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Androgens correlate with increased erythropoiesis in women with congenital adrenal hyperplasia

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ABSTRACT

Objective: Hyperandrogenism in congenital adrenal hyperplasia (CAH) provides an *in vivo* model for exploring the effect of androgens on erythropoiesis in women. We investigated the association of androgens with haemoglobin (Hb) and haematocrit (Hct) in women with CAH.

Design: Cross-validation study

Patients: Women with CAH from Sheffield Teaching Hospitals, UK (cohort 1, the training set: n=23) and National Institutes of Health, USA (cohort 2, the validation set: n=53).

Measurements: Androgens, full blood count and basic biochemistry, all measured on the same day. Demographic and anthropometric data.

Results: Significant age-adjusted correlations (P<0.001) were observed for Ln testosterone with Hb and Hct in cohorts 1 and 2 (Hb r=0.712 & 0.524 and Hct r=0.705& 0.466), and remained significant after adjustments for CAH status, glucocorticoid treatment dose and serum creatinine. In the combined cohorts Hb correlated with androstenedione (P=0.002) and 17-hydroxyprogesterone (P=0.008). Hb and Hct were significantly higher in cohort 1 than those in cohort 2, while there were no group differences in androgen levels, glucocorticoid treatment dose or body mass index. In both cohorts women with Hb and Hct in the highest tertile had significantly higher testosterone levels than women with Hb and Hct in the lowest tertile.

Conclusions: In women with CAH, erythropoiesis may be driven by androgens and could be considered a biomarker for disease control.

INTRODUCTION

The effect of androgens on erythropoiesis is well described and initially came to light through the observation that men have higher levels of haemoglobin (Hb) than women [1]. Pre-pubertal boys and girls have similar levels of Hb but boys acquire higher Hb levels following puberty that coincides with the surge in testosterone levels [2]. Lower Hb levels in women are not due to chronic menstrual blood loss as this gender difference persists in non-menstruating women [3, 4]. The evidence for an erythropoietic effect of testosterone led to its use as a treatment for anaemia in renal failure [4] and bone marrow failure [5] in the past before the invent of recombinant erythropoietin. In men intramuscular testosterone replacement, is often associated with polycythemia [6], which reverses with a dose reduction or discontinuation of therapy [7]. Conversely, androgen deprivation therapy for prostate cancer leads to a reduction in Hb levels [8].

Congenital adrenal hyperplasia (CAH) is the commonest genetic endocrine disorder and 21-hydroxylase deficiency accounts for more than 95% of the cases [9]. In this condition, defective cortisol synthesis in the adrenal glands leads to the loss of negative feedback inhibition of ACTH secretion by the pituitary. The elevated ACTH leads to hyperplasia of the adrenal glands and excess production of adrenal androgens [9]. Treatment with glucocorticoids aims to control the androgen excess and replace the steroid deficiencies; however it is challenging to achieve the correct balance between over and under-treatment. When patients are under-replaced, adrenal androgens are

elevated and women are affected by symptoms of hyperandrogenism. With over-replacement adrenal androgens are suppressed.

The effect of elevated adrenal androgens on erythropoietic markers in patients with CAH has been assumed but not studied in detail. Polycythaemia is seen in neonates with CAH [10] and there have been a few case studies reporting polycythemia in untreated men and women with CAH and androgen excess [11, 12]. To the best of our knowledge, there are no studies examining the relationship of androgens and erythropoiesis in women with CAH. The present study investigates the association of androgens with Hb and haematocrit (Hct) in women with CAH in a cross-validated study.

METHODS

Study population

This was a retrospective analysis of data from two cohorts of CAH patients managed in two tertiary centers with expertise on the management of CAH. Cohort 1 comprised of patients from Sheffield Teaching Hospitals, UK and cohort 2 from National Institutes of Health, Bethesda, USA.

Data gathering

Demographic, anthropometric, biochemical, haematological and hormonal data measured on the same day were recorded. A total of 83 women (cohort 1: n = 30, cohort 2: n = 53) with CAH were eligible for recruitment. Seven women were excluded from cohort 1 prior to the analysis (four due to incomplete biochemical data and three due to medical conditions or

medications known to affect the erythropoiesis or red cell parameters i.e. iron deficiency anaemia, vitamin B_{12} deficiency and methotrexate treatment). After screening for completeness, data of 76 women were used in the final analysis, 23 in cohort 1 and 53 in cohort 2.

Biochemical data for androgens [total testosterone, androstenedione and 17-hydroxyprogesterone (17-OHP)], full blood count, serum urea, creatinine and electrolytes were retrieved from electronic data systems. In cohort 1 the majority of samples were measured between 0800-1400hrs during clinic visits, after the morning dose of glucocorticoids, whereas for cohort 2 most samples were measured before the morning dose of glucocorticoids between 0700-0900hrs. The two laboratories had different reference ranges for Hb (cohort 1 110-147g/L, cohort 2 112g/L-157g/L). Hence, the tertiles were used for comparison between two cohorts in analysis. Age, height, weight, glucocorticoid treatment dose, CAH phenotype and smoking, medical and drug history were obtained from medical case notes. Body mass index (BMI) was calculated; weight (kg) divided by height (m) squared (kg/m^2). Since patients were treated with different glucocorticoid regimens (hydrocortisone, prednisolone/prednisone and dexamethasone), those glucocorticoid doses were converted to hydrocortisone equivalent dose using the ratio hydrocortisone: prednisolone: dexamethasone of 1:5:80 [13]. The values used to calculate the hydrocortisone equivalent doses vary widely and we chose to use 5 times potency for prednisolone/prednisone, which is the widely accepted. For dexamethasone we chose that originally proposed by Wilkins in 1965 "The potency of this glucocorticoid in suppressing adrenal steroid

biosynthesis relative to cortisol is about 80: 1" and partially evaluated in CAH by Rivkees [13].

Hormonal assays

In cohort 1, 17-OHP was measured by the Siemens Coat-a-Count radioimmunoassay (RIA) [inter-assay coefficient of variance (CV) 5.0-11%] until October 2014 and thereafter with Diasource RIA (inter-assay CV 6.3-16%). Androstenedione was measured using the Siemens Immulite 2000 chemiluminescence immunoassay (CLIA) (inter-assay CV 8.5-12.0%) until February 2014 and using the Beckman Coulter Active RIA (inter-assay CV 4.5-16.9%) thereafter. Total Testosterone was measured using the Siemens Advia Centaur CLIA (inter-assay CV 6.8-13.3%) until January 2011 and by the Roche Cobas e602 electrochemiluminescence immunoassay (ECLIA) (inter-assay CV 3.5-7.3%).

In cohort 2 all the androgens were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). From 2005 to 2012 assays were performed at Mayo Medical Laboratories, Rochester, MN; The androstenedione assay had a sensitivity of 15ng/dl; inter-assay CV of 7.9, 7.2, 8.7%; intra-assay CV of 13.9, 5.9, 2.6 at mean concentration of 112, 916, and 2281ng/dl respectively, and normal range of 40-150ng/dl for males and 30-200ng/dl for females. The 17-OHP assay had an analytical sensitivity of 40ng/dl, inter-assay CV of 9.7, 8.7, 6.8%; intra-assay CV of 6.8, 2.9, 4.4% with a mean concentration of 111, 751, and 2006ng/dl, respectively, and normal range of less than or equal to 220ng/dl for males and less than or equal to 285ng/dl for

females; 2012 onwards androstenedione and 17-OHP were measured by LC-MS/MS at National Institutes of Health, Bethesda MD; intra-assay CV ranged from 2.5-9.5% and inter-assay CV from 2.9 - 11.1%.

Statistical analysis:

Data were analyzed using SPSS v22. Group differences were determined by Student's t-tests. Relationships of Hb and Hct with androgens were assessed by partial correlations to enable adjustments for confounding factors including age, study cohorts, glucocorticoid treatment dose, CAH status and renal function. Data for androgens and glucocorticoid treatment dose were logarithmically transformed due to being positively skewed.

RESULTS

Characteristics of the study populations

Mean age of women in cohort 1 was 35.3 (SD ±14) years (Table 1). Among this cohort of women, 17 (73.9%) had classic CAH, in whom 13 (73%) were salt wasting and 4 (23%) simple virilizing subtypes, and 6 (26.1%) had non-classic CAH. The mean age of women in cohort 2 was 30.8 (SD ±11.4) years. This cohort comprised mostly of women with classic CAH (n = 51, 96.2%), of whom 33 (65%) had the salt wasting and 18 (35%) the simple virilizing type. There was one (1.9%) patient with non-classic CAH and one (1.9%) with 11-β hydroxylase deficiency.

In cohort 1 the majority received either hydrocortisone alone (n=10, 43.47%) administered twice or thrice daily, or prednisolone alone (n=9, 39.1%)

administered once or twice daily. The remaining patients were treated with either dexamethasone once daily (n=2, 8.69%), or hydrocortisone and dexamethasone combined (n=2, 8.69%). In cohort 2, the majority was treated with prednisone (n=21, 39.6%) administered twice daily, followed by hydrocortisone (n=14, 26.4%) thrice daily, and dexamethasone once daily (n=12, 22.64%). Hydrocortisone combined with either prednisone or dexamethasone and prednisolone alone was given in one patient each (1.89%).

Correlations of androgens with erythropoiesis

The associations of testosterone with Hb and Hct in the two cohorts are shown in Figures 1 and 2. The regression slopes were similar in both cohorts but the intercepts were lower in cohort 2. In cohort 1, age adjusted Ln testosterone correlated positively with Hb and Hct (P <0.001) (Table 2). These relationships remained significant (P <0.01) after further adjustments for CAH status, glucocorticoid treatment dose and serum creatinine levels. The results from the cohort 2 confirmed these relationships but were less strong. These associations continued to persist after the two cohorts were analyzed together (Table 2). In both cohorts, the androgen precursors androstenedione and 17-OHP also correlated with Hb and Hct but the correlations were weaker than for testosterone.

Androgens, glucocorticoid treatment dose and anthropometry of women with erythropoietic markers in the highest tertile were compared with those of women in the lowest tertile (Table 3). Women with Hb or Hct in the highest tertile had significantly higher testosterone levels compared with women with

Hb or Hct in the lowest tertile in both cohorts. The same was true for androstenedione and 17-OHP in cohort 1 but only for androstenedione and Hb in cohort 2. In cohort 2 women in the highest tertile of Hb and Hct had a higher BMI and higher glucocorticoid treatment dose.

Comparisons between cohort 1 and cohort 2

There were no group differences in age, anthropometric, BMI or glucocorticoid treatment dose between cohorts 1 and 2 (Table 1). Women in cohort 1 had significantly higher mean Hb ($P = 0.031$) and Hct ($P = 0.035$) levels than those in cohort 2 (Table 1). Similarly, substantially higher proportions of women had Hb and Hct above the upper limit of the reference range in cohort 1 (Hb: 30.4%, Hct: 47.8%) compared with cohort 2 (Hb, Hct <4%). The levels of total testosterone and its precursors, androstenedione and 17-OHP and creatinine levels did not differ significantly between the two study cohorts.

DISCUSSION

We have demonstrated that androgen levels in women with CAH are positively associated with Hb and Hct, suggesting that these markers of erythropoiesis are a potential biomarker of androgen control in women with CAH. The findings strengthen the evidence for an action of androgens on erythropoiesis in women.

The mechanism by which androgens promote erythropoiesis is not established [1, 14]. There are conflicting results on the effect of testosterone on erythropoietin, the major regulator of erythropoiesis. Some studies have

suggested that testosterone increases erythropoietin production [1, 15], while others found no evidence to support these findings [6, 16]. Other possible mechanisms by which testosterone might induce erythropoiesis include a direct effect on the bone marrow hematopoietic stem cells by stimulating insulin-like growth factor 1 and erythrocyte colony forming units [17], and increasing intestinal iron absorption and incorporation into erythrocytes [14].

Exogenous androgens have been associated with an increase in erythropoiesis. Supra-physiologic pharmaceutical doses of androgens cause an increase in Hb and Hct in men [18], which is dose-dependent and polycythaemia is a common but unwanted side-effect of testosterone therapy in hypogonadal men [6]. Similarly in women, androgen therapy was associated with an increase in Hb and erythroid cell hyperplasia in bone marrow aspirates [19]. In gender reassignment, hormone therapy raising testosterone levels in female-to-male reassignment leads to an increase in Hb levels while suppressed testosterone levels in male-to-female reassignment leads to a decrease in Hb levels [20]. The levels of endogenous androgens has also been associated with erythropoiesis; healthy adult men with low free testosterone levels have a lower haematocrit than men with normal free testosterone [21] and Hb levels correlate with total and bioavailable testosterone in men and women older than 65 years [22].

Conditions associated with significant hyperandrogenism such as Cushing's disease and androgen producing ovarian tumors may present with polycythaemia [23, 24]. We hypothesized that lower chronic elevations of

androgens may be associated with more subtle increases in erythropoietic markers. Women with CAH have elevated levels of adrenal androgens if inadequately treated with glucocorticoids [25] and provide a free-living model for exploring the effect of androgens on erythropoiesis. Cortisol has been implicated to play a mediating role in erythropoiesis [26, 27]. Activation of the glucocorticoid receptor promotes 'stress erythropoiesis' and maturation of erythroid progenitors *in vitro* [28]. It is well documented that anaemia occurs in patients with hypocortisolism, *e.g.* Sheehan's syndrome [29] and polycythemia in women with hypercortisolism [30]. Correcting hypocortisolism with glucocorticoid replacement [31] or hypercortisolism by surgery [24] leads to normalization of Hb levels. Hypogonadal men with active Cushing's disease have low erythroid parameters that improve slowly after correction of hypercortisolism in parallel with improvements in testosterone levels. In our study, glucocorticoid equivalent doses did not differ between women with normal and those with elevated haematological parameters. A previous study of testosterone replacement in two men with aromatase deficiency has shown that the action of testosterone on erythropoiesis does not require its aromatization to oestrogen [32].

In our study, androgen precursors androstenedione and 17-OHP were weakly associated with erythropoietic markers compared with testosterone. Androgenic precursors exert their androgenic effect through conversion to testosterone and do not directly activate the androgen receptor, which may explain the weaker relationship with erythropoiesis. Free testosterone may have a stronger association with erythropoiesis but was not calculated in the

present study because sex hormone binding globulin (SHBG) was not measured. We have however adjusted our data for body mass index, which relates inversely to SHBG levels. It would be of interest to examine the association of Hb and Hct with dihydrotestosterone, which has tenfold greater affinity for androgen receptor than testosterone [33]. However, dihydrotestosterone is not routinely measured in the clinical setting and therefore was not available in the present study. Chronic kidney disease is also associated with anaemia due to the reduction in renal production of erythropoietin [34]. In the two cohorts presented here there were no subjects with chronic kidney disease and the relationship between androgens and markers of erythropoiesis continued to persist after adjusting for creatinine.

The two cohorts of women could potentially have differences in genotypes and exposure to lifestyle factors, which could affect the outcomes, but our results were reproducible in the two cohorts. This is evident by the parallel regression slopes for the association of testosterone with Hb and Hct in the two study cohorts. Interestingly, mean Hb and Hct were higher in the UK cohort than in the US cohort with no differences in androgen levels, body mass index or glucocorticoid treatment dose between the two cohorts. This may indicate underlying genetic differences between the two cohorts that could affect the action of testosterone on erythropoiesis *e.g.* differences in androgen receptor CAG repeat lengths. Lifestyle factors such as smoking and dietary iron intake and menstruation status may be some other factors to consider, however both cohorts had similar mean age. Compliance with

304 glucocorticoid treatment or error in reporting of treatment dose may also
305 explain this difference.
306
307 Strengths and limitations of the present study: The strengths of the present
308 study lie in its robust cross-validation study design and adjustments for a
309 number of major confounding factors. The study is limited by its retrospective
310 nature and sampling bias might have been introduced as data collection
311 spanned across approximately ten years. Different assays had been used
312 during this period, which might have affected the accuracy of the biochemical
313 data and also the two cohorts have used different assay techniques for
314 androgens, which limits the comparison between the two cohorts. Another
315 limitation of the study is wide variation of androgen levels observed in both
316 cohorts. However, this reflects the previous observations with poor disease
317 control on current therapeutic regimens [9] and potentially affected by
318 differences in time of blood sampling. Lifestyle factors such as diet and
319 smoking history were not available given this was a retrospective study.
320
321 In conclusion, the strong association of adrenal androgens with Hb and Hct in
322 two cohorts of women with CAH suggests that these markers of
323 erythropoiesis may be considered as biomarkers of disease control in women
324 with CAH and in those with polycythaemia or anemia under or over
325 suppression of adrenal androgens should be considered as a cause. Chronic
326 over and under-treatment of CAH patients may have an effect on
327 erythropoiesis, which can also potentially impact physical performance [35].
328

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Tables

Table 1. Characteristics of women with congenital adrenal hyperplasia in cohort 1, UK (n = 23) and cohort 2, US (n = 53).

	Cohort 1 (n = 23) Mean (SD)	**Cohort 2** (n = 53) Mean (SD)	**Group difference** (cohort 1 minus cohort 2) Mean (95% CI)	*P*
Age (years)	35.3 (13.9)	30.8 (11.4)	4.4 (-1.6, 10.5)	0.148
Haemoglobin (g/L)	140.4 (13.3)	134.1 (10.5)	6.3 (0.6, 11.9)	0.031
Haematocrit (%)	41.7 (04.0)	39.9 (3.1)	1.8 (0.1, 3.5)	0.035
17-OHP (nmol/L)	98.3 (151.4)	127.1 (150.1)	-28.9 (-110.7, 53.0)	0.484
Androstenedione (nmol/L)	12.4 (13.3)	15.4 (19.6)	-3.0 (-12.3, 6.3)	0.519
Testosterone (nmol/L)	3.2(6.1)	2.7 (5.5)	0.5 (-2.4, 3.4)	0.748
Height (m)	1.58 (0.08)	1.57 (0.08)	0.00 (-0.03, 0.05)	0.687
Weight (kg)	86.4 (27.2)	78.2 (29.0)	8.1 (-8.8, 24.4)	0.323
Body mass index (kg/m^2)	34.6 (11.4)	31.7 (12.1)	2.9 (-3.9, 9.7)	0.396
Serum creatinine (µmol/L)	66.5 (13.1)	73.6 (14.4)	-7.1 (-14.3, 0.10)	0.053
Glucocorticoid treatment dose (mg/day)	28.2 (11.2)	29.4 (13.4)	-1.3 (-7.7, 5.1)	0.692

Table 2. Partial correlations of haemoglobin and haematocrit with androgens in women from two separate study cohorts. All analyses were adjusted for age. Further adjustments were made for glucocorticoid treatment dose, CAH status and serum creatinine.

	Ln 17-OHP		Ln Androstenedione		Ln Testosterone	
	r	***P***	***r***	***P***	***r***	***P***
Cohort 1: Adjusted for age						
Haemoglobin	0.472	0.056	0.352	0.129	0.712	<0.001
Haematocrit	0.508	0.037	0.485	0.030	0.705	0.001
Cohort 2: Adjusted for age						
Haemoglobin	0.508	0.037	0.372	0.007	0.524	<0.001
Haematocrit	0.176	0.211	0.298	0.032	0.466	<0.001
Cohort 1: Adjusted for age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.524	0.066	0.555	0.032	0.797	<0.001
Haematocrit	0.570	0.042	0.724	0.002	0.778	0.001
Cohort 2: Adjusted for age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.301	0.038	0.363	0.011	0.491	<0.001
Haematocrit	0.168	0.253	0.259	0.075	0.415	0.003
Both cohorts: Adjusted for study group + age						
Haemoglobin	0.316	0.008	0.357	0.002	0.545	<0.001
Haematocrit	0.260	0.031	0.349	0.003	0.497	<0.001
Both cohorts: Adjusted for study group + age + CAH status + Ln glucocorticoid treatment dose + serum creatinine						
Haemoglobin	0.294	0.019	0.325	0.008	0.490	<0.001
Haematocrit	0.225	0.076	0.314	0.010	0.438	<0.001

Table 3. Independent t-tests to assess differences in androgens, glucocorticoid treatment dose and anthropometry of CAH women with Hb or Hct in the highest tertile compared with those in the lowest tertile (Hb cut-offs at 137 and 147 g/l in cohort 1 and at 130 and 138 g/l in cohort 2; Hct cut-offs at 41.0 and 43.7% in cohort 1 and at 38.8 and 41.1% in cohort 2).

	Hb: highest tertile minus lowest tertile Mean difference (95% CI)	***P***	**Hct: highest tertile minus lowest tertile** Mean difference (95% CI)	***P***
Cohort 1				
Ln 17-OHP (nmol/L)	2.79 (0.94, 4.64)	0.007	2.61 (0.82, 4.39)	0.006
Ln Androstenedione (nmol/L)	1.83 (0.36, 3.30)	0.018	2.15 (0.72, 3.57)	0.006
Ln Testosterone (nmol/L)	1.67 (0.20, 3.14)	0.029	1.59 (0.14, 3.03)	0.034
Ln Glucocorticoid treatment dose (mg/day)	0.04 (-0.43, 0.51)	0.848	0.08 (-0.41, 0.57)	0.781
Height (m)	0.01 (-0.08, 0.11)	0.755	0.03 (-0.07, 0.13)	0.509
Body mass index (kg/m^2)	1.9 (-16.7, 20.4)	0.824	-3.4 (-15.2, 8.4)	0.522
Cohort 2				
Ln 17-OHP (nmol/L)	1.44 (-0.12, 3.00)	0.069	0.54 (-0.97, 2.05)	0.472
Ln Androstenedione (nmol/L)	1.44 (0.54, 2.34)	0.003	0.76 (-0.17, 1.70)	0.105
Ln Testosterone (nmol/L)	1.75 (1.02, 2.48)	<0.001	1.27 (0.52, 2.02)	0.002
Ln Glucocorticoid treatment dose (mg/day)	0.19 (-0.09, 0.48)	0.181	0.25 (0.01, 0.50)	0.043
Height (m)	-0.04 (-0.10, 0.02)	0.193	-0.04 (-0.10, 0.01)	0.140
Body mass index (kg/m^2)	10.1 (3.7, 16.5)	0.003	10.0 (3.7, 16.2)	0.003

Figures and Legends

Figure 1. Relationship between haemoglobin and testosterone levels in women with CAH (● and solid line indicate cohort 1; △ and dashed line indicate cohort 2). Regression equations for cohort 1: Haemoglobin = 4.6 (95%CI: 1.5-7.8) x Ln Testosterone + 141 (95% CI: 137-145) (r^2 = 31.5%) and for cohort 2: Haemoglobin = 4.4 (95%CI: 2.4-6.5) x Ln Testosterone + 133 (131-136) (r^2 = 27.5%). The slopes of regression did not differ between the two cohorts.

Figure 2. Relationship between haematocrit and testosterone levels in women with CAH (● and solid line indicate cohort 1; △ and dashed line indicate cohort 2). Regression equations for cohort 1: Haematocrit = 1.4 (95%CI: 0.4-2.4) x Ln Testosterone + 42.0 (95% CI: 40.7-43.4) (r^2 = 30.3%) and for cohort 2: Haematocrit = 1.2 (95%CI: 0.5-1.8) x Ln Testosterone + 39.7 (39.0-40.5) (r^2 = 21.9%). The slopes of regression did not differ between the two cohorts.