

P R A C E K A Z U I S T Y C Z N E
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Spuriously high androstendione concentrations due to assay interference as a cause of diagnostic conundrum in women with oligomenorrhoea

Fałszywie wysokie wartości stężeń androstendionu spowodowane interferencją jako przyczyna trudności w diagnostyce różnicowej zaburzeń miesiączkowania

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Abstract

Polycystic ovary syndrome (PCOS) is a diagnosis of exclusion. We present two cases of women with oligomenorrhoea and high concentration of androstendione, suggestive of possible androgen-secreting tumour, caused by assay interference. The first patient, investigated for oligomenorrhoea, had no significant hirsutism or acne. Androstendione concentration was above 10.0 ng/ml (rr: 0.3-3.3 ng/ml). In order to rule out possible androgen-secreting tumour or hypercortisolaemia we performed 48-hour low dose dexamethasone suppression test (LDDST). This failed to demonstrate adequate suppression of androstendione (6.05 ng/ml and 9.32 ng/ml after the first and the second day, respectively). Pelvic ultrasound examination showed polycystic ovaries, while abdominal CT scan failed to show any ovarian or adrenal lesion. Despite such high androstendione concentrations, urinary steroid profile (gas chromatography/mass spectrometry method) yielded normal results. Hence a possibility of androstendione assay interference was raised.

The second patient was also admitted for investigations of oligomenorrhoea. Clinical examination was unremarkable. There was a high concentration of testosterone 0.78 ng/ml (rr: 0.084-0.481 ng/ml) and androstendione above 10.0 ng/ml (rr: 0.3-3.3 ng/ml). LDDST failed to demonstrate any suppression of androstendione, while recalculated concentrations of androstendione after serial dilutions were markedly lower in comparison to initial values. Therefore, such high androstendione concentrations (i.e. above the upper limit of the assay) must have resulted from assay interference. In both cases a final diagnosis of PCOS was established.

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Krzysztof C. Lewandowski et al. *Spuriously high androstendione concentrations due to assay interference as a cause of diagnostic conundrum in women with oligomenorrhoea.*

Conclusions: *In the absence of clinical features, contrasting with unusually high androgen levels, a possibility of androgen assay interference should be considered in differential diagnosis of hyperandrogenism or PCOS.*

Key words: **hyperandrogenism / androgens / androstendione / assay interference /**

Streszczenie

W diagnostyce różnicowej zespołu policystycznych jajników (PCOS) należy wykluczyć pozostałe przyczyny hiperandrogenizacji. W pracy przedstawiono przypadki dwóch kobiet z zaburzeniami miesiączkowania i wysokimi wartościami stężeń androstendionu, to jest w zakresie stężeń sugerujących możliwość obecności hormonalnie czynnego guza wydzielającego androgeny. W pierwszym przypadku u pacjentki bez klinicznych cech hiperandrogenizacji odnotowano stężenie androstendionu powyżej 10,0 ng/ml (norma: 0,3-3,3 ng/ml). W celu wykluczenia obecności guza hormonalnie czynnego przeprowadzono 48 godzinny test hamowania z deksametazonem (0,5 mg co sześć godzin), w którym nie stwierdzono zadowalającego spadku stężeń androstendionu (wartości 6,05 ng/ml oraz 9,32 ng/ml, odpowiednio po pierwszym i po drugim dniu testu). W wykonanym badaniu TK jamy brzusznej nie stwierdzono patologii nadnerczy, zaś w badaniu ultrasonograficznym opisano morfologię jajników policystycznych. W związku z powyższym zlecono oznaczenie dobowego profilu steroidowego moczu metodą chromatografii gazowej i spektrometrii masowej. W ocenie dobowego profilu steroidów nie odnotowano nieprawidłowości, co wskazuje na możliwość interferencji w oznaczeniu androstendionu.

