ORIGINAL ARTICLE

Women with oligo-/amenorrhoea and polycystic ovaries have identical responses to GnRH stimulation regardless of their androgen status: comparison of the Rotterdam and Androgen Excess Society diagnostic criteria

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Abstract

OBJECTIVE: As increased frequency of gonadotrophin-releasing hormone (GnRH) pulses is characteristic for polycystic ovary syndrome (PCOS), we assessed gonadotrophin response to GnRH in women with PCOS with normal and raised androgens and in regularly menstruating controls. **DESIGN, PATIENTS AND METHODS:** The study involved 155 subjects: PCOS, n=121, age (mean±SD) 24.8±5.4 yrs, BMI 24.5±6.0 kg/m^2, all with oligo-/amenorrhoea and PCO morphology, and 34 controls. Gonadotrophins were measured in early follicular phase after GnRH stimulation (0, 30 and 60 minutes). **RESULTS:** Fifty four (41.9%) women with PCOS had androgens (testosterone, androstendione, dihydroepiandrosterone sulphate) within the reference range, and would fulfil the "Rotterdam", but not the Androgen Excess Society PCOS criteria. Baseline and stimulated LH concentrations were higher in PCOS (9.09±5.56 vs 4.83±1.71 IU/l, 35.48±31.4 vs 16.30±6.68 IU/l, 33.86±31.8 vs 13.45±5.2 IU/l, at 0, 30 and 60 min post GnRH, respectively, $p<0.0001$). An LH/FSH ratio in PCOS increased further after GnRH stimulation. ROC analysis revealed that LH30min/FSH30min >2.11 or LH60min/FSH60min >1.72 had 78.3% and 87.5% sensitivity and 81.7% and 81.3% specificity for diagnosis of PCOS. Both baseline and GnRH-stimulated LH and FSH concentrations were similar in women with PCOS and raised androgens and with androgens within the reference range ($p=0.71$ and $p=0.20$ for LH and FSH, respectively). **CONCLUSIONS:** Regardless of their androgen status, women with PCO morphology and oligo-/amenorrhoea have higher baseline and GnRH-stimulated LH concentrations and higher GnRH-stimulated LH/FSH ratio than controls, suggestive of similar underlying mechanism accounting for menstrual irregularities. These observations support validity of PCOS diagnostic criteria based on the Rotterdam consensus.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a multifaceted disorder that can present as oligo-/amenorrhoea and/or hyperandrogenism, as well as fertility problems (Azziz *et al.* 2004; Ehrmann 2005; Conway *et al.* 1989). Current definition of PCOS is based on the European Society of Human Reproduction and Embryology (ESHRE) and American Society for Reproductive Medicine (ASRM) consensus, i.e., the so called ESHRE/ASRM Rotterdam criteria (2004), where a women can be diagnosed with PCOS as long as she fulfils two out of three criteria, i.e., oligo-/anovulation, clinical or biochemical hyperandrogenism and polycystic ovaries on ultrasound imaging, on condition that other causes of menstrual irregularities/hyperandrogenism (hyperprolactinaemia, Cushing syndrome, late onset congenital adrenal hyperplasia, etc.) have been ruled out (The Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group 2004). There are, however, still controversies as to the most appropriate definition of PCOS. This is reflected in Androgen Excess Society Guidelines (Azziz *et al.* 2006), where the authors suggest that women with polycystic ovarian morphology and oligo-anovulation – but without clinical or biochemical hyperandrogenemia – should not be classified as having PCOS. Furthermore, standard androgen assays were not designed to measure androgens accurately in the female range (Fears *et al.* 2000; Boots *et al.* 1998), while results of the largest UK series demonstrated raised total testosterone only in about 40% women with PCOS (Balen *et al.* 1995).

Increased androgen synthesis in PCOS, both of ovarian and adrenal contribution (Milewicz *et al.* 1983), results from various abnormalities of the hypothalamo-pituitary-ovarian axis, including increased frequency of LH pulses, that – in turn – reflect an increased frequency of pulsatile hypothalamic gonadotrophin releasing hormone (GnRH) secretion (Taylor *et al.* 1997). This phenomenon leads to a relative overproduction of LH, which is one of the factors responsible for excessive androgen synthesis, i.e., one of the characteristic features of PCOS (Taylor *et al.* 1997; Blank *et al.* 2006). Over twenty years ago it was also demonstrated that administration of GnRH analogue allowed to demonstrate certain abnormalities of steroidogenesis in PCOS, that resulted in an increased 17-hydroxy-progesterone to androstendione ratio in about 16–24 hours after GnRH stimulation (Barnes *et al.* 1989). The authors subsequently named these abnormalities as functional ovarian hyperandrogenism (Ehrmann *et al.* 1995), that – in their opinion – was a characteristic feature of PCOS (Ehrmann *et al.* 1995). The mentioned authors (Barnes *et al.* 1989; Ehrmann *et al.* 1995) also described the increased LH release following GnRH analogue stimulation, though in terms of a qualitative rather than a quantitative phenomenon.

In such circumstances we have endeavoured to quantitatively assess LH and FSH secretion after GnRH stimulation in women with PCOS and in healthy, regularly menstruating controls. The aim of our study was to determine whether assessment of gonadotrophin secretion after GnRH stimulation could provide further information potentially useful in the diagnosis of PCOS. We hypothesised that GnRH stimulation might reveal a relative excess of pituitary LH in comparison to FSH that is the result of an increased activity of hypothalamic GnRH pulse generator. We also have endeavoured to specifically assess GnRH-stimulated gonadotrophin secretion in women who fulfil diagnostic criteria for PCOS, according to the Rotterdam consensus (2004), but fail the diagnostic criteria of the Androgen Excess Society (Azziz *et al.* 2006), i.e., in women with oligo-/amenorrhoea and polycystic ovarian morphology, however with androgen concentrations within the reference range.

SUBJECTS AND METHODS

The study involved 155 women admitted to the Department of Endocrinology and Metabolic Diseases of the Medical University of Lodz, Polish Mother's Memorial Hospital – Research Institute in Lodz, between 2006 and 2009. Of those, 121 women of 24.8±5.4 years of age (mean±SD) were diagnosed with PCOS, according to the Rotterdam criteria (The Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group 2004). All these women had oligo- or amenorrhoea and polycystic ovaries on pelvic (intravaginal) ultrasound, while the majority also had clinical hyperandrogenism (acne and/or hirsutism). Control group consisted of 34 healthy, regularly menstruating women aged 26.6±5.1 years.

All hormonal investigations were performed between the third and sixth day of either a spontaneous or a progestagen-induced menstruation (in the latter case menstrual bleeding was typically obtained after 10-day administration of dydrogesterone (Duphaston®) or micronized progesterone (Luteina®). Endocrine investigations involved measurements of LH, FSH, oestradiol, total testosterone, androstendione, dihydro-epinadrosterone sulphate (DHEAS), 17-hydroxy-progesterone, TSH, free T_3 and free T_4. The presence of hyperprolactinaemia was excluded after assessment of a nine time point prolactin day curve, as described before (Karasek *et al.* 2006). Hypercortisolaemia was excluded either on a basis of a midnight serum cortisol below 50 nmol/l (1.8 μg/dl) or cortisol suppression below 50 nmol/l after an overnight 1.0 mg dexamethasone suppression test. Furthermore, in all subjects we have performed a 75 gram oral glucose tolerance test (OGTT) with glucose and insulin measurements at 0, 60 and 120 minutes. Subsequently, insulin resistance parameters were calculated, i.e., HOMA, (where HOMA=fasting insulin (μU/ml) x fasting glucose (mmol/l)/22.5) (Matthews *et al.* 1985) and an Insulin Resistance Index (IRI) that is based on the assessment of glycaemia and insulinaemia

during OGTT. In the latter model, the product of the glucose area under the plasma glucose curve and insulin area under plasma insulin curve is used as an index of insulin resistance, calculated through the formula: 2/[1/(INSp x GLYp)]+1, where INSp and GLYp are the measured insulin and glycaemic areas. This method has a good correlation with the „golden standard" of assessment of insulin resistance, i.e., an euglycaemic hyperinsulinaemic clamp technique (Matsuda & DeFronzo 1999).

Hirsutism was assessed according to the Ferriman-Galwey scale (Ferriman & Gallwey 1961) where the score above eight was considered significant. All pelvic ultrasound examinations were performed in the early follicular phase at the Department of Ultrasound Diagnostics of the Polish Mother's Memorial Hospital – Research Institute, where diagnosis of polycystic ovaries was based on the presence of either 12 or more follicles measuring 2–9 mm in diameter, or the increased ovarian volume (>10 cm^3) (Balen *et al.* 2003). In order to limit variability in interpretation of ovarian ultrasound imaging, we ensured that all ultrasound scans were performed in follicular phase by the same observer in a tertiary referral centre (Polish Mother's Memorial Hospital – Research Institute) that is considered as one of the highest ranking institutions in the field of obstetrics and gynaecology in Poland.

In all patients and controls we performed a GnRH test that involved intravenous administration of 100 μg of synthetic GnRH (Relisorm®, Serono or LHRH®, Ferring). Blood samples for the measurements of LH and FSH [electrochemiluminescence method (ECLIA) by Elecsys 2010 analyser] were taken before (0 minutes) and at 30 and 60 minutes after GnRH administration. The study was approved by the Ethical Committee of the Polish Mother's Memorial Hospital – Research Institute.

Statistical analysis

The data were analysed by means of simple descriptive statistics of location and dispersion and tests of significance for comparison of distributions in different groups, parametric (the t-test) or non-parametric (Mann-Whitney's test), depending on distribution of analysed variables. Between group comparisons for levels of selected characteristics before and after GnRH stimulation were compared by Friedman's ANOVA for repeated measures design. Correlation analysis was performed by the means of Spearman rank correlation method. Statistical significance was assumed for $p<0.05$. All analyses were performed by the means of Statistica 8.0 software.

RESULTS

Women with PCOS and Controls were matched for their age and BMI (24.4±5.0 years *versus* 26.8±5.1 years, BMI 24.1±5.5 kg/m^2 *versus* 24.6±5.6 kg/m^2, for PCOS (n=121) and controls (n=34), respectively, p=ns). Mean 24-hour prolactin concentrations (based on 9-point prolactin profiles) were non-significantly higher in women with PCOS (data not shown). As expected, women with PCOS had significantly higher concentrations of total testosterone, androstendione, 17-hydroxy-progesterone and oestradiol in comparison to Controls (Table 1). Interestingly, however, androgen concentrations were within reference ranges in a significant proportion of women with PCOS. Namely, raised total testosterone concentrations were found in 38.6%, androstendione in 34.7%, while DHEAS concentrations were raised only in 28.8% women with PCOS. Altogether, raised concentration of at least a single androgen was observed in 58.1% women with PCOS. Women with PCOS had higher fasting insulin ($p<0.05$) and were more insulin resistant [higher Insulin Resistance Index (IRI), $p<0.05$]. HOMA index was also higher in women with PCOS, but it just failed to reach statistical significance ($p=0.06$) (Table 1). There was a significantly raised 17-hydroxy-progesterone/androstendione ratio 0.50±0.57 versus 0.26±0.12 ($p=0.0014$) in women with PCOS versus controls. Given that over 40% of women diagnosed with PCOS had concentrations of androgens within the reference range, we have divided women with PCOS into two groups, i.e., with androgens within the reference range (PCOS-Normal Androgens – PCOS-NA, n=54) and with raised androgens (PCOS-Not Normal Androgens – PCOS-NNA, n=67). With exception of androgens and 17-hydroxy-progesterone, hormonal and insulin resistance indices did not differ between women with normal androgens (PCOS-NA) and those with raised androgens (PCOS-NNA) (Table 2). Women with raised androgens (PCOS-NNA group) had raised 17-hydroxy-progesterone concentrations ($p=0.008$), however, without significant differences in 17-hydroxy-progesterone/androstendione ratio ($p=0.14$) (Table 2). Both these groups, however, had significantly ($p<0.001$) raised 17-hydroxy-progesterone/androstendione ratio in comparison to controls (Figure 1). As described above, women from PCOS-NA group would be diagnosed as having PCOS according to the Rotterdam consensus criteria (2004), but they would not fulfil diagnostic criteria for PCOS according to the Androgen Excess Society guidelines (Azziz *et al.* 2006).

Gonadotrophin responses to GnRH are presented in Table 3. Women with PCOS had significantly higher LH concentrations before and after GnRH stimulation ($p<0.001$). There were, however, no differences in FSH levels. There were no differences in the ratio of stimulated (i.e., after GnRH administration) gonadotrophin concentrations in relation to their respective baseline values between the groups (i.e., LH_{30min}/LH_{0min}, LH_{60min}/LH_{0min}, FSH_{30min}/FSH_{0min}, FSH_{60min}/FSH_{0min}) (Table 3). There was, however, a highly significant increase in LH/FSH ratio in women with PCOS after GnRH stimulation, i.e., from 1.59±1.0 to 4.09±2.99 and 3.56±2.58 at 30 and 60 minutes post GnRH stimulation

Tab. 1. Descriptive statistics for concentrations of selected hormones and glucose, insulin and insulin resistance indices during oral glucose tolerance test (OGTT) in women with PCOS (n=121) and Controls (CONTR, n=34). *p*-value of appropriate test for comparison of distributions between groups (Mann-Whitney's or Friedman's ANOVA for repeated measures).

	Group	Mean	Median	SD	Min	Max	*p*-value
Oestradiol [pmol/l]	PCOS	237.1	172.8	224.0	64	1476.4	0.018
	CONTR	155.1	127.3	115.1	59	689.7	
Testosterone [nmol/l]	PCOS	2.77	2.59	1.44	0.75	5.45	0.0002
	CONTR	1.86	2.06	1.02	0.56	4.25	
DHEAS* [nmol/l]	PCOS	7.86	7.14	3.50	1.91	18.04	0.38
	CONTR	7.18	6.81	3.12	1.93	14.02	
Androstendione** [nmol/l]	PCOS	10.72	9.13	19.6	3.30	33.45	0.0021
	CONTR	7.36	7.20	3.06	2.61	16.43	
17OH-progesterone [nmol/l]	PCOS	4.16	3.33	3.09	0.99	10.6	<0.00001
	CONTR	1.78	1.53	0.76	0.33	4.29	
Glucose 0′ [mmol/l]	PCOS	4.44	4.50	0.52	3.71	5.55	0.569
	CONTR	4.48	4.53	0.38	3.66	5.33	
Glucose 60′ [mmol/l]	PCOS	6.48	6.31	2.00	4.89	13.78	
	CONTR	6.47	6.37	1.72	4.96	11.83	
Glucose 120′[mmol/l]	PCOS	5.44	5.29	1.65	2.96	14.45	
	CONTR	5.23	5.26	1.32	2.76	8.81	
Insulin 0′ [µmol/l]	PCOS	62.65	50.01	44.45	12.67	293.2	0.344
	CONTR	51.38	39.56	45.78	14.36	224.0	
Insulin 60′ [µmol/l]	PCOS	469.0	311.8	396.8	75.8	2183.0	
	CONTR	362.9	254.9	283.5	114.8	1349.2	
Insulin 120′ [µmol/l]	PCOS	348.8	256.4	332.9	53.9	2520.9	
	CONTR	309.8	224.0	307.6	91.2	2120.0	
Insulin Resistance Index (IRI)	PCOS	0.93	0.84	0.38	0.15	1.88	0.045
	CONTR	0.78	0.69	0.34	0.33	1.68	
HOMA [mmol/l × µIU/ml]	PCOS	1.81	1.41	1.36	0.07	8.37	0.060
	CONTR	1.48	1.16	1.32	0.36	5.99	

*upper reference range for DHEAS for women in reproductive age is 12.24 nmol/l
**upper reference range for androstendione for women in reproductive age is 11.55 nmol/l

respectively ($p<0.01$), and both baseline and GnRH-stimulated LH/FSH ratio was higher in women with PCOS than in Controls ($p<0.001$) (Table 3).

In contrast, concentrations of LH and FSH were not different before and after GnRH stimulation in women with PCOS and raised androgens (PCOS-NNA) and women with PCOS and androgens within the reference range (PCOS-NA), $p=0.71$ and $p=0.20$ for LH and FSH, respectively (Table 3). In both groups, however, both baseline and GnRH-stimulated LH concentrations were higher than in controls ($p<0.001$) (Figure 2A). There were also no differences in both baseline and GnRH stimulated LH/FSH ratio between those groups (i.e., PCOS-NNA and PCOS-NA, $p=0.61$) (Table 3), however, again in both groups, both baseline and GnRH stimulated LH/FSH ratio was significantly higher than in controls ($p<0.001$) (Figure 2B).

Receiver operating characteristic analysis (ROC curves) was employed in order to define the best discriminatory cut-off point for GnRH-stimulated LH/FSH ratio. Optimal sensitivity and specificity for the diagnosis of PCOS was obtained for $LH_{30min}/FSH_{30min}>2.118$ (sensitivity 78.3%, specificity 87.5%) or $LH_{60min}/FSH_{60min}>1.72$ (sensitivity 81.7%, specificity 81.3%) (Figures 3A and 3B). In contrast for baseline LH/FSH ratio>2.0 (the value most often quoted in literature – see Discussion), we obtained a high (100%) specificity for the diagnosis of PCOS, but at the expense of very low sensitivity (23%). We subsequently applied receiver operating characteristic analysis for LH/FSH

Tab. 2. Descriptive statistics for glucose, insulin and insulin resistance indices during oral glucose tolerance test (OGTT), as well as selected hormonal parameters in women with PCOS and androgens within the reference range (PCOS-Normal Androgens – PCOS-NA, n=54), and at least a single androgen (testosterone, androstendione and/or DHEAS) above the reference range (PCOS-Not Normal Androgen – PCOS-NNA, n=67). P-value of appropriate test for comparison of distributions between groups (Mann-Whitney's or Friedman's ANOVA for repeated measures).

