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Original Research

Steroid hormone profiling reveals altered adrenal androgen production in endometriosis

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Abstract

Objective **Endometriosis is a chronic, hormone-dependent condition affecting an estimated 190 million women worldwide. Our understanding of hormonal contributions to endometriosis pathophysiology is incomplete, hindering the identification of diagnostic biomarkers and novel therapeutic targets. Although the role of estrogens is well established, research on androgens in endometriosis is limited and the contribution of adrenal-derived 11-oxygenated androgens remains largely unknown.**

Methods **We performed steroid androgen profiling to measure androgen concentrations in serum from healthy controls and women with laparoscopically confirmed endometriosis. We found that women with endometriosis had a distinct hormone signature characterized by systemic differences in adrenal androgen concentrations and 11-ketotestosterone excess.**

Using metabolomic data, we generated statistical models that showed robust discrimination between healthy controls and women with endometriosis (AUC = 0.99; positive predictive power = 96.84%, negative predictive power = 92.86%) consistent with an endometriosis-specific signature. Data were partitioned into train and validation groups to assess diagnostic potential and a refined model identified >95% of endometriosis patients in a blinded sample set.

Conclusions **Collectively, these data reframe endometriosis as an androgen-dependent disorder and highlight 11-oxygenated androgens as potential diagnostic biomarkers and future therapeutic targets.**

Keywords androgen, endometriosis, 11-oxygenated androgens, adrenal

Significance

Endometriosis occurs when cells similar to those lining the womb are found elsewhere in the body. These cells can grow and change in response to hormones, causing pain and inflammation. While estrogens are known to affect endometriosis, the role of other hormones, such as androgens, isn't as well understood. In this study, we measured androgens in blood and found that women with endometriosis have a distinct hormone pattern, including higher levels of an androgen called 11-ketotestosterone. We used this information to create a statistical model that could accurately identify women with endometriosis based on androgen concentrations. These findings could help with earlier diagnosis and the development of new treatments for endometriosis.

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Introduction

Endometriosis is a chronic, hormone-dependent condition that affects an estimated 190 million women worldwide.[1] It is characterized by the presence of "endometrial-like" tissue (referred to as "lesions") outside the uterus, most commonly within the pelvic peritoneal cavity. Women experience life-altering symptoms including chronic pelvic pain and infertility. In addition to impacts on quality of life, endometriosis also has serious financial consequences limiting health, well-being, and life potential.[2]

Our understanding of hormonal contributions to endometriosis pathophysiology is incomplete, which limits our capacity to identify disease-relevant biomarkers and therapeutic targets. Endometriosis is primarily an estrogen-dependent condition, with research focused on the role of local hormones within endometriotic lesions.[3] These studies demonstrate that local estrogen production is a key driver of disease by promoting inflammation and tissue growth within lesions. In contrast, systemic hormonal influences, particularly those of androgens, are less well understood.

Androgens play established roles in regulating endometrial function as hormone precursors as well as direct mediators.[4] Androgen precursors, such as dehydroepiandrosterone (DHEA) and androstenedione (A4), are locally activated in the endometrium during the establishment of pregnancy where they promote tissue remodeling.[5] Active androgens, such as testosterone (T) and dihydrotestosterone (DHT), regulate endometrial proliferation, tissue remodeling, angiogenesis, and inflammation, which are processes associated with establishment and growth of endometriosis lesions.[6-8] Concentrations of T are increased in endometriosis lesions, and this is associated with changes in androgen-regulated genes[9] indicating the potential for direct androgen action within lesions. Androgens can also have indirect roles through conversion to estrogens and activation of pro-disease pathways. Thus, changes in systemic androgen bioavailability have the potential to directly and indirectly alter disease processes in endometriosis. However, profiling of androgens in endometriosis has been limited, both in range of analytes and in sample size, making it difficult to determine their role in endometriosis pathophysiology.

11-Oxygenated androgens have historically been overlooked in endometriosis, but they are increasingly recognized as important components of the androgen pool, particularly in women.[10] 11-Oxygenated androgens are a group of adrenal-derived steroids that include both inactive precursors and potent androgens.[11] They are characterized by the presence of an oxygen atom at carbon 11 and are among the most abundant androgens produced by the adrenal gland. 11-Ketotestosterone (11KT), the most potent metabolite, circulates at concentrations equivalent to or greater than T in women and has similar androgenic activity, consistent with a potential role in regulating androgen-dependent processes.[12] Changes in 11-oxygenated androgens have been described in polycystic ovarian syndrome (PCOS),[13] but whether they are also altered in endometriosis is yet to be determined. Given the potential importance of androgen signaling to endometriosis pathophysiology, characterizing the abundance of 11-oxygenated androgens is crucial in order to understand if they are an important feature of the condition.

In this study, we performed comprehensive androgen profiling to measure and compare androgen concentrations in serum from healthy controls and women with laparoscopically confirmed endometriosis. We aimed to identify systemic differences that could be used to characterize a distinct hormone signature in endometriosis. Serum samples from women with laparoscopically confirmed endometriosis (= 159) and healthy controls (n = 57) were profiled using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) assay to simultaneously measure classic and 11-oxygenated androgens. We combined androgen profiling with statistical modeling to characterize hormonal differences, hypothesizing that women with endometriosis exhibit a distinct androgen profile and that adrenal-derived 11-oxygenated androgens are key to this unique signature.

Results

Endometriosis is characterized by altered concentrations of androgenic hormones

Androgens and their precursors were quantified using LC-MS/MS in serum samples from healthy controls (HC; $n = 57$) and women with endometriosis (ENDO; $n = 159$). The concentrations of "classic" androgens, DHEA, A4, T, and DHT, and adrenal-derived 11-oxygenated androgens, 11OHA4, 11OHT, 11KA4, and 11KT, were determined (Figure 1 and Table 1).

Concentrations of DHEA (Figure 1A; $P < .0001$), A4 (Figure 1B; $P < .0001$), and T (Figure 1C; $P < .0001$) were significantly higher in serum samples from women with endometriosis compared to HC (Table 1 "All'). Concentrations of DHT were not significantly different between groups (Figure 1D). The 11-oxygenated androgen precursor metabolites, 11OHA4 (Figure 1E; $P < .05$) and 11KA4 (Figure 1F; $P < .0001$), were decreased, and the active 11-oxygenated androgens, 11OHT (Figure 1G; $P < .0001$) and 11KT (Figure 1H; $P < .0001$), were increased in women with endometriosis (Table 1 "All'). Consistent with previous reports,[14] 11-oxygenated androgens did not differ by menstrual cycle phase (Table S1). Neither classic nor 11-oxygenated androgen concentrations correlated with age, although 11OHT trended to positive correlation ($r = 0.1267$, $P = .0636$). Only 11KA4 negatively correlated with body mass index (BMI; $r = -0.2029$, $P = .0037$; Table S2).

Altered adrenal androgen production is detected in all stages of endometriosis

To assess whether androgen concentrations were associated with disease severity in endometriosis, data were stratified according to the revised American Society for Reproductive Medicine (rASRM) staging. Broadly, there were no significant differences in steroid concentrations between the rASRM stages for any of the analytes assessed. Analytes that were increased in the unstratified group (ENDO/"All") were also increased in each rASRM stage compared to HC (Figure 1I-P; Table 1). Notably, the median concentrations of DHEA ($P < .0001$), A4 ($P < .0001$), and T ($P < .0001$) were highest in Stage I endometriosis and significantly higher than healthy controls (Figure 1I-P; Table 1). Similarly, concentrations of 11OHT ($P < .001$) and 11KT ($P < .0001$) were highest in Stage I endometriosis and significantly higher than healthy controls (Figure 1I-P; Table 1).

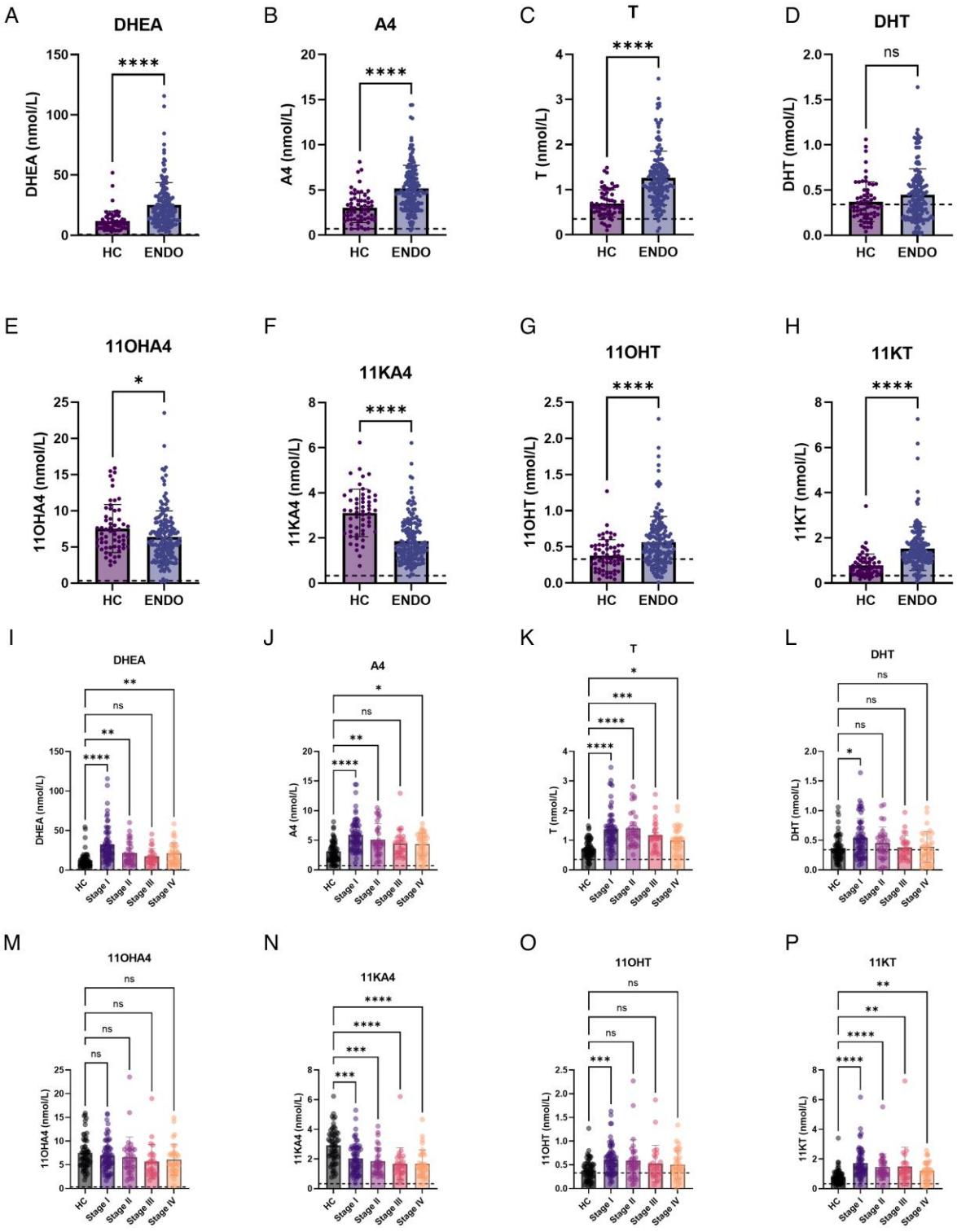


Figure 1. Serum concentrations of classic and 11-oxygenated androgens are altered in women with endometriosis. Serum concentrations of DHEA, A4, T, 11OHA4, 11KA4, 11OHT, and 11KT were determined by LC-MS/MS. DHEA, A4, and T were significantly elevated in women with endometriosis (ENDO) compared with heathy controls (HCs; A-C). Concentrations of DHT did not differ between groups (D). 11-oxygenated androgen precursors, 11OHA4 (E) and 11KA4 (F), were decreased in ENDO, while potent 11-oxygenated androgens, 11OHT (G) and 11KT (H) were significantly elevated in ENDO. Patients with ENDO were stratified according to rASRM stage (I-P). DHEA and A4 were significantly elevated in Stage I, II, and IV endometriosis (I, J). T was elevated in all endometriosis stages (K), and DHT was significantly elevated in Stage I endometriosis (L). Concentrations of 11OHA4 did not significantly differ between groups (M) while 11KA4 was decreased in all stages (N). In contrast, 11OHT was significantly elevated in Stage I endometriosis (O) while 11KT was elevated in all endometriosis stages (P). Median serum steroid concentrations (25th-75th centile range; nmol L-1) for each stage (I). Error bars represent the SD of the mean. Dotted lines denote LLOQ for each analyte. Undetected steroids or those detected below LLOQ were replaced by 0.5×LLOQ for statistical purposes. Statistical comparison using Mann–Whitney *U* test or Kruskal–Wallis test with multiple comparisons. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001. ns, non-significant.

Table 1 Serum steroid concentrations of classic and 11-oxygenated androgens in healthy controls and women with endometriosis. Age (years), median (min-max), BMI (kg/m^2), median (min-max), serum steroid concentrations (median and 25th-75th centile range; nmol/L). Steroid concentrations below the LLOQ were replaced by 0.5×LLOQ for statistical purposes.

	Controls	Endometriosis				
	All	All	Stage I	Stage II	Stage III	Stage IV
n	57	159	66	34	26	33
Age	30 (22-48)	32.00 (19-50)	28.00 (19-47)	36.00 [a] (22-46)	37.00 [a] (22-50)	34.00 [a] (23-47)
BMI	22.04 (17.3-37.3)	**25.00 (18.0-62.0) *****	**25.00** (18.0—62.0)**	**25.25** (18.0—37.5)**	**25.00* (19.2—35.0)**	25.65 (18.0—39.5)
Cycle stage pro/sec/ND	19/22/16 33.3%/38.6%/28%	75/67/17 47.2%/42.1%/10.7%	32/30/4 48.5%/45.5%/6.1%	16/14/4 47.0%/41.2%/11.2%	17/9/0 65.4%/34.6%/0%	10/15/8 30.3%/45.5%/24.2%
DHEA	9.6 (6.02-14.63)	**20.80 (12.23-32.48) ******	**25.40**** (16.35-39.45)**	**18.50*** (11.70-33.55)**	14.65[a] (10.00-22.40)	**19.40** (10.20-30.60)**
A4	2.83 (1.76-4.01)	**4.72 (3.37-6.59) ******	**5.55**** (3.71-7.51)**	**4.52*** (3.25-7.41)**	4.56 (2.88-5.51)	**4.61* (2.76-5.83)**
T	0.63 (0.50-0.93)	**1.15 (0.86-1.49) ******	**1.26**** (0.97-1.59)**	**1.25**** (1.00-1.80)**	**1.07*** (0.80-1.44)**	**1.03* [b] (0.69-1.33)**
DHT	0.17 (0.17-0.50)	0.40 (0.17-0.60)	**0.48* (0.17-0.70)**	0.44 (0.17-0.59)	0.26 (0.17-0.47)	0.36 (0.17-0.57)
11OHA4	7.26 (5.35-10.12)	**5.67 (3.80-8.21) ****	6.27 (4.49-8.54)	6.34 (4.11-8.34)	**5.38* (3.08-7.10)**	5.18 (3.78-7.35)
11KA4	3.18 (2.28-3.75)	**1.64 (1.11-2.39) ******	**1.88**** (1.24-2.72)**	**1.63**** (1.07-2.32)**	**1.53**** (1.08-2.05)**	**1.42**** (0.97-2.28)**
11OHT	0.36 (0.17-0.52)	**0.51 (0.17-0.72) ******	**0.57*** (0.36-0.73)**	**0.53* (0.31-0.68)**	0.43 (0.17-0.67)	0.44 (0.25-0.71)
11KT	0.69 (0.44-0.97)	**1.3 (0.99-1.80) ******	**1.49**** (1.12-2.19)**	**1.25**** (0.96-1.80)**	**1.18*** (0.87-1.72)**	**1.15** (0.72-1.71)**

P value vs healthy control; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$. Bold values present statistically significant difference from controls, specific *P* values are denoted by *.
[a]vs Stage I.
[b]vs Stage II.

Concentrations of T and 11KT were significantly elevated in women with Stage I, II, III, and IV endometriosis compared to healthy controls, whereas 11KA4 was significantly reduced in women with all stages of endometriosis compared to healthy controls (Figure 1I-P). The greatest difference in androgen concentrations compared to healthy controls was in patients classified as Stage I. Median concentrations of T, 11KA4, and 11KT were modestly, inversely decreased with stage, but overall, there was no distinct steroid signature associated with a given rASRM stage.

Endometriosis is characterized by altered adrenal androgen metabolism that is biased toward production of 11-ketotestosterone

The correlation among different serum androgen analytes was assessed in healthy controls and women with endometriosis using Pearson correlation (Figures 2A and B, Table S3). In healthy controls, closely related metabolites exhibited a significant positive correlation. This included classic androgens A4 and T ($r = 0.8$, $P = 1.59E-12$) and T and DHT ($r = 0.5$, $P = 6.37E-06$), as well as 11-oxygenated androgens 11OHA4 and 11KA4 ($r = 0.6$, $P = 8.2E-07$) and 11OHT and 11KT ($r = 0.9$, $P = 5.53E-20$). In women with endometriosis, additional metabolites that were not correlated in healthy controls were found to be positively correlated. This included a significant positive correlation between classic and 11-oxygenated androgens, as well as a greater correlation amongst all 11-oxygenated androgen metabolites. This was characterized by DHEA, A4, T, and DHT each being significantly positively correlated with all 11-oxygenated androgen metabolites (Figures 2A and B, Table S3). Additionally, there was a significant positive correlation between 11KT and 11OHA4 ($r = 0.6$, $P = 1.82E-19$), 11KT and 11KA4 ($r = 0.7$, $P = 7.68E-22$), 11OHT and 11OHA4 ($r = 0.7$, $P = 2.14E-24$), and 11OHT and 11KA4 ($r = 0.6$, $P = 3.94E-17$) in women with endometriosis that was absent in healthy controls (Figures 2A and B, Table S3).

To investigate if these changes were indicative of altered metabolism in women with endometriosis, we estimated the activities of enzymes required for 11-oxygenated androgen production by calculating product to substrate ratios (Figure 2; 11-β-hydroxylase (CYP11B1), 11β-hydroxysteroid dehydrogenase type 2 (HSD11B2) and Aldo-keto reductase family 1 member C3 (AKR1C3)).

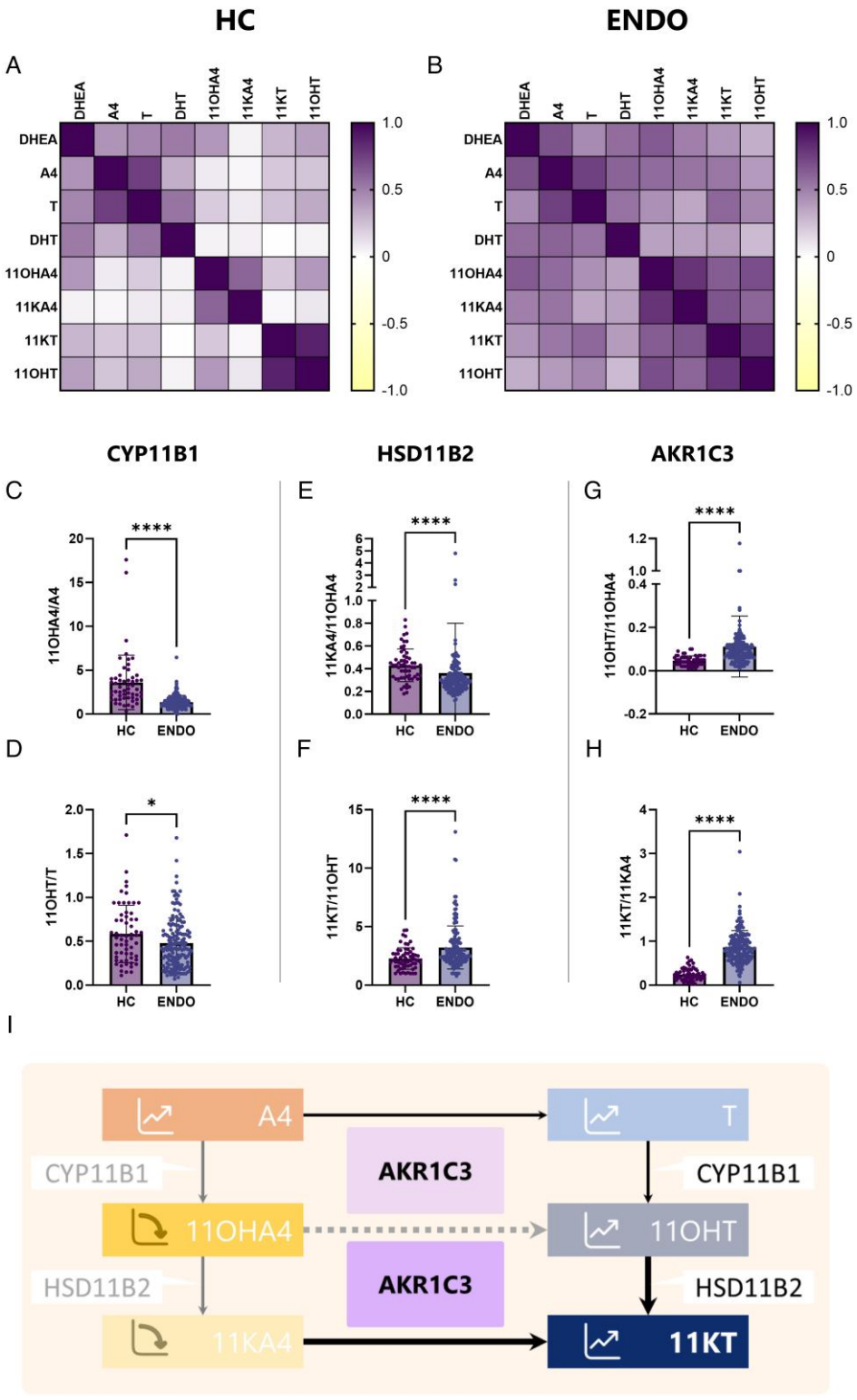


Figure 2. Endometriosis is associated with altered androgen metabolism characterized by increased production of 11KT. (A) Pearson correlation matrix of key analytes in HC demonstrates strong correlation between related steroids within relevant subgroupings including A4 and T, 11OHA4 and 11KA4 as well as 11KT and 11OHT. (B) In patients with ENDO, there was a strong correlation between both classic and 11-oxygenated androgen metabolites indicative of altered metabolism. Enzyme activity based on substrate to product ratios (product/substrate) were calculated based on measured serum concentrations in healthy control women (HC) and patients diagnosed with endometriosis (ENDO). The ratios required for sequential production of 11KT were assessed including CYP11B1 (C, D), HSD11B2 (E, F), and AKR1C3 (G, H). The ratios of 11OHA4/A4 (C), 11OHT/T (D), and 11KA4/11OHA4 (E) were significantly decreased in ENDO compared with HC. In contrast, the ratios of 11KT/11OHT (F), 11OHT/11OHA4 (G), and 11KT/11KA4 (H) were significantly increased in ENDO compared with HC. Collectively, these metabolism changes are associated with increased production of 11KT (I). Statistical comparison using Mann–Whitney *U* test. **P*<0.05, *****P*<0.0001.

CYP11B1 and HSD11B2 enzymes mediate interconversion and activation of 11-oxygenated androgens. The ratios of 11OHA4/A4 and 11OHT/T (mediated by CYP11B1) were significantly decreased in women with endometriosis (Figures 2C and D), consistent with decreased bioavailability of the precursors 11OHA4 and 11OHT. The ratio of 11KA4/11OHA4 (mediated by HSD11B2) was significantly decreased in women with endometriosis (Figure 2E, $P < .0001$), consistent with decreased bioavailability of the precursor 11KA4. In contrast, the ratio of 11KT/11OHT was modestly increased between healthy controls and women with endometriosis (Figure 2F, $P < .0001$), suggesting that metabolism favors the production of 11KT. Ratios for the reciprocal inactivation of 11KA4 and 11KT, mediated by HSD11B1,[15] were also calculated. The ratio of 11OHA4/11KA4 was increased (Figure S1A, $P < .0002$) and 11OHT/11KT controls were decreased (Figure S1B, $P < .0002$) in women with endometriosis compared to healthy controls. The ratio of T/A4 did not differ between groups (Figure S1C).

AKR1C3 is a multifunctional enzyme that plays a pivotal role in all pathways to androgen production including that of T, 11OHT and 11KT. The ratio of T/A4 (mediated by AKR1C3) did not differ between groups (Figure S1C), but other AKR1C3 activities determined by the ratios of 11OHT/11OHA4 and 11KT/11KA4 were significantly higher in women with endometriosis (Figures 2G and H; $P < .0001$), suggestive of greater production of 11OHT and 11KT in women with endometriosis. Taken together, these data are consistent with a shift in metabolism towards production of 11KT (Figure 2I).

Women with endometriosis have a disease-specific hormone signature characterized by altered androgen concentrations

Receiver operating characteristic (ROC) curve analysis of serum androgen concentrations was performed to identify if differences in androgen metabolites or ratios were robust enough to discriminate between healthy controls and women with endometriosis (Table S4). The androgen analytes T, 11KA4, and 11KT exhibited best sensitivity and specificity with area under the ROC curve (AUC) values of 0.815 ($P < .001$), 0.822 ($P < .001$), and 0.815 ($P < .001$), respectively (Figure 3A-C).

ROC curve analysis of calculated enzyme ratios was also performed as these data have reduced inter-sample variability and provide a better indicator of the proposed altered metabolism found in women with endometriosis. Enzyme ratios associated with metabolism of 11-oxygenated androgens were assessed (Table S4). The ratios 11OHA4/A4, 11OHT/11OHA4, and 11KT/11KA4 had best sensitivity and specificity with AUC values of 0.857 ($P < .001$), 0.817 ($P < .001$), and AUC of 0.968 ($P < .001$), respectively (Figure 3D-F). The ratio of 11KT/11KA4 provided robust discrimination between healthy controls and women with endometriosis with a sensitivity of 92.36% and specificity of 90.91% and a likelihood ratio of 10.16 (Figure 3G). These data suggest that differences in androgen metabolism in endometriosis represent a consistent, disease-specific hormone signature.

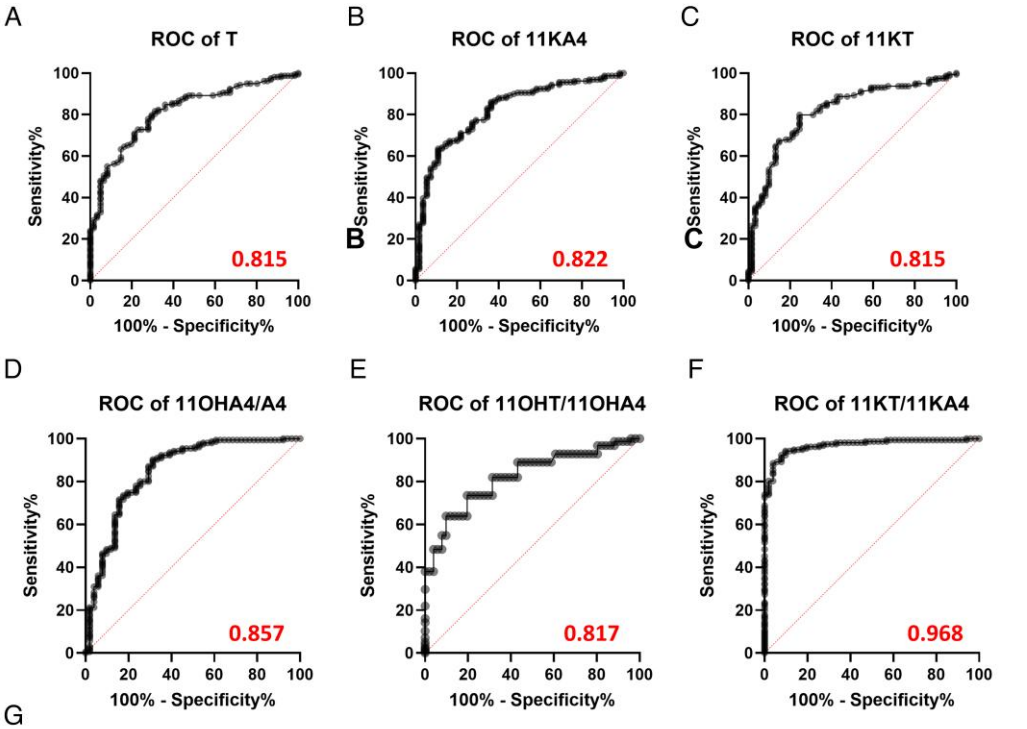
Development of a predictive model using supervised machine learning confirms specificity of androgen signature to endometriosis diagnosis

To complement and extend these findings, androgen data were used to train a predictive model using multiple logistic regression. Androgen concentrations, DHEA, A4, T, DHT, 11OHA4, 11OHT, and 11KT, were used as dependent variables, and diagnosed endometriosis (as a binary) was the outcome variable. BMI was also included in the model to account for any independent effects of this variable (Figure 3H). ROC curve analysis demonstrated excellent discrimination between healthy controls and women with endometriosis with AUC of 0.99 (Figure 3I; $P < .0001$, CI 0.9878-1.000), providing further improvement on discrimination informed by single analytes or ratios (Figure 3A-G). The predicted probability for outcome was calculated for each sample using a classification cut-off of 0.5. The negative predictive power of the multiple logistic regression model was 92.86% and the positive predictive power was 96.84% (Figures 3H and J) consistent with androgen concentrations providing a robust and accurate model for predicting diagnostic outcome in endometriosis.

Validation of a refined statistical model highlights potential for androgen concentrations to robustly predict diagnostic outcome in a blinded cohort

To assess how a predictive model could perform when blinded to outcome, the whole data set was partitioned into training and validation groups. The training set contained dependent and outcome variables for an arbitrary subset of healthy controls ($n = 21$) and women with endometriosis ($n = 21$), and the validation set contained dependent variables but was blinded to outcome variable ($n = 138$). Comparative modeling was used initially to identify the most robust analytes to include in a refined predictive model (not shown). Multiple logistic regression was used to fit a predictive model using BMI, 11OHA4, 11KA4, and 11KT as dependent variables and diagnosed endometriosis as the outcome variable (Figures 4A and B). ROC curve analysis of the simplified model demonstrated excellent discrimination between healthy controls and women with endometriosis with an AUC of 0.97 (Figure 4C; $P < .0001$, CI 0.9124-1.000). The estimated negative predictive power of the model was 94.74% and the positive predictive power was 95.24% (Figures 4A and D).

The actual performance of the model was assessed using the remaining data with outcome blinded (validation set) and included separate performance estimates according to stratification by rASRM stage (Figure 4E). The predicted outcome was calculated for each sample, using a classification cut-off of 0.5. In the validation dataset, the sensitivity of the refined model was 95.65% with accurate prediction observed for all disease



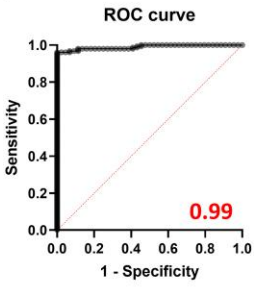
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	Area	P value	Cut-off	Sensitivity%	95% CI	Specificity%	95% CI	Likelihood ratio
11KT/11KA4	**0.9676**	**<0.001**	**> 0.4550**	**92.36**	**87.12% to 95.57%**	**90.91**	**80.42% to 96.05%**	**10.16**
11OHT/11OHA4	0.8174	<0.001	> 0.05500	79.11	72.12% to 84.72%	67.27	54.10% to 78.19%	2.417
11OHA4/A4	0.8566	<0.001	< 1.825	78.48	71.44% to 84.17%	76.36	63.65% to 85.63%	3.32
T	0.8152	<0.001	> 0.8350	77.85	70.76% to 83.62%	72.13	59.83% to 81.81%	2.793
11KT	0.8145	<0.001	> 0.9450	78.62	71.61% to 84.27%	75.41	63.32% to 84.49%	3.197
11KA4	0.8219	<0.001	< 2.450	76.1	68.91% to 82.06%	72.73	59.77% to 82.72%	2.79

H

Area under the ROC curve				
Area	0.9898			
Std. Error	0.005301			
95% confidence interval	0.9794 to 1.000			
P value	<0.0001			
Classification table	Predicted 0	Predicted 1	Total	% Correctly classified
Observed 0	39	5	44	88.64
Observed 1	3	153	156	98.08
Total	42	158	200	96.00
Negative predictive power (%)	92.86			
Positive predictive power (%)	96.84			
Classification cutoff	0.5			

Odds ratios	Variable	Estimate	95% CI (profile likelihood)
β0	Intercept	0.006340	1.053e-005 to 2.180
β1	BMI	1.274	1.031 to 1.624
β2	DHEA	1.092	0.9514 to 1.291
β3	A4	1.276	0.5926 to 2.889
β4	T	4.550	0.07186 to 379.0
β5	DHT	0.01404	0.0001399 to 0.8244
β6	11OHA4	0.8590	0.4417 to 1.560
β7	11KA4	0.1106	0.01920 to 0.4151
β8	11OHT	0.03000	5.311e-005 to 16.76
β9	11KT	383.3	16.71 to 26404



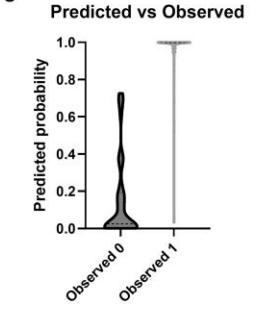


Figure 3. Androgen concentrations can be used to robustly discriminate between HC and ENDO. ROC curve analysis of serum analyte concentrations (A-C) and ratios (D-F) was assessed (G). Robust discrimination between HC and ENDO was observed for single analytes; T (AUC 0.8152; $P<0.001$), 11KA4 (AUC 0.8145; $P<0.001$) and 11KT (AUC 0.8219; $P<0.001$), and for each ratio; 11OHA4/A4 (AUC 0.8556; $P<0.001$), 11OHT/11OHA4 (AUC 0.817; $P<0.001$), and 11KT/11KA4 (AUC 0.9676; $P<0.001$). Sensitivity, specificity, and likelihood ratio were summarized, and the ratio of 11KT/11KA4 provided the most robust discrimination between HