($6{\cdot}48 \pm 1{\cdot}24$ *vs* $10{\cdot}12 \pm 0{\cdot}79$, $P < 0{\cdot}005$). The latter difference was mainly caused by the results in the postpubertal group, where no significant increase in testosterone after hCG administration was found; in the other groups, no significant differences were observed. We did not find a significant difference in ASI between AAD(+) and AAD(−) patients.

Sex chromosome DSD

The karyotype of 18 of 24 patients contained Y-chromosome material; five of these patients and the six patients who did not carry any Y-material were raised as females. All patients without a Y-chromosome containing karyotype and with an EMS score of 1 were adults at the time of referral (Table 1).

Hormonal profiles of the patients with a Y-chromosome material containing karyotype showed the same tendencies as found in patients with 46,XY gonadal dysgenesis particularly in the postpubertal group (>12 years), that is high levels of LH and FSH, and low levels of AMH, inhibin B and testosterone (Table S5).

Histology

In 16 patients, gonadal tissue was available for histology following gonadectomy or biopsy. Due to specimen quality issues, the analysis could only be performed on tissue samples of 13 patients. As reported separately,[20] a detailed study of gonadal histology, morphology and immunohistochemistry was undertaken. Three of seven patients with 46,XY DSD showed Leydig cell hyperplasia, while the other four showed the following: carcinoma in situ (CIS), CIS with seminoma, streak gonad and gonadoblastoma. Five patients with sex chromosome DSD showed an ovary with multiple cysts (46,XY/46,XX), a normal ovary (46,XY/46,XX), Sertoli cells only phenotype (46,XY/45,X), Leydig cell hyperplasia (46,Xunusual idicY) and seminoma (46, XY/45,X).[19] A hormone-producing ovarian Leydig cell tumour was identified in a 46,XX patient, who was earlier provisionally diagnosed as a late onset CAH patient.[21]

Discussion

We studied a large cohort of patients with a wide variety of genital anomalies referred to a single centre in Indonesia. The age distribution differs from what is observed in developed countries.[26] Patients referred after the age of 6 months, and adults were dominant among our patients. This confirms the observation of Warne *et al.* [26,27] that in many underprivileged Asian countries a child born with ambiguous genitalia will grow up bearing the congenital anatomic sex features, which remain surgically untreated until adolescence or adulthood. The observation of a preference of male sex of rearing in our total patient group is of specific interest as the last three decades have shown a temporal trend in western societies pointing towards an increased likelihood of affected infants being raised as boys.[28] This trend reflects a shift away from the influence of genital appearance. However, in our patient group, socio-economic and cultural aspects may have been predominant motivations.

In parallel with the current study, an investigation was conducted in patients with DSD in comparison with healthy control subjects matched for gender, age and residential setting on psychosexual development and psychological well-being. The results indicate that a DSD condition has great impact on many psychological aspects across gender and developmental stage. Patients with DSD who had received limited or no treatment experienced gender-related, sexuality related as well as emotional and behaviour problems.[29–31]

The patients were grouped in accordance with the recommendations following a consensus meeting on DSD in 2005.[8] CAH was the most common diagnosis among 46,XX DSD patients. Following clinical and sonographic evaluation of these patients, the diagnosis of CAH was established with 17-hydroxyprogesterone and androgen measurements in serum and confirmed by gene mutation analysis. Sensitivity and specificity of the results of the 17-hydroxyprogesterone assays were slightly better than for the androstenedione determinations.

It should be noted that in 27/67 (40%) of the 46,XX DSD patients, other causes of ambiguity or atypical genital development had to be considered, which is slightly more often than reported recently by Ocal *et al.*.[32] As a first step, PCR was performed to investigate the presence of the SRY gene on one of the other chromosomes, this was observed in 6 of our patients. Another possibility is the presence of a Y chromosome in a low mosaic form. Both situations can be missed with routine karyotyping, indicating that this technique is not sufficient in DSD diagnostics. Two of these patients had clinical characteristics of MRKH, 1 was classified as having gonadal dysgenesis and the remaining 3 as cloacal malformation. Among three patients with

unclassified androgen excess, in one patient an ovarian Leydig cell tumour was diagnosed. In two patients, glucocorticoid resistance was suspected based on hormonal data, but this could not be confirmed by glucocorticoid receptor gene mutation analysis, as has been described before.[33] We conclude that serum measurements of 17-hydroxyprogesterone and androstenedione are the most predictive parameters in determining the underlying cause in 46,XX DSD patients, as CAH was the most common diagnosis. LH and FSH, testosterone and AMH levels are the subsequent parameters to assess gonadal function in the non-CAH patients.