	Group	Mean	Median	SD	Min	Max	*p*-value
Glucose 0′ [mmol/l]	PCOS – NA	4.44	4.46	0.43	3.71	5.50	0.13
	PCOS – NNA	4.42	4.44	0.61	3.74	5.50	
Glucose 60′ [mmol/l]	PCOS – NA	5.86	5.56	1.94	4.89	12.28	
	PCOS – NNA	6.47	6.06	2.11	4.98	13.78	
Glucose 120′ [mmol/l]	PCOS – NA	5.17	5.11	1.67	2.96	10.33	
	PCOS – NNA	5.46	5.50	1.63	3.11	14.45	
Insulin 0′ [µmol/l]	PCOS – NA	58.52	52.31	37.80	12.67	170.1	0.08
	PCOS – NNA	65.60	46.97	49.60	14.43	293.2	
Insulin 60′ [µmol/l]	PCOS – NA	427.8	295.4	344.1	75.8	1624.0	
	PCOS – NNA	506.8	303.1	462.0	77.9	2183.0	
Insulin 120′ [µmol/l]	PCOS – NA	294.5	239.3	224.0	53.9	1253.4	
	PCOS – NNA	399.2	248.7	403.5	57.8	2520.9	
Insulin Resistance Index (IRI)	PCOS – NA	0.86	0.82	0.36	0.15	1.62	0.12
	PCOS – NNA	1.29	0.86	2.46	0.34	18.80	
Oestradiol [pmol/l]	PCOS – NA	234.2	163.3	259.2	64	1476.4	0.13
	PCOS – NNA	225.1	177.9	178.5	68	1303.2	
17OH-progesterone [nmol/l]	PCOS – NA	3.37	2.90	2.27	0.99	11.22	0.008
	PCOS – NNA	4.85	4.39	3.63	1.33	17.6	
17-OHP/androstendione ratio	PCOS – NA	0.53	0.35	0.39	0.10	1.50	0.14
	PCOS – NNA	0.53	0.30	0.74	0.06	4.07	
HOMA [mmol/l × µIU/ml]	PCOS – NA	1.66	1.37	1.18	0.33	5.76	0.56
	PCOS – NNA	1.87	1.35	1.50	0.07	8.37	

ratio after GnRH stimulation for women with PCOS who had androgens concentrations within the reference range (PCOS-NA). The calculated values were very similar as for the group of women with PCOS analysed as a whole (LH_{30min}/FSH_{30min}>2.118, sensitivity 72.7%, specificity 84.4%, LH_{60min}/FSH_{60min}>1.766, sensitivity 72.7%, specificity 87.5%). Given the highly significant difference in the baseline 17-hydroxy-progesterone/androstendione ratio for women with PCOS versus controls, we have also performed sensitivity and specificity analysis for this parameter. Interestingly, for 17-hydroxy-progesterone/androstendione ratio 0.244 we obtained a 68.4% sensitivity and 64.3% specificity for the diagnosis of PCOS (and 76.3% sensitivity, with 64.3% specificity for PCOS-NA).

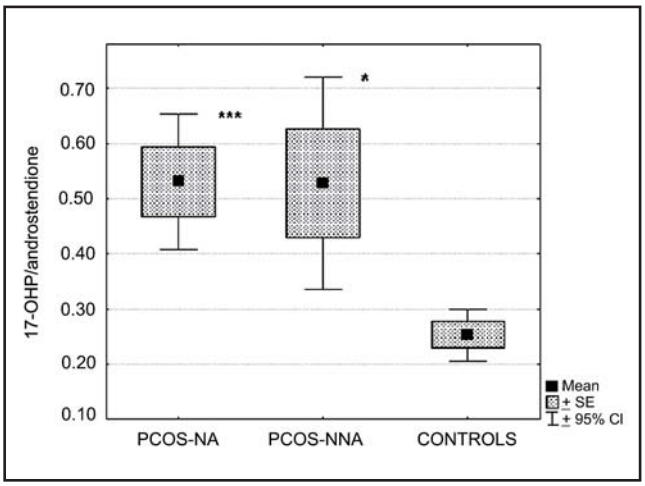

Figure 1. 17-hydroxyprogesterone [nmol/l] to androstendione [nmol/l] ratio in women with PCOS and normal androgens (PCOS-NA) and not-normal androgens# (PCOS-NNA) and in healthy, regularly menstruating Controls. Significant differences between PCOS and Controls, assessed by means of Mann-Whitney's U test, are indicated by asterisks: *($p \leq 0.05$); ***($p \leq 0.001$).
#Implies that plasma concentration of at least a single androgen, i.e., total testosterone, androstendione or DHEAS was above the reference range.

DISCUSSION

Given the high prevalence of PCOS among women of reproductive age, the issue of the optimal diagnostic criteria for this condition is under intensive debate. The main finding of our study was demonstration that

Table 3. Descriptive statistics for concentrations of LH, FSH and appropriate ratios in comparison to respective baseline values, as well as LH/FSH ratio before and after GnRH stimulation and appropriate ratios in comparison to respective baseline values - in women with PCOS (n=121) and Controls (n=34). Descriptive statistics for LH and FSH concentrations before and after GnRH stimulation in women with PCOS and androgens within the reference range (PCOS-NA, n=54), and at least a single androgen (testosteron, androstendione and/or DHEAS) above the reference range (PCOS-NNA, n=67). P-value of Friedman's ANOVA for repeated measures design.

	Group	Mean	Median	SD	Min	Max	*p*-value
LH 0′ [IU/l]	PCOS	9.04	7.80	5.81	0.46	34.00	0.0004
	CONTR	4.83	4.57	1.68	1.08	9.22	
LH 30′ [IU/l]	PCOS	35.38	24.00	31.32	5.92	200.00	
	CONTR	16.30	16.23	6.68	5.34	32.59	
LH 60′ [IU/l]	PCOS	33.86	21.67	31.78	4.87	200.00	
	CONTR	13.45	12.79	5.22	5.01	28.51	
FSH 0′ [IU/l]	PCOS	5.80	5.45	2.48	1.14	24.49	0.891
	CONTR	6.44	6.01	2.02	2.15	12.11	
FSH 30′ [IU/l]	PCOS	8.90	7.83	4.84	3.24	42.58	
	CONTR	8.84	8.11	2.73	3.61	15.28	
FSH 60′ [IU/l]	PCOS	9.95	8.43	8.21	3.58	73.24	
	CONTR	9.04	8.61	3.00	4.05	17.36	
LH 30′/0′	PCOS	3.99	2.13	7.83	1.08	12.99	0.465
	CONTR	3.89	3.30	2.88	1.59	17.56	
LH 60′/0′	PCOS	3.76	3.06	2.00	1.10	11.89	
	CONTR	3.28	2.78	2.59	0.94	15.91	
FSH 30′/0′	PCOS	1.52	1.41	0.53	0.81	5.80	0.079
	CONTR	1.38	1.33	0.21	1.08	1.98	
FSH 60′/0′	PCOS	1.68	1.47	1.14	0.86	12.96	
	CONTR	1.42	1.39	0.25	0.96	2.13	
LH/FSH 0′	PCOS	1.59	0.99	1.00	0.23	5.28	0.00003
	CONTR	0.76	0.76	0.20	0.26	1.06	
LH/FSH 30′	PCOS	4.09	3.18	2.99	0.51	16.99	
	CONTR	1.89	1.75	0.79	0.72	5.25	
LH/FSH 60′	PCOS	3.56	2.77	2.58	0.43	13.65	
	CONTR	1.55	1.44	0.63	0.60	4.24	
LH 0′ [IU/l]	PCOS-NA	8.67	7.49	5.03	1.89	28.75	0.71
	PCOS-NNA	8.93	7.77	6.03	0.10	26.90	
LH 30′ [IU/l]	PCOS-NA	36.06	24.80	31.56	5.96	200.00	
	PCOS-NNA	34.51	22.93	31.96	5.92	185.70	
LH 60′ [IU/l]	PCOS-NA	34.71	21.09	33.88	4.87	200.00	
	PCOS-NNA	32.73	22.16	30.57	5.61	155.30	
FSH 0′ [IU/l]	PCOS-NA	6.08	5.92	2.21	2.08	13.29	0.20
	PCOS-NNA	5.47	5.05	2.78	1.29	24.49	
FSH 30′ [IU/l]	PCOS-NA	9.79	8.84	4.65	4.25	29.77	
	PCOS-NNA	8.14	7.38	4.98	3.24	42.58	
FSH 60′ [IU/l]	PCOS-NA	10.28	9.27	5.34	3.58	31.61	
	PCOS-NNA	9.62	7.94	10.14	4.24	73.24	
LH 30′/0′	PCOS-NA	3.86	3.43	1.60	1.69	8.71	0.31
	PCOS-NNA	5.46	3.49	10.64	1.08	86.90	
LH 60′/0′	PCOS-NA	3.72	2.85	2.04	1.27	10.46	
	PCOS-NNA	4.76	3.56	7.82	1.10	64.30	
FSH 30′/0′	PCOS-NA	1.60	1.44	0.69	0.93	5.80	0.75
	PCOS-NNA	1.53	1.39	0.57	0.81	4.99	
FSH 60′/0′	PCOS-NA	1.65	1.48	0.55	0.86	3.41	
	PCOS-NNA	1.79	1.48	1.56	0.93	12.96	
LH/FSH 0′	PCOS-NA	1.53	1.24	0.94	0.29	4.47	0.61
	PCOS-NNA	1.62	1.33	0.97	0.08	4.58	
LH/FSH 30′	PCOS-NA	3.88	2.84	3.15	0.75	15.05	
	PCOS-NNA	4.20	3.41	2.92	0.51	16.99	
LH/FSH 60′	PCOS-NA	3.39	2.52	2.75	0.61	13.65	
	PCOS-NNA	3.67	2.92	2.48	0.43	12.82	

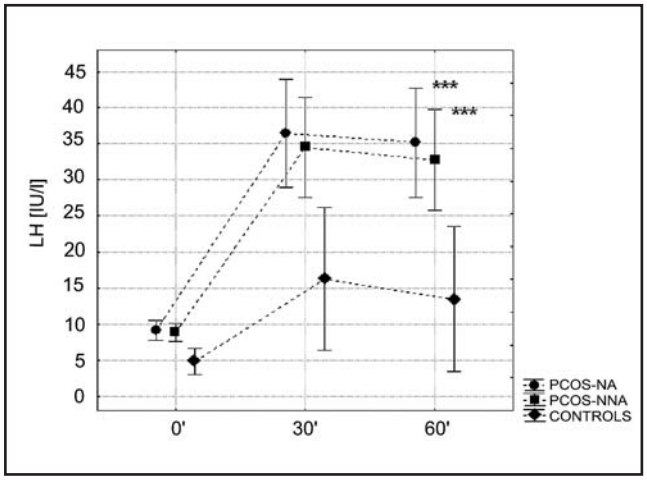

Fig. 2A. Mean LH concentrations [IU/l] during GnRH test in women with PCOS and normal androgens (PCOS-NA) and not-normal androgens (PCOS-NNA) and in regularly menstruating controls. Vertical bars represent 95% confidence intervals in the respective time-points. Significant differences between PCOS and Controls, assessed by means of Friedman's ANOVA for repeated measures design, are indicated by asterisks: ***($p \leq 0.001$).

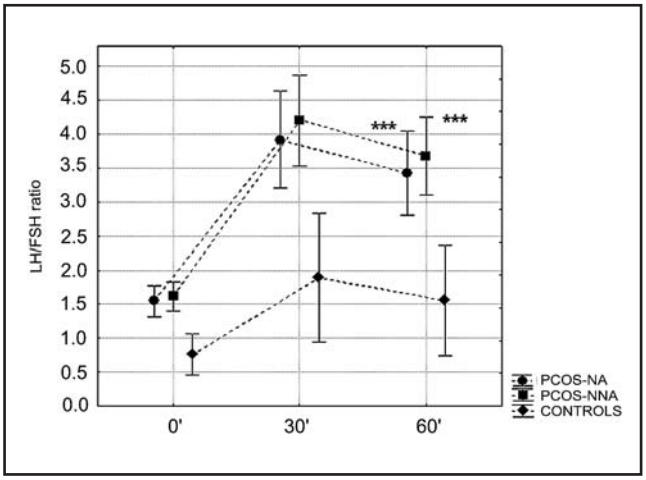

Fig. 2B. Mean values of LH/FSH ratio during GnRH test in women with PCOS and androgens within the reference range (PCOS-NA) and at least a single androgen concentration higher than the reference range (PCOS-NNA) and in regularly menstruating controls. Vertical bars represent 95% confidence intervals in the respective time-points. Significant differences between PCOS and Controls, assessed by means of Friedman's ANOVA for repeated measures design, are indicated by asterisks: ***($p \leq 0.001$).

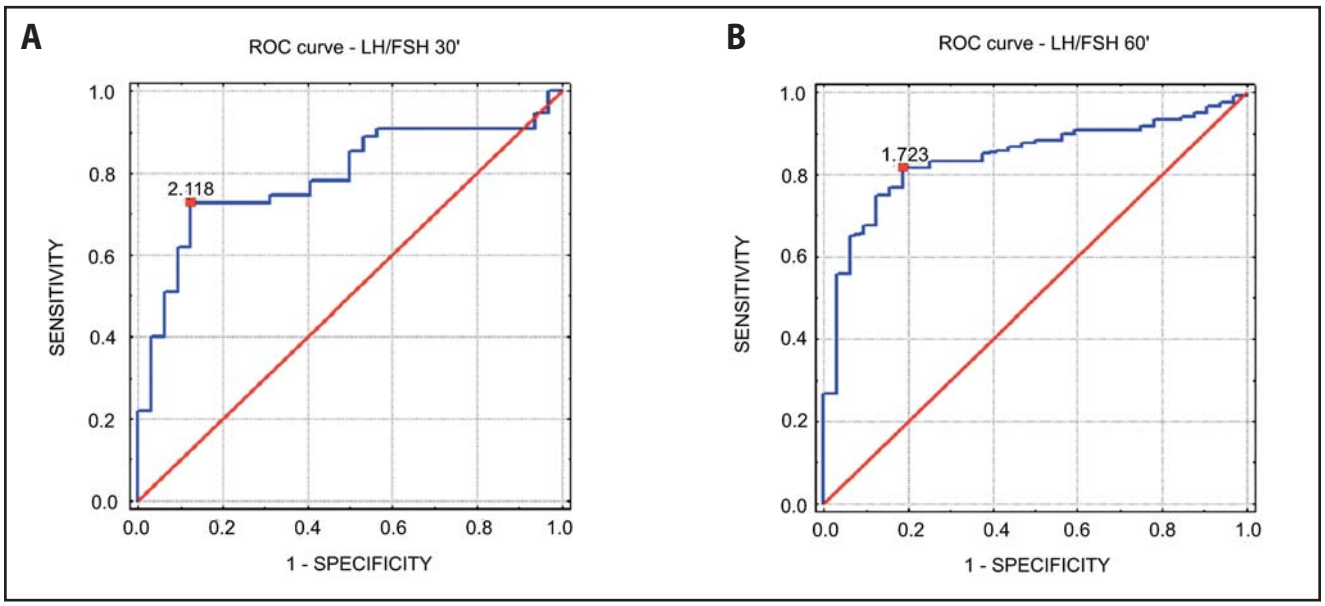

Fig. 3A,B. Receiver operating characteristic (ROC) curve analysis for the finding the cutoff for optimal diagnostic accuracy (considered as the highest proportion of correctly classified subjects) of LH/FSH ratio during GnRH test. The highest sensitivity (78.3% and 81.3%) is obtained for LH/FSH>2.110 at 30 minutes of GnRH test (**Figure 3A**) and for LH/FSH>1.723 at 60 minutes of GnRH test (**Figure 3B**). Respective sensitivity is 87.5% and 81.3%.

women with PCOS defined as oligo-/amenorrhoea, polycystic ovaries and raised androgens (ie., fulfilling both the "Rotterdam" and AES criteria) have virtually identical gonadotrophin response to GnRH stimulation as women with oligo-/amenorrhoea, polycystic ovaries, but androgens within the reference range (i.e., those who fulfill the "Rotterdam" criteria only). Furthermore, there is a clear-cut difference in both baseline and stimulated LH concentrations in comparison to controls. In our opinion the issue of the choice of the most appropriate criteria is highly relevant to clinical practice, as a significant number of women (i.e., about 40%) with oligo-/amenorrhoea and polycystic ovarian morphology and androgens within the reference range would fulfill the "Rotterdam" criteria for the diagnosis of PCOS, but fail the criteria according to the AES consensus. This is in keeping with the recent data (March *et al.* 2010) that show about a 40% increase of prevalence

of PCOS among women of reproductive age when "Rotterdam" rather than AES criteria are used (17.8±2.8% versus 10.2±2.4%).

In such context, the findings of our study might, in our opinion, contribute to the debate as to the choice of the optimal diagnostic criteria for PCOS. It is well known that relative proportions of pituitary secretion of LH and FSH are determined by both frequency and amplitude of hypothalamic GnRH pulses (Ferris & Shupnik 2006; Marshall *et al.* 1991). In normal menstrual cycle GnRH pulse frequency increases from about 90–100 minutes to about once in 60 minutes through a follicular phase. Gradual increase in GnRH pulse frequency facilitates LH secretion culminating in ovulatory LH surge (Marshall & Eagleson 1999). Progesterone secretion in a luteal phase results in an increase in FSH synthesis as a result of less frequent hypothalamic GnRH secretion, through mechanisms involving opioid receptors (Marshall & Eagleson 1999; Nippoldt *et al.* 1989) and possibly other factors, such as kisspeptin (Neal-Perry *et al.* 2009).

Polycystic ovary syndrome is characterised by an increase in both frequency and amplitude of hypothalamic GnRH pluses that are generated with frequency of about once per 60 minutes, i.e., the frequency observed only in a late follicular phase in healthy women (Kazer *et al.* 1987). This is accompanied by resistance to inhibitory effects of progesterone on the frequency of hypothalamic GnRH secretion (Pastor *et al.* 1998). Furthermore, similar situation is observed in hyperandrogenic adolescent girls, even in the setting of regular menstrual cycles (Chhabra *et al.* 2005). As a result of the above described phenomena, PCOS is characterized by a relative overproduction of LH in relation to FSH that can be revealed by GnRH stimulation.

We have demonstrated that – once other causes of oligo-/amenorrhoea have been ruled out – women with polycystic ovaries have identical responses to GnRH in terms of LH secretion. This suggests the existence of similar underlying mechanism, most likely related to an increased hypothalamic GnRH pulse frequency, resulting in a relative pituitary LH excess in both women with raised androgens and with androgens within the reference range, i.e., regardless of their androgen status. In our opinion, our data indirectly support the use of the "Rotterdam" criteria in clinical setting, given that assessment of androgen concentrations within the female range by the means of standard androgen assays is fraught with various methodological problems, including very high coefficients of variation reaching as much as around 35% (Boots *et al.* 1998; Fears *et al.* 2000). If such assay-dependent variability were employed, e.g., for the diagnosis of diabetes, than for fasting glucose concentration of 136 mg/dl, the obtained results would range from 88 mg/dl (entirely normal) to as much as 183 mg/dl (poorly controlled diabetes)!

In our opinion, the results of our study also cast some new light on application of the long-abandoned use of LH/FSH ratio for the diagnosis of PCOS. In the past, a relative LH overproduction in PCOS was said to be reflected in a raised baseline LH/FSH ratio (Taylor *et al.* 1997; Taylor 1998). There was, however, no agreement as to the optimal cut-off point, and various authors suggested several cut-off values for baseline LH/FSH ratio [e.g., LH/FSH>1 (Steward *et al.* 1993), LH/FSH>2 (Koskinen *et al.* 1996) or even LH/FSH>3 (Jasonni *et al.* 1991)]. Meta-analysis of several studies demonstrated very poor positive predictive value for baseline LH/FSH ratio, i.e., only 18% (Barth *et al.* 2007), that effectively precluded the use of that index as a valid diagnostics tool. In our series, for the most frequently quoted cut-off point of LH/FSH>2 (Koskinen *et al.* 1996), we obtained only a 23% sensitivity (and 43% sensitivity for LH/FSH>1.5), despite high (100%) specificity for the diagnosis of PCOS. The failure of baseline LH/FSH ratio as a diagnostic tool in PCOS was said to be related to the fact that women with PCOS and more pronounced insulin resistance and hyperandrogenism have relatively lower LH concentrations, despite preserved increased GnRH pulse frequency (Lawson *et al.* 2008). In keeping with these data, in our study there was a weak (r=–0.21), but still significant ($p<0.05$) negative correlation between BMI and baseline LH concentrations, as well as between LH and insulin resistance index (r=–0.31). Though not the main purpose of our study, our results suggest that – in contrast to the baseline LH/FSH ratio – the assessment of GnRH-stimulated LH/FSH ratio might be potentially useful for diagnosis of PCOS. A relative increase of LH concentrations is proportional to (higher) baseline LH concentrations in women with PCOS, resulting in higher stimulated LH concentrations in this group, however, without significant differences in both baseline and stimulated FSH levels. This is in accordance with results of other studies (Lawson *et al.* 2008; Patel *et al.* 2004). Such phenomenon results in a more pronounced increase of GnRH-stimulated LH/FSH ratio in women with PCOS than in controls. This, in turn, leads to an almost four-fold increase in diagnostic sensitivity of GnRH-stimulated LH/FSH ratio. For instance, results of ROC analysis reveal 81.7% sensitivity and 81.3% specificity for LH/FSH ratio above 1.72 at 60 minutes after GnRH stimulation. This is much better than sensitivity of total testosterone assays (around 40%) (Balen *et al.* 1995) and it is higher than the combined sensitivity of all androgen assays (58.1%) observed in our study. Therefore, we suggest that the use of GnRH stimulated LH/FSH ratio as an additional diagnostic tool for diagnosis of PCOS at least merits some further research and should not be discarded outright as clinically useless.

As we have already described, Barnes *et al.* (1989) and Ehrmann *et al.* (1995) suggested that GnRH test might be useful for the diagnosis of PCOS, according to the protocol involving assessment of 17-hydroxyprogesterone/androstendione ratio around 24-hours after GnRH agonist stimulation. As it turns out, however,

increased GnRH-stimulated 17-hydroxy-progesterone/androstendione ratio is observed only about 50% of women with PCOS (Pasquali *et al.* 2007; Rosenfield *et al.* 1993). That is also in accordance with earlier studies, involving ovarian and adrenal vein catherisation (Milewicz *et al.* 1983), where frank ovarian hyperandrogenism was observed only in around 50% (10/21) women with PCOS. Interestingly, this is actually lower than diagnostic utility of the baseline 17-hydroxy-progesterone/androstendione ratio, where we observed 68.4% sensitivity and 64.3% specificity for the diagnosis of PCOS, for the cut-off point above 0.244. We can also conclude that women with oligo-/amenorrhoea and polycystic ovaries have not only raised baseline and Gn-RH-stimulated LH concentrations, but also an elevated baseline 17-hydroxy-progesterone/androstendione ratio regardless of their androgen status (see Figure 1), that might reflect abnormalities of ovarian steroidogenesis typical for PCOS (Ehrmann *et al.* 1995, Rosenfield *et al.* 1993, White *et al.* 1995).

In summary, our study demonstrated virtually identical LH responses to GnRH stimulation in women with oligo-/amenorrhoea and polycystic ovarian morphology regardless of their androgen status, that were clearly different in comparison to regularly menstruating controls. There was also a marked increase in GnRH-stimulated LH/FSH ratio in comparison to controls. This suggests an identical hypothalamic/pituitary abnormality in women with PCOS, diagnosed according to the Rotterdam consensus criteria (2004), once other causes of oligo-/amenorrhoea and hyperandrogenism have been ruled out. In our opinion, the results of our study support the use of the "Rotterdam" PCOS criteria in clinical practice, while potential application of GnRH-stimulated LH/FSH ratio at least merits some consideration and further research.

REFERENCES

1 Azziz R, Carmina E, Dewailly D, Diamanti-Kandarakis E, Escobar-Morreale HF, Futterweit W, Janssen OE, Legro RS, Norman RJ, Taylor AE, Witchel SF (2006). Androgen Excess Society. Positions Statement: criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: an Androgen Excess Society guideline. J Clin Endocrinol Metab **91:** 4237–4245.

2 Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO (2004). The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab **89:** 2745–2749.

3 Balen AH, Conway GS, Kaltsas G, Techatrasak K, Manning PJ, West C, Jacobs HS (1995). Polycystic ovary syndrome: The spectrum of the disorder in 1741 patients. Hum Reprod **10:** 2705–2712.

4 Balen AH, Laven JS, Tan SL (2003). Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update **9:** 505–514.

5 Barnes RB, Rosenfield RL, Burstein S, Ehrmann DA (1989). Pituitary-ovarian responses to nafarelin testing in the polycystic ovary syndrome. New Engl J Med **320:** 559–565.

6 Barth JH, Yasmin E, Balen AH (2007). The diagnosis of polycystic ovary syndrome: the criteria are insufficiently robust for clinical research. Clin Endocrinol (Oxford) **67:** 811–815.

7 Blank SK, McCartney CR, Marshall JC (2006). The origins and sequelae of abnormal neuroendocrine function in polycystic ovary syndrome. Hum Reprod Update **12:** 351–361.

8 Boots LR, Potter S, Potter D, Azziz R (1998). Measurement of total serum testosterone levels using commercially available kits: high degree of between-kit variability. Fertility Steril **69:** 286–292.

9 Chhabra S, McCartney CR, Yoo RY, Eagleson CA, Chang RJ, Marshall JC (2005). Progesterone inhibition of the hypothalamic gonadotropin-releasing hormone pulse generator: evidence for varied effects in hyperandrogenemic adolescent girls. J Clin Endocrinol Metab **90:** 2810–2815.

10 Conway GS, Honour JW, Jacobs HS (1989). Heterogeneity of the polycystic ovary syndrome: clinical, endocrine and ultrasound features in 556 patients. Clin Endocrinol (Oxford) **30:** 459–470.

11 Ehrmann DA (2005). Polycystic ovary syndrome. New Engl J Med **352:** 1223–1236.

12 Ehrmann DA, Barnes RB, Rosenfield RL (1995) Polycystic ovary syndrome as a form of functional ovarian hyperandrogenism due to dysregulation of androgen secretion. Endocrine Rev **16:** 322–353.

13 Fears TR, Ziegler RG, Donaldson JL, Falk RT, Hoover RN, Stanczyk FZ, Vaught JB, Gail MH (2000). Reproducibility studies and interlaboratory concordance for androgen assays in female plasma. Cancer Epidemiol Biomarkers Preview **9:** 403–412.

14 Ferriman D, Gallwey JD (1961). Clinical assessment of body hair growth in women. J Clin Endocrinol Metab **21:** 1440–1447.

15 Ferris HA, Shupnik MA (2006). Mechanisms for pulsatile regulation of the gonadotropin subunit genes by GnRH1. Biol Reprod **74:** 993–998.

16 Jasonni VM, Bulletti C, Naldi S, Di Cosmo E, Cappuccini F, Flamigni C (1991). Treatment of hirsutism by an association of oral cyproproterone acetate and transdermal 17-β-estradiol. Fertil Steril **55:** 742–745.

17 Karasek M, Pawlikowski M, Lewinski A (2006). Hyperprolactinemia: causes, diagnosis, and treatment. Endokrynol Pol - Polish J Endocrinol **57:** 656–662.

18 Kazer RR, Kessel B, Yen SS (1987). LH pulse frequency in women with PCO. J Clin Endocrinol Metab **65:** 233–236.

19 Koskinen P, Penttilä TA, Anttila L, Erkkola R, Irjala K (1996). Optimal use of hormone determinations in the biochemical diagnosis of the polycystic ovary syndrome. Fertil Steril **65:** 517–522.

20 Lawson MA, Jain S, Sun S, Patel K, Malcolm PJ, Chang RJ (2008). Evidence for insulin suppression of baseline luteininising hormone in women with polycystic ovarian syndrome and normal women. J Clin Endocrinol Metab **93:** 2089–2096.

21 March WA, Moore VM, Willson KJ, Phillips DI, Norman RJ, Davies MJ (2010). The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. Hum Reprod **25:** 544–551.

22 Marshall JC, Dalkin AC, Haisenleder DJ, Paul SJ, Ortolano GA, Kelch RP (1991). Gonadotropin releasing hormone pulses: regulators of gonadotropin synthesis and ovolulatory cycles. Rec Prog Horm Res 1991 **47:** 155–187.

23 Marshall JC, Eagleson CA (1999). Neuroendocrine aspects of polycystic ovary syndrome. Endocrinol Metab Clin North Am **28:** 295–324.

24 Matsuda M, DeFronzo R (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing. Diabet Care **9:** 1462–1470.

25 Matthews DR, Hosker JP, Rudenski AS (1985). Homeostasis model assessment: insulin resistance and cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia **28:** 412–419.

26 Milewicz A, Silber D, Mielecki T (1983) The origin of androgen synthesis in polycystic ovary_syndrome. Obstetr Gynecol **62:** 601–614.

27 Neal-Perry G, Lebesgue D, Lederman M, Shu J, Zeevalk GD, Etgen AM (2009). The excitatory peptide kisspeptin restores the luteinizing hormone surge and modulates amino acid neurotransmission in the medial preoptic area of middle-aged rats. Endocrinology **150:** 3699–3708.

28 Nippoldt TB, Reame NE, Kelch RP, Marshall JC (1989). The roles of estradiol and progesterone in decreasing luteinizing hormone pulse frequency in the luteal phase of the menstrual cycle. J Clin Endocrinol Metab **69:** 67–76.
29 Pasquali R, Patton L, Pocognoli P, Cognigni GE, Gambineri A (2007). 17-Hydroxyprogesterone responses to gonadotropin-releasing hormone disclose phenotypes of functional ovarian hyperandrogenism and polycystic ovary syndrome. J Clin Endocrinol Metab **92:** 4208–4217.
30 Pastor CL, Griffin-Korf ML, Aloi JA, Evans WS, Marshall JC (1998). Polycystic ovary syndrome: Evidence for reduced sensitivity of the gonadotropin-releasing hormone pulse generator to inhibition by estradiol and progesterone. J Clin Endocrinol Metab **83:** 582–590.
31 Patel K, Coffler MS, Dahan MH, Malcom PJ, Deutsch R, Chang RJ (2004). Relationship of GnRH-stimulated LH release to episodic LH secretion and baseline endocrine-metabolic measures in women with polycystic ovary syndrome. Clin Endocrinol *(Oxford)* **60:** 67–74.
32 Rosenfield RL, Ehrmann DA, Barnes RB, Sheikh Z (1993). Gonadotropin-releasing hormone agonist as a probe for the pathogenesis and diagnosis of ovarian hyperandrogenism. Ann New York Acad Sci **687:** 162–181.
33 Stewart PM, Penn R, Holder R, Parton A, Ratcliffe JG, London DR (1993). The hypothalamo-pituitary-adrenal axis across the normal menstrual cycle and in polycystic ovary syndrome. Clin Endocrinol (Oxford) **38:** 387–391.
34 Taylor AE (1998). Polycystic ovary syndrome. Endocrinol Metabc Clin N Am **27:** 877–902.
35 Taylor AE, McCourt B, Martin KA, Anderson EJ, Adams JM, Schoenfeld D, Hall JE (1997). Determinants of abnormal gonadotrophin secretion in clinically defined women with polycystic ovary syndrome. J Clin Endocrinol Metab **82:** 2248–2256.
36 The Rotterdam ESHRE/ASRM-sponsored PCOS Consensus Workshop Group (2004). Revised 2003 consensus on diagnostic criteria and longterm health risks related to polycystic ovary syndrome (PCOS). Hum Reprod **19:** 41–47.
37 White D, Leigh A, Wilson C, Donaldson A, Franks S (1995). Gonadotrophin and gonadal steroid response to a single dose of a long-acting agonist of gonadotrophin-releasing hormone in ovulatory and anovulatory women with polycystic ovary syndrome. Clin Endocrinol (Oxford) **42:** 475–481.