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Measuring salivary androgens as a useful tool in the diagnosis of polycystic ovary syndrome

Pomiar androgenów w ślinie jako przydatne narzędzie w diagnostyce zespołu policystycznych jajników

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is one of the commonest endocrinopathies. Clinically it can present as oligo-/amenorrhoea, hyperandrogenism and/or fertility problems.

Material and methods: The study involved 60 women admitted to the Department of Internal Medicine and Endocrinology at the Medical University of Warsaw. The initial evaluation, including case history and two-dimensional vaginal ultrasound, was performed by gynaecologists. All hormonal investigations (fT, free testosterone; bioT, bioavailable testosterone; T, total testosterone; T EQ, free testosterone by equilibrium dialysis; A, androstenedione; A EQ, free androstenedione by equilibrium dialysis; salA, salivary androstenedione; salT, salivary testosterone) were performed. Anthropometrical data, excess facial and body hair, acne, and menstrual cycle frequency were also assessed.

Results: Increased levels of T, fT, T EQ and A were noted in 20.0%, 89.8%, 100% and 28.3% of women, respectively. A very high correlation was found between salivary androstenedione and free androstenedione estimated by EQ in plasma ($p < 0.05$, $r = 0.67$), and total androstenedione in plasma ($p < 0.05$, $r = 0.71$). Correlation between salT and T was $r = 0.31$, $p < 0.05$ and salT and T EQ was $r = 0.26$, $p = 0.04$. Correlation between salA/salT and T, A in plasma (respective values $r = 0.39$ and $r = 0.28$, $p < 0.01$) and between salA/salT and A EQ, T EQ (respectively $r = 0.34$ and $r = 0.48$, $p < 0.01$) was evident.

Conclusions: SalA/salT ratio may be a good indicator of hyperandrogenism in women. We also confirm that measurement of androstenedione in plasma may be useful in making a diagnosis of PCOS. **(Pol J Endocrinol 2012; 63 (3): 183–190)**

Key words: *polycystic ovary syndrome, salivary androgens, hyperandrogenism, anovulation*

Streszczenie

Wstęp: Zespół policystycznych jajników (PCOS) jest jedną z najczęstszych endokrynopatii. Charakteryzuje się zaburzeniami miesiączkowania o charakterze rzadkich miesiączek, niepłodnością, hiperandrogenizmem.

Materiał i metody: Do badania włączono 60 kobiet hospitalizowanych w Klinice Chorób Wewnętrznych i Endokrynologii Warszawskiego Uniwersytetu Medycznego. Przeprowadzono badanie podmiotowe i przedmiotowe, łącznie z badaniem ultrasonograficznym narządu rodnego. Analizie poddano panel badań hormonalnych (fT, wolny testosteron; bioT, testosteron biodostępny; T, całkowity testosteron; T EQ, wolny testosteron wyznaczony metodą dializy równowagowej; A, androstendion; A EQ, wolny androstendion wyznaczony metodą dializy równowagowej; salA, androstendion w ślinie; salT, testosteron w ślinie) wykonanych w godzinach porannych, między 3. a 6. dniem cyklu. Oceniano parametry antropometryczne, stopień hirsutyzmu oraz charakter cyklów miesiączkowych.

Wyniki: Podwyższone stężenie androgenów: T, fT, T EQ i A odnotowano u 20,0, 89,8, 100,0 i 28,3% badanych kobiet. Odnotowano wysoką korelację między salA i A EQ ($p < 0,05$, $r = 0,67$) i A ($p < 0,05$, $r = 0,71$). Korelacja między salT i T wynosiła $r = 0,31$, $p < 0,05$ oraz salT i T EQ była równa $r = 0,26$, $p = 0,04$. Wykazano zależność pomiędzy salA/salT oraz T, A (odpowiednio $r = 0,39$ i $r = 0,28$, $p<0,01$) oraz między salA/salT i A EQ, T EQ (odpowiednio $r = 0,34$ i $r = 0,48$, $p < 0,01$).

Wnioski: Wydaje się, że oznaczanie wskaźnika salA/salT może być dobrym wyznacznikiem hiperandrogenizacji u kobiet. Ocena stężenia androstendionu w surowicy u kobiet może być pomocna w diagnostyce zespołu hiperandrogenizacji u kobiet. **(Endokrynol Pol 2012; 63 (3): 183–190)**

Słowa kluczowe: *zespół policystycznych jajników, androgeny w ślinie, hiperandrogenizm, zaburzenia miesiączkowania*

Introduction

Polycystic ovary syndrome (PCOS) is one of the commonest endocrinopathies, affecting about 4–7% of women of reproductive age [1, 2]. Clinically it is presented as oligo-/amenorrhoea, clinical and/or laboratory hyperandrogenism, and/or fertility problems [1–3]. According to the ESHRE/ASMR consensus, PCOS can be diagnosed when a woman fulfills at least two out of three criteria: oligo/anovulation, clinical or biochemical

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hyperandrogenism, and/or polycystic ovaries found by ultrasound imaging, after exclusion of other causes of menstrual irregularities and hyperandrogenaemia [4]. Hyperandrogenism is manifested by hirsutism, persistent acne, alopecia and biochemical abnormalities, such as increased levels of total (T) and free testosterone (fT) [5–7]. Hyperandrogenaemia in PCOS can be assessed in various ways. Commonly, this is done by measuring the level of T and/or fT and sex hormone binding globulin (SHBG) and calculating the bioavailable fraction using free androgen index (FAI) or mass equation [8, 9]. Synthesised in the adrenal cortex and ovarian theca cells, androstenedione (A) may be also used to diagnose hyperandrogenaemia. However, only a few prospective studies on androstenedione in PCOS are available [10]. Dehydroepiandrosterone (DHEA) derived from the adrenal glands, has limited diagnostic value. It requires accurate and sensitive assay which cannot be achieved because of its diurnal variations and technological difficulties [11]. Nevertheless, DHEA sulfate (DHEAS) has been widely used, mainly as a marker for adrenal androgen excess, because it is almost exclusively synthesised in the adrenal cortex. Additionally, its level is stable throughout the day and the menstrual cycle, making it easily measured [12]. Peripheral androgen synthesis may also contribute to hyperandrogenism in PCOS [13, 14]. Testosterone level is important in the diagnosis and management of a number of diseases or syndromes in females, including precocious puberty, androgen-secreting tumours, and PCOS [15]. However, there are several limitations to their use. Most measurements based on immunoassays are not designed or validated for the relatively low levels normally presented in women [9]. Moreover, the range considered as normal, not changing with age, is broad and includes also hyperandrogenic women, even those with severe hirsutism [16].

All these factors raise questions about the diagnostic value of testosterone level measurement in women with hyperandrogenism [16]. In 20–40% of patients with PCOS, serum analysis fails to identify biochemical hyperandrogenism [17]. Most testosterone assays show poor sensitivity and accuracy in the female ranges, which, even when elevated under conditions such as PCOS, still tend to be well below normal male ranges [18]. Additionally, circulating levels in females vary according to reproductive maturity, phase of the menstrual cycle, time of day, and feeding [19]. There are no age and gender normal ranges, and in fact there is no testosterone standard on which an assay could be based [20]. Other steroids with similar configurations may cross-react with the antibody used in immunoassay. Methods that improve the performance of testosterone immunoassays, such as extraction of testosterone and chromatographic purification before the assay, are not supposed to be used in a commercial setting. From the other side, simpler immunoassay methods such as direct RIA or ELISA are criticised because of poor accuracy in the female range or the overestimation of results [19]. Measurement of total testosterone by mass spectrometry has been recommended as an alternative to avoid the above mentioned problems [21]. Unfortunately, the method is slower and more expensive than RIA. Salivary testosterone represents the unbound serum fraction, therefore levels are much lower than those in serum [22].

The aim of this study was to determine the usefulness of salivary androgens as a new diagnostic tool and to evaluate their applicability for the diagnosis of women with PCOS.

Material and methods

The study involved 60 women admitted to the Department of Internal Medicine and Endocrinology at the Medical University of Warsaw. All women, characterised in Table I, included in the study fulfilled the Rotterdam criteria [4]. The exclusion criteria included: hyperprolactinaemia, congenital adrenal hyperplasia, thyroid disease, other causes of amenorrhea such as premature ovarian failure, Cushing's syndrome, and androgen-secreting tumour. No subject was on study medication at the time of assay. The protocol was approved by the local ethics committee, and written informed consent was obtained from each patient. The initial evaluation, including case history and two-dimensional vaginal ultrasound, was performed by gynaecologists. All hormonal investigations were performed after an overnight fast, 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 ten-day administration of dydrogesterone (Duphaston®). Blood samples for endocrine measurements were obtained between 7.30 am and 8.30 am and stored

Table I. *Anthropometric data in PCOS women*
Tabela I. *Dane antropometryczne badanych kobiet z PCOS*

Anthropometric data	Mean ± SD (n = 60)
Age [years]	28.69 ± 5.23
BMI [kg/m^2]	25.04 ± 6.78
WHR	0.80 ± 0.06
SBP [mm Hg]	117.12 ± 12.12
DBP [mm Hg]	73.62 ± 8.97

BMI — body mass index; WHR — waist to hip ratio; SBP — systolic blood pressure; DBP — diastolic blood pressure; SD — standard deviation

at –80 °C until assayed. Age, height, body weight, body mass index (BMI), excess facial and body hair, acne, and menstrual cycle frequency were also assessed. BMI was calculated as weight divided by height squared. Amenorrhea was defined as absent menstrual bleeding in the past 90 days. Oligomenorrhea was defined as more than 35 days between cycles with fewer than eight menstrual periods in the past year. Hirsutism was assessed according to the Ferriman-Galwey scale [23], where a score above eight was considered significant. Diagnosis of polycystic ovaries using pelvic ultrasound examinations was based on the presence of either 12 or more follicles measuring 2–9 mm in diameter, or increased ovarian volume (> 10 cm^3) [24]. fT and bioT were carried out using the formula available on the website of the International Society for the Study of the Ageing Male (ISSAM) (http://www.issam.ch/freetesto.htm) from concentrations of T, SHBG and albumin values measured in the same sample from each woman. This method is described in detail by Vermeulen et al. [25]. Free testosterone and free androstenedione levels in serum were measured by an equilibrium dialysis (T EQ, A EQ). Equilibrium dialysis was carried out to assess free testosterone and free androstenedione fraction using a 96-well Equilibrium Dialyser, Membrane MWCO 5 kDa (Harvard Apparatus, USA) [26]. Radioactive testosterone (PerkinElmer, Life and Analytical Sciences, USA) or androstenedione (American Radiolabelled Chemicals, Inc, USA) respectively was used as a tracer. Tracers were thin-layer purified (HPTLC-Alu Silica Gel 60) according to the manufacturer's instruction using toluene:ethyl acetate 2:1, and used no longer than one month after purification. Serum was diluted 1:1 with 0.9% saline solution, with 30 kBq/ml of tracer added. Samples were incubated for 30 min at 37 °C with agitation. Immediately, 290 microlitres of sample was transferred to the appropriate compartment of the dialyser with opposite cell filled with 290 microlitres of saline solution. Equilibrium was reached during dialysis within 20 hours at 37 °C with gentle agitation. Subsequently, radioactivity of samples from each compartment was measured. Free Androgen Index (FAI) was measured as a ratio of the total testosterone concentration divided by the SHBG level. Endocrine investigations involved luteinising hormone (LH), follicle-stimulating hormone (FSH), oestradiol, total testosterone (T), DHEAS, thyrotrophin-stimulating hormone (TSH), insulin, and SHBG being measured by electrochemiluminescence immunoassays (Roche Diagnostics, Germany) using an Elecsys 2010 analyser (Hitachi, Japan). Androstenedione (A) and 17-OH-progesterone (17-OH-P) concentrations were measured by chemiluminescence immunoassays (Siemens Healthcare Diagnostics Products Ltd, UK) using Immulite 2000. Salivary testosterone (sal T) and androstenedione (sal A) were measured using enzyme immunoassay kits (Salimetrics, USA).

All statistical analyses were performed using Statistica 9.0 software. Data was presented as mean and standard deviation (± SD). Correlations between androgens in women with PCOS were tested by analysis of covariance (ANOVA test). Depending on distribution characteristics of the analysed parameters, we employed either a *t*-test in cases of normal distribution or a Mann-Whitney test if distribution characteristics were not normal. Correlation analysis was performed by means of the Pearson rank correlation method. Statistical significance was assumed for $p < 0.05$.

Results

Androgen concentrations in PCOS women are shown in Table II. Increased levels of T, fT, T EQ, A and DHEA-S were noted in 20.0%, 89.8%, 100%, 28.3% and 30.4% of women, respectively. More than 27% of women (n = 16) showed an elevated (i.e. over 7) concentration of FAI. The mean level of SHBG was 47.32 nmol/l. In more than 80% of women (n = 49), SHBG concentration was close to the down range. Figure 1 shows different concentrations of free androgens measured or calculated in women with PCOS by using different

Table II. *Hormonal characteristics of women with PCOS*
Tabela II. *Charakterystyka hormonalna badanych kobiet z PCOS*

Hormonal tests	Mean value ± SD, n = 60
T [ng/mL]	0.66 ± 0.32
sal T [pg/mL]	90.81 ± 25.71
fT [pg/mL]	10.34 ± 6.63
fT [%]	1.57 ± 0.52
FAI	6.6 ± 5.97
bioT [pg/mL]	258.47 ± 165.42
bioT [%]	39.23 ± 12.75
T EQ [pg/mL]	42.7 ± 27.09
T EQ [%]	6.19 ± 1.50
DHEA-S [μg/dL]	312.38 ± 128.4
A [ng/mL]	4.34 ± 1.85
sal A [pg/mL]	391.91 ± 173.29
A EQ [pg/mL]	1,069.64 ± 506.3
A EQ [%]	24.66 ± 6.74

T — total testosterone; salT — salivary testosterone; fT — free testosterone; FAI — free androgen index; bioT — bioactive testosterone; T EQ — free testosterone by equilibrium dialysis; DHEA-S — dehydroepiandrosterone sulfate; A — androstendione; salA — salivary androstendione; A EQ — free androstenedione by equilibrium dialysis; SD — standard deviation; n — number of patients

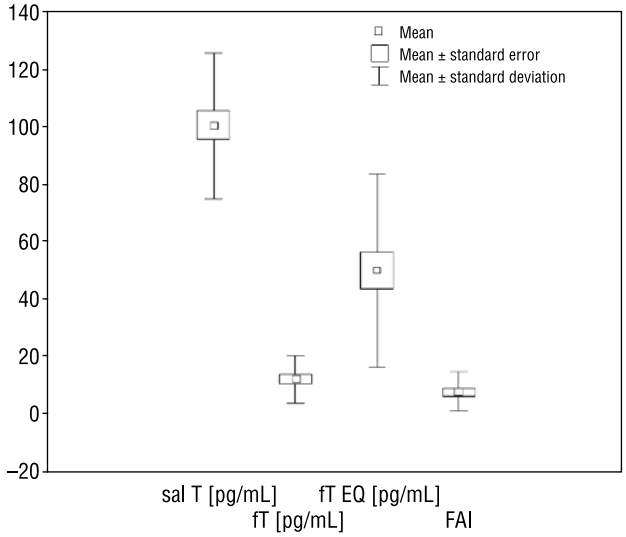

Figure 1. *Comparison of androgen level measured/calculated with different methods in PCOS women; salT — salivary testosterone; fT — free testosterone calculated; fT EQ — free testosterone by equilibrium dialysis; FAI — free androgen index*

Rycina 1. *Porównanie androgenemii ocenianej różnymi metodami u kobiet z PCOS*

diagnostic tools. The level of free testosterone calculated was similar to FAI; however, it was about 5-6-fold lower than salT, and 4-fold lower than T EQ. The very high correlation between salivary androstenedione and free androstenedione estimated by EQ in plasma ($p < 0.05$, $r = 0.67$), and total androstenedione in plasma ($p < 0.05$, $r = 0.71$) is shown in Figure 2. Figure 3 shows the correlation between salT and T which is $r = 0.31$, $p < 0.01$ and between salT and T EQ which is $r = 0.26$, $p = 0.04$. A correlation between T and T EQ, and A and A EQ was evident: $p < 0.01$, $r = 0.88$, $p < 0.01$, $r = 0.85$, respectively (Figure 4). Figure 5 shows a correlation between salA/salT and total androgens T, A in plasma (respective values $r = 0.39$ and $r = 0.28$, $p < 0.01$). Finally, Figure 6 shows a correlation between salA/salT and A EQ, T EQ, which was respectively $r = 0.34$ and $r = 0.48$, $p < 0.01$.

Discussion

Testosterone circulates in blood mostly (98%) as bound to serum proteins, primarily to SHBG and albumin. Only 1–2% of serum testosterone is free of bounded proteins binding [27]. Because SHBG binds T with high affinity, the dissociation is very long, so SHBG-bound T is not thought to be available for dissociation into target tissues for action via classical androgen receptor mechanisms [28]. In contrast, albumin binds T with low affinity, and its dissociation is rapid [29]. The combination of weakly albumin-bounded T and free T is referred to as bioavailable or non-SHBG-bound T available for target tissues to enable androgen action. Using the free T by equilibrium dialysis as a gold standard, previous studies have evaluated the accuracy of other free and bioavailable T assays in men, but only to a limited extent in women [30–33]. Free T concentration in women is approximately 10- or 20-fold lower than T. Because oestrogens increase SHBG concentrations and may bind to SHBG with high affinity, SHBG level in women

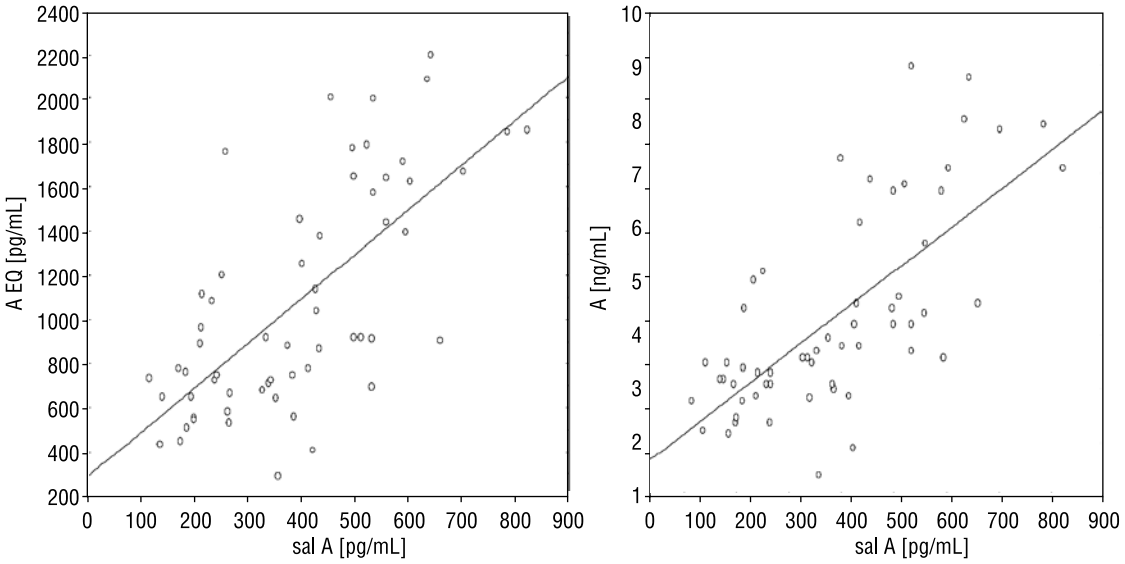

Figure 2. *Pearson's correlation between salA (pg/mL) and A EQ (pg/mL), $p < 0.05$, $r = 0.67$ and salA (pg/mL) and A (ng/mL), $p < 0.05$, $r = 0.71$; salA — salivary androstendione; A EQ — free androstenedione by equilibrium dialysis; A — androstendione*

Rycina 2. *Ocena zależności między salA (pg/mL) a A EQ (pg/mL), $p < 0,05$, $r = 0,67$ i salA (pg/mL) a A (ng/mL), $p < 0,05$, $r = 0,71$ w korelacji Pearsona*

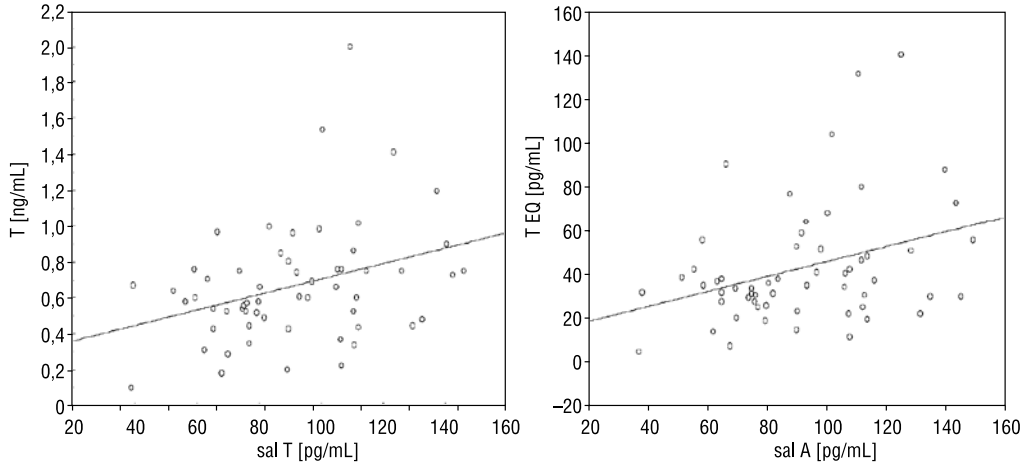

Figure 3. *Pearson's correlation between salT (pg/mL) and T (ng/mL), p = 0.01, r = 0.31 and salT (pg/mL) and T EQ (pg/mL), r = 0.26, p = 0.04; T — total testosterone; salT — salivary testosterone; fT EQ — free testosterone by equilibrium dialysis*

Rycina 3. *Ocena zależności pomiędzy salT (pg/mL) a T (ng/mL), p = 0,01 r = 0,31 i salT (pg/mL) a T EQ (pg/mL), r = 0,26, p = 0,04 w korelacji Pearsona*

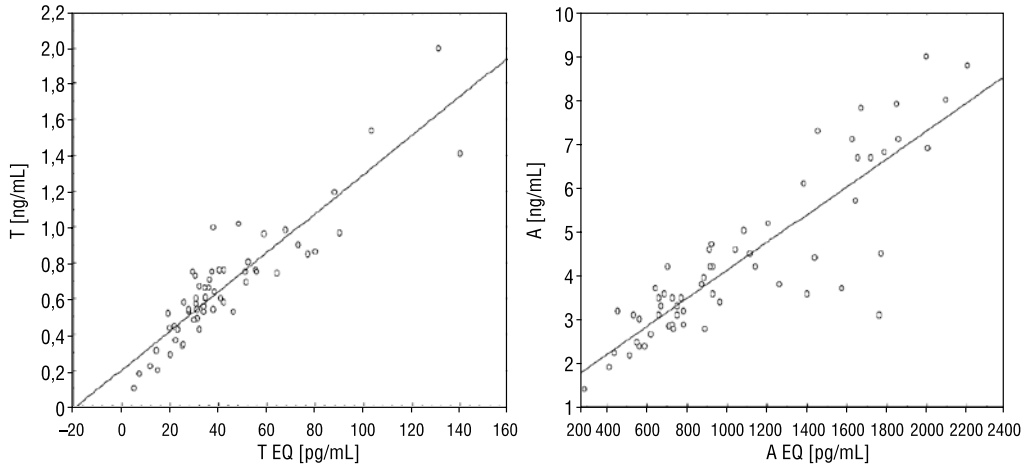

Figure 4. *Pearson's correlation between T (ng/mL) and T EQ (pg/mL), p < 0.01, r = 0.88 and A (ng/mL) and A EQ (pg/mL), p < 0.01, r = 0.85; T EQ — free testosterone by equilibrium dialysis; T — total testosterone; A EQ — free androstenedione by equilibrium dialysis; A — androstendione*

Rycina 4. *Ocena zależności między T (ng/mL) a T EQ (pg/mL), p < 0,01, r = 0,88 i A (ng/mL) a A EQ (pg/mL), p < 0,01, r = 0,85 w korelacji Pearsona*

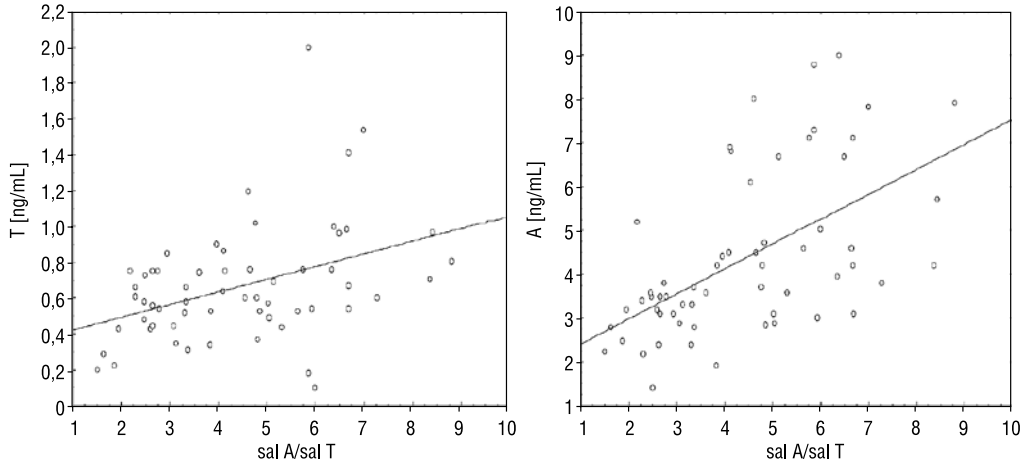

Figure 5. *Pearson's correlation between salA/salT and T (ng/mL), p < 0.01, r = 0.39 and salA/salT and A (ng/mL), p < 0.01, r = 0.28; T — total testosterone; salT — salivary testosterone; salA — salivary androstendione; A — androstendione*

Rycina 5. *Ocena zależności między salA/salT a T (ng/mL), p < 0,01, r = 0,39 i salA/salT a A (ng/mL), p < 0,01, r = 0,28 w korelacji Pearsona*

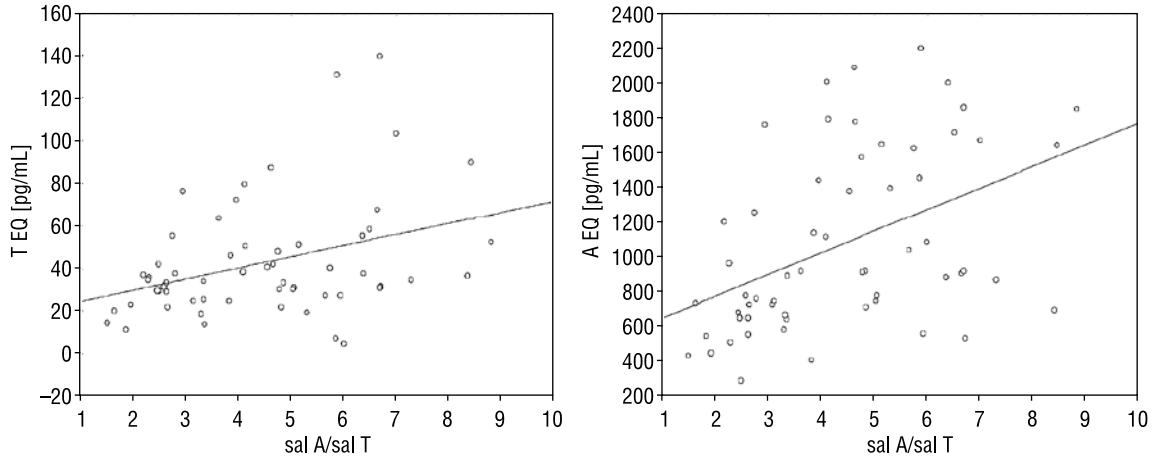

Figure 6. *Pearson's correlation between salA/salT and T EQ (pg/mL), p < 0.01, r = 0.34 and salA/salT and A EQ (pg/mL), p < 0.01, r = 0.48; T EQ — free testosterone by equilibrium dialysis; salT — salivary testosterone; salA – salivary androstendione; A EQ — free androstenedione by equilibrium dialysis*

Rycina 6. *Ocena zależności między salA/salT a T EQ (pg/ml), p < 0,01 r = 0,34 i salA/salT a A EQ (pg/ml), p < 0,01, r = 0,48 w korelacji Pearsona*

is highly variable and affects measurements of total T. Calculated free T values were nearly identical to those measured by equilibrium dialysis in PCOS women. However, the level of calculated fT strongly depends on the specific total T and SHBG assays used. Most assays for T are immunobased and were not designed or validated for the relatively low levels normally present in women [9]. Serum analysis fails to identify biochemical hyperandrogenism in 20–40% of patients with PCOS [17]. Every investigator should remember that samples for the estimation of plasma levels testosterone should be carried out in the morning, before 9 am, in order to obviate the effect of the diurnal variation in testosterone production. Salivary testosterone offers a non-invasive estimation of free testosterone concentration, but there does not appear to be an immediate demand for a routine salivary testosterone service.

It is important to note that free T measured by equilibrium dialysis requires a sensitive, specific, precise and accurate assay for total T. Recent studies have shown that the current methods of measurement of total T are not sensitive for samples with very low T concentrations, such as occur in women, testosterone-deficient men, or children. However, sensitivity has been improved by new methods based on mass spectrometry [34–36]. Furthermore, the free T analogue–based assay should not be used in practice because of poorly assessed and quality controlled hormone assays [37, 38].

That is why scientists, physicians, investigators, clinical chemists, pathologists and editors of medical journals insist on better surveillance of assays used in research and patient care. Many women with PCOS were not hyperandrogenic at baseline in total testosterone assay report. This may be due to a variety of factors. Testosterone assays of varying and lesser quality may lead to overestimation of the number of normoandrogenic women. Our study did not include any healthy women, so it is difficult to define sensitivities and specificities of specific testosterone levels to make a diagnosis of PCOS. Similarly, a multicentre representative cohort of normal women would have definitely strengthened the study. We therefore examined the correlation between different ways of measuring the levels of androgens.

The question is why the amount of testosterone in saliva is higher than free level in plasma, in contrast to androstenedione. We know that the submandibular and parotid glands metabolise the steroids. Intrinsic 17-ketooxidoreductase activity in the salivary gland has already been demonstrated *in vitro* in several species [39–42]. The main metabolites are 20-alpha-hydroxy-4-pregnen-3-one for progesterone and androstenedione for testosterone. This is why the progesterone and testosterone levels in saliva may not be identical with the unbound steroid fraction circulated, because a part of the steroids are metabolised by salivary glands [43]. However, it has also been suggested that high salivary testosterone concentration may result from the transfer of a small amount of albumin from plasma to saliva [44]. The different ratio in women and men makes this hypothesis unlikely given earlier observations [45–47]. Moreover, the small transfer of 0.1% of the plasma proteins does not explain such a difference between salivary and plasma hormones concentration [48]. That is why metabolism of androgen precursors during their passage through the salivary gland may be an explanation for this difference. Additionally, it was noted in a study of 22 healthy women that the ratio of free androstenedione to free testosterone in

plasma was about 4-fold the salivary ratio between androstenedione and testosterone [49]. This is why we support the hypothesis that metabolic conversion of testosterone precursors in the salivary gland might be added to the value of salivary testosterone measurements as a marker of androgen excess, especially when increased peripheral metabolism of such precursors is the main mechanism causing hirsutism.

Therefore, we suggest that the best solution is to measure salivary androstenedione to salivary testosterone ratio. The correlation between salA/salT and A EQ was better ($p < 0.01$, $r = 0.48$) than the correlation between salA/salT and T EQ ($p < 0.01$, $r = 0.34$). We found a strong correlation between salA and A EQ ($r = 0.67$). Moreover, the assessment of salivary androgens has several practical advantages, e.g. non-invasive character, repeatability, and simplicity in use.

Conclusions

We conclude that salT and salA may be good indicators of hyperandrogenism in women. Our results indicate that the salA/salT ratio is a good representation of the clinical status of androgenicity. We also confirm that measurement of androstenedione in plasma may be useful in making a diagnosis of hyperandrogenism in PCOS women.

Founding source

'Mazowieckie Stypendium Doktoranckie' project realised by Marschal Department Mazovian Area in Warsaw.

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