In our patients, 46,XY DSD was most prominent (68·2% of the cases). Various studies from Western and non-Western countries have shown different results with respect to the proportion of 46,XY DSD, ranging from 31% to 52%.[34–37]

The diagnostic management of 46,XY DSD patients remains the greatest challenge. We made a distinction between patients with a suspected androgen synthesis or androgen action disorder (AAD), and patients with unclassified male undervirilization (UMU) on the basis of the EMS score as suggested by Rodie *et al.*.[22] These authors found an EMS score <9 in patients with disorders of androgen synthesis or androgen action in a group of 572 Scottish patients with DSD. However, in our AAD patient group we did not find patients with androgen synthesis disorders; post-hCG concentrations of progesterone, 17-hydroxyprogesterone, androstenedione, testosterone and dihydrotestosterone and the ratios between the concentrations of these steroids did not suggest defective action of 17-hydroxylase, 17,20 lyase, 17β-hydroxysteroid dehydrogenase or 5α-reductase, although we were not able to measure dihydrotestosterone in all patients. The stimulation protocol used here was described by Ahmed *et al.* [38] to be as effective as the other protocols investigated in their study. The AAD and UMU groups did not differ with respect to hormone levels before the age of 12 years, whereas in the postpubertal age group, both testosterone and LH levels were significantly higher in the AAD group compared with the UMU patients, indicating the usefulness of the distinction between the AAD and UMU groups. AR gene sequencing was performed in all patients with AAD or UMU and mutations were found in 25/152 patients, all but one being diagnosed within the group of AAD. The 24 patients with androgen insensitivity, in whom indeed an AR mutation was found, did not differ from patients in whom no AR gene mutation was established, with the exception of a slightly lower score for scrotal fusion in the EMS, a lower standard deviation score for the level of inhibin B and a lower increase in testosterone after administration of hCG. The latter observation can be explained on basis of absence of increase in testosterone in the postpubertal AAD(+) group, where testosterone production apparently was maximally stimulated by endogenous LH. Most of our patients were classified as partial androgen insensitive. We did confirm the observation of Zuccarello *et al.* that the product of the levels of LH and T forms an index for mild androgen insensitivity.[23] There was a negative correlation between this ASI and severity of virilization (EMS) in 46, XY DSD with suspected AAD and UMU in postpubertal patients. Subsequent micro-array and MLPA analyses in the AR mutation-negative patients revealed no further diagnostic information.

At a young age, levels of gonadotrophins were increased in patients with gonadal dysgenesis, but decreased concentrations of inhibin B and AMH had a better value for the discrimination between gonadal dysgenesis on the one hand and AAD and UMU on the other (Table 3). Our results suggest that there is a further discriminatory value of the determination of AMH and inhibin B levels in the diagnosis of gonadal dysgenesis in the older patient groups. However, it needs to be emphasised that PPV and NPV of the various hormone concentrations have to be interpreted with caution in view of the relatively small numbers of patients in the various groups. Subsequent gene mutation analysis yielded the confirmation of a mutation of genes involved in gonadal development and differentiation in 10/31 patients. However, a definitive diagnosis for this group is the pathological examination of the gonads, which is cumbersome for logistical, socio-economic and cultural reasons.[4] Importantly, in spite of the significantly lower risk of gonadal germ cell tumours in the general Asian population, a preliminary study indicated that this risk is increased in Indonesian patients with DSD and is likely to be at the same level observed in Caucasian patients with DSD.[20]

From our study, we conclude that, depending on age category, LH, FSH and basal testosterone are the most important parameters to distinguish between the various groups of patients with 46,XY DSD, followed by AMH and inhibin B. The hCG test was shown to be of limited value as we did not establish the diagnosis of androgen synthesis disorders in our patient population, although we did not measure post-hCG dihydrotestosterone in all patients. We hypothesize that one of the reasons for not detecting deficiencies of androgen synthesis might be the absence of consanguinity in our population in contrast to other studies.[34–37,39,40] Consanguinity is socially not accepted in Indonesia and, moreover, family trees in which grandparents were included were constructed for all patients.

It is not recommended to sequence the AR gene in patients with 46,XY DSD with an EMS score ≥9 because of the very low yield. The aetiology of male undervirilization, also confusingly termed PAIS-like phenotype, remains unclear, raising questions as to whether additional factors, such as (epi)genetic variations (DNA methylation, histone modifications or other, yet-unknown related genes) or environmental factors, may play a role.[41,42]

Regarding sex chromosome DSD, karyotyping is the most important method to establish aneuploidy but arrays or sequence-based techniques can yield additional information. In this group of patients, hormonal data largely depend on the degree of gonadal differentiation. Measurement of AMH and inhibin B at a prepubertal age and the additional measurement of postpubertal FSH are the most indicative for the quality of gonadal function.

In conclusion, the aetiological spectrum of DSD in Indonesia is broad, with 46,XY DSD being most common with a surprising absence of patients with androgen synthesis disorders. Among the 46,XY DSD patients, the distinction of androgen action disorder and male undervirilization syndrome cannot be made based on hormonal evaluation, except for the difference in LH

and testosterone levels after the age of 12 years. The number of AR gene mutations in UMU is very low. In 46,XX DSD, the levels of 17-hydroxyprogesterone and androstenedione are the most important hormones that should be examined and the number of patients with *CYP21A2* or *CYP11B1* variants is high. Using conventional Sanger sequencing, CNV microarrays, MLPA and histology, a final molecular diagnosis could be made in approximately 30% of the patients in our cohort. These results are in the same order as reported recently.[43,44]

Disclosure Statement

Nothing to declare.

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