Podobnie w drugim opisywanym przypadku wysokiemu stężeniu testosteronu (0,78 ng/ml przy normie: 0,084-0,481 ng/ml) i androstendionu powyżej 10,0 ng/ml (norma: 0,3-3,3 ng/ml) nie towarzyszyły kliniczne cechy hiperandrogenizacji. W przeprowadzonym 48-godzinym teście hamowania z deksametazonem nie stwierdzono spadku stężenia androstendionu. Dodatkowo wartości stężeń androstendionu uzyskane po rekalkulacji wartości uzyskanych po rozcieńczeniach były znikomo niższe od wartości wyjściowych, co pozwoliło na stwierdzenie interferencji w oznaczeniu androstendionu. W obu przypadkach postawiono ostateczne rozpoznanie zespołu policystycznych jajników.

Wnioski: *W diagnostyce różnicowej zespołu PCO w przypadku zaskakująco wysokiego stężenia androgenów, przy braku nasilonych objawów klinicznych, należy uwzględnić możliwość wystąpienia interferencji w oznaczaniu androgenów.*

Key words: **hiperandrogenizacja / androgeny / androstendion /
/ interferencje w oznaczaniu hormonów /**

Introduction

Though polycystic ovary syndrome (PCOS) is the most common cause of hyperandrogenism and/or oligomenorrhoea, diagnosis of PCOS according to current criteria implies that PCOS is essentially a diagnosis of exclusion [1]. In particular, other causes of clinical and/or biochemical hyperandrogenism and/or menstrual irregularities must be excluded [2, 3]. Proper exclusion of other causes of hyperandrogenism is based on assumption of reliability of measured androgen concentrations. These, i.e. testosterone, androstendione and dihydroepiandrosterone sulphate (DHEAS), are typically measured by immunoassays, as availability of tandem mass spectrometry method is still limited. It is, however, well recognised that immunoassays are prone to interference, including androgen assays [4, 5]. As clinician must be aware of possible interference in measurements of serum androgens, then, reliability of the obtained results must be always analysed in an individual clinical context. In our paper we present two cases of interference with androstendione and possibly testosterone assays, where very high concentrations, i.e. out of proportion to clinical presentation, precluded a straightforward diagnosis of PCOS and prompted us to perform several costly investigations in order to rule out an androgen secreting tumour.

Case 1

A 34 years old woman (BMI: 19.2 kg/m²) was admitted for investigations of oligomenorrhoea of several years duration. Clinical examination did not reveal any significant hirsutism or acne. She had normal follicular phase gonadotrophin and estradiol concentrations [FSH 6.93 IU/ml (reference range (rr): 2.8-11.3 IU/ml), LH 14.6 IU/ml (rr: 1.1-11.6 IU/ml) and oestradiol 42.90 pg/ml (rr: 0-160.0 pg/ml)]. There were normal concentrations of prolactin 10.12 ng/ml (rr: 3.9-25.4 ng/ml), DHEAS 208.6 ug/dl (rr: 98.8-340 ug/dl), 17-OH-progesterone 0.95 ng/ml (rr: 0.3-1.0 ng/ml), early morning cortisol 10.21 ug/dl (rr: 6.2-19.4 ug/dl), TSH 4.02 IU/l (rr: 0.27-4.2 mIU/l), free thyroxine (free T4) 1.29 ng/dl, (rr: 0.93-1.7 ng/dl) and free 3-iodothyronine (free T3) 3.35 pg/ml (rr: 2.6-4.4 pg/ml).

Testosterone concentration (0.71 ng/ml) was, however, significantly above the reference range (rr: 0.084-0.481 ng/ml), while androstendione was strikingly high and out of proportion to patient's clinical presentation, i.e. above 10 ng/ml (rr: 0.3-3.3 ng/ml), that is above the upper limit of the assay. The above mentioned androstendione measurements were performed with commercially available Immulite 2000 androstenedione kits (Siemens Healthcare Diagnostics Products Ltd), by using an automated chemiluminescence assay system (Immulite 2000XPI,

Krzysztof C. Lewandowski et al. *Spuriously high androstendione concentrations due to assay interference as a cause of diagnostic conundrum in women with oligomenorrhoea.*

Siemens, USA), with intra-assay CV (range %): 6.2%-15.1%, inter assay CV: 8.5%-17.8%, analytical sensitivity: 0.3 ng/ml and assay range: 0.3- 10ng/ml. Serum concentrations of FSH, LH, estradiol, prolactin, testosterone, DHEAS, cortisol, TSH, free T4 and free T3 were measured by electrochemiluminescence immunoassay (ECLIA) method using Elecsys - Cobas e 601 immunoassay analyzer, Roche Diagnostics GmbH Mannheim Germany. Concentrations of 17-OH progesterone were assessed by enzyme linked immunosorbent assay (ELISA) method based on the principle of competitive binding, using commercially available 17-OH Progesterone ELISA kits (IBL International).

In order to rule out possible androgen-secreting tumour, we performed a 48-hour dexamethasone suppression test (0.5 mg every 6-hours). This failed to demonstrate suppression of androstendione (6.05 ng/ml after one day and 9.32 ng/ml after two days) (see Table I), where criteria for adequate suppression of androgens during 48hr dexamethasone suppression test are described by Kaltsas et al. 2002 [6]. Pelvic ultrasound examination showed polycystic ovaries, while abdominal CT scan failed to show any ovarian or adrenal lesion. A possibility of androstendione assay interference was therefore raised. Given the lack of clinical features consistent with such high androstendione concentrations, we assessed urinary steroid profile by the means of gas chromatography/mass spectrometry method. This yielded normal results.

We concluded that such high androstendione concentrations, out of proportion to clinical presentation, must have resulted from assay interference. Unfortunately, a direct test for possible heterophilic antibodies was not possible in our Department. A final diagnosis of PCOS was established.

Case 2

A 21 years old woman (BMI: 20.1 kg/m²) was admitted for investigations of oligomenorrhoea, persisting since menarche (at the age of 14). Clinical examination was unremarkable, and in particular there was no evident hirsutism according to Ferriman-Gallwey score. Initial hormonal tests revealed: FSH 5.62 IU/l (rr: 2.8-11.3 IU/ml), LH 27.67 U/l (rr: 1.1-11.6 IU/ml), oestradiol 127.8 pg/ml (rr: 0-160.0 pg/ml). The above described hormonal profile corresponded with luteal phase of menstrual cycle. Concentration of 17-OH-progesterone was also consistent with luteal phase (2.38 ng/ml (rr: 0.2-2.9 ng/ml)). Further investigations revealed normal concentrations of prolactin [13.01 ng/ml (rr: 3.9-25.4 ng/ml)], DHEAS 239.1 µg/dl (rr: 148-407 µg/dl), early morning cortisol 25.44 µg/dl, TSH (0.6 mIU/l), as well as free T4 and free T3.

Serum testosterone and androstendione concentrations were, however, significantly above the reference range, i.e. testosterone 0.78 ng/ml, (rr: 0.084-0.481 ng/ml) and androstendione above 10.0 ng/ml (rr: 0.3-3.3 ng/ml). Both testosterone and androstendione concentrations were therefore strikingly high and out of proportion to patient's clinical presentation. In order to rule out possible androgen-secreting tumour or hypercortisolaemia we performed 48-hour dexamethasone suppression test (0.5 mg every 6-hours). This failed to demonstrate any suppression of androstendione (concentrations above 10 ng/ml either after one and two days) (see Table II). The pelvic ultrasound examination showed polycystic ovaries, while abdominal ultrasounds examination failed to demonstrate any adrenal lesion. A possibility

of androstendione assay interference was raised. Given the lack of clinical features consistent with such high androstendione concentrations we assessed the concentration of androstendione after 1:4 and 1:16 dilutions (Table II). Recalculated concentrations of androstendione were still raised but significantly lower than predilution concentrations (Table II). Thus, we concluded that such high androstendione concentrations (i.e. above the upper limit of the assay) must have resulted assay interference. A final diagnosis of PCOS was established.

Discussion

Polycystic ovary syndrome is the most common endocrinopathy in women in reproductive age and is the principal cause of hyperandrogenism. According to the Rotterdam criteria, one can recognize PCOS when a patient fulfils two out of three criteria, i.e. oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovaries, however, only on condition that other causes of oligo-/amenorrhoea and hyperandrogenism have been excluded [1,2,3,7,8]. These include disorders of androgen excess, such as like non-classical congenital adrenal hyperplasia, Cushing's syndrome, hyperprolactinaemia, acromegaly, syndromes of severe insulin resistance, side effects of certain medications [2, 3]. The last but not the least, androgen-secreting neoplasms must be also excluded [2, 3, 9]. The principal hormonal evaluation in our patients included serum androgens (testosterone, androstendione, dehydroepiandrosterone sulfate), prolactin, thyroid function tests and 17-hydroxyprogesterone levels. In both patients we observed very high concentrations of androstendione (i.e. above the upper limit of the assay) as well as raised concentrations of testosterone, that prompted us to perform investigations in order to rule out androgen-secreting tumour.

Our first patient had history of oligomenorrhoea but did not have any significant hirsutism, acne or clitoromegaly, defined as a clitoral diameter of greater than 4 mm [10]. Very high androgen concentrations, and androstendione in particular, prompted us to undertake tests in order to rule out an androgen-secreting tumours. Though these are rare, for instance constitute only 0.2% cases of hyperandrogenism according to Azziz et al., they are of paramount clinical importance, while a diagnosis of PCOS cannot be established, if there is a possibility of an androgen-secreting tumour [11]. In order to rule out possible androgen-secreting tumour or hypercortisolaemia we performed 48-hour low dose dexamethasone suppression test (LDDST), where first blood samples are collected in the morning, then dexamethasone is administered at a dose of 0.5 mg every six hours for 48 hours according to the standard protocol [12]. According to Kaltsas et al., this test could be also applied to investigation of possible androgen-secreting tumours [6]. Adequate androgen suppression (i.e. above 50% of initial values or within the reference range) in response to glucocorticoid administration effectively excludes androgen secreting adrenal or ovarian tumour. In particular, all patients who harboured an androgen-secreting tumour failed to achieve normalization of elevated androgen levels during LDDST or had a greater than 40% reduction from baseline [6]. With respect to the above criteria, the LDDST was associated with 100% sensitivity and 88% specificity in identifying patients with androgen-secreting tumours [6].

In our first patient we observed satisfactory suppression of cortisol [i.e. below 1.8 µg/dl (50 nmol/l)] and DHEAS but

Krzysztof C. Lewandowski et al. *Spuriously high androstendione concentrations due to assay interference as a cause of diagnostic conundrum in women with oligomenorrhoea.*

Table I. Androgen and cortisol concentrations during low dose dexamethasone suppression test (0.5 mg every six hours for 48 hours) in patient no. 1.

	Before the test	Day 1	Day 2	Reference ranges
Cortisol	12.87	0.47	0.26	6.2–19.4 µg/dl
Testosterone	0.68	0.41	0.68	0.084-0.481 ng/ml
DHEAS	147.9	65.57	53.69	60.9-337 µg/dl
Androstendione	>10.0 (12.7*)	6.05	9.32	0.3-3.3 ng/ml

*Concentration recalculated after 1:4 dilutions (4 x 3.175 ng/ml=12.7)

Table II. Androgen and cortisol concentrations during low dose dexamethasone suppression test (0.5 mg every six hours for 48 hours) – patient no. 2.

	Before the test	Day 1	Day 2	Reference ranges
Cortisol	25.44	0.70	0.58	6.2–19.4 µg/dl
Testosterone	0.78	0.53	0.56	0.084-0.481 ng/ml
DHEAS	239.0	83.67	53.55	60.9-337 µg/dl
Androstendione	>10.0*	>10.0**	>10.0***	0.3-3.3 ng/ml
Androstendione dilution 1:4	1.73 (Recalculated concentration 4x1.73=6.92)	1.08 (Recalculated concentration 4x1.08=4.32)	1.29 (Recalculated concentration 4x1.29=5.16)	
Androstendione dilution 1:16		0.31 (Recalculated concentration 16x0.31=4.96)	0.342 (Recalculated concentration 16x0.342=5.47)	

we did not observe adequate suppression of testosterone and androstendione (0.68 ng/ml compared to 0.68 ng/ml after LDDST) and androstendione (12.7 ng/ml compared to 9.32 ng/ml after LDDST). The results of the test, therefore, allowed us to rule out Cushing's syndrome, but suggested a possibility of an androgen secreting tumour. Pelvic ultrasonography and abdominal computed tomography imaging were - therefore - performed. The ultrasound scan showed polycystic ovaries, while abdominal CT scan failed to show any ovarian or adrenal lesion. Given the patient clinical presentation, and in particular an absence of obvious signs of hyperandrogenism or virilisation, a possibility of androstendione and testosterone assay interference was raised. Thus, we assessed a urinary steroid profile by the means of gas chromatography/mass spectrometry method. Gas chromatography–mass spectrometry (GC-MS) enables to precisely identify various substances within a test sample, produced during a ionisation process, according to their mass-to-charge-ratio. Mass spectrometry-based methods are currently the most specific quantitative analytical methods for steroids determination [13]. In contrast to immunoassays, this method is independent of matrix effects or cross-reactivity [13]. Taking all this into account, an analysis of urinary steroid metabolite excretion was performed according to the method described by Arlt et al. and Ambroziak et al., i.e. by a quantitative gas chromatography/mass spectrometry (GC-MS) selected ion-monitoring method [14, 15]. Steroids quantified according to this method include corticosterone metabolites [tetrahydrocorticosterone (THB), 5αTHB, tetrahydro-11-dehydrocorticosterone (THA), and 5αTHA], the progesterone metabolite pregnanediol (PD), 17OHP

metabolites [pregnanetriol (PT), and 17-hydroxypregnanolone (17HP)], the 17-hydroxypregnenolone metabolite pregnenetriol (5-PT), the 21-desoxycortisol metabolite pregnanetriolone (P'TONE), cortisol metabolites [tetrahydrocortisol (THF), 5αTHF, tetrahydrocortisone (THE)], and androgen metabolites [androsterone, etiocholanolone, dehydroepiandrosterone (DHEA), and 16-hydroxy-DHEA]. Despite very high androstendione concentrations (i.e. above the upper limit of the immunoassay), all steroids metabolites were within normal range when measured by GC-MS method, while in case of such very high androstendione concentrations one might expect an increase in androstendione metabolites, i.e. androsterone, epitestosterone and etiocholanolone. Hence, we concluded that such high androstendione concentrations must have been caused by assay interference.

In the second presented case, again there was an absence of significant clinical features or hyperandrogenism or virilisation, contrasting with high testosterone, and particularly very high androstendione concentrations. There was also an inadequate suppression of testosterone and androstendione concentrations during LDDST. In this case, however, we performed serial dilutions of androstendione sample, where recalculated androstendione concentrations were significantly lower, i.e. there was very poor recovery of initial androstendione concentrations. Such result, in our opinion, confirmed an interference in androgen assay.

There are several possible causes of interference with androgen assays. For instance, the presence of heterophilic antibodies can cause interference with several immunoassays,

Krzysztof C. Lewandowski et al. *Spuriously high androstendione concentrations due to assay interference as a cause of diagnostic conundrum in women with oligomenorrhoea.*

including androgen assays [16]. Unfortunately, in our Department, we had no access to blocking antibodies in order to confirm or refute this hypothesis (i.e. to measure androstendione and testosterone concentrations before and after addition of blocking antibodies). Thus, though a possibility of heterophilic antibody interference is highly likely, it still remains speculative. It is also recognised that cross-reactivity with other metabolites can disturb the androgen evaluation. The most common metabolite which might cause interference is spironolactone. Namely, treatment with 100-200 mg/d of spironolactone was associated with spuriously high androstendione concentrations measured by an immunoassay [17]. Also norethisterone has recently been reported to cause interference in androgen assays [18]. However, our patients were not receiving spironolactone or norethisterone, though obviously we cannot fully exclude a possibility of interference by other steroid metabolites. It should be also noted that our second patient (aged 21) had already passed her adolescent age, so standard criteria for diagnosis of PCOS could be applied [19].

Conclusions

In the absence of clinical features, contrasting with unusually high androgen levels, a possibility of androgen assay interference should be considered in differential diagnosis of hyperandrogenism and/or PCOS. Such interference may prompt costly, and eventually unnecessary, investigations in order to rule out and androgen-secreting tumour. It is also a reminder that grossly abnormal results of laboratory investigations must not be interpreted outside a clinical context.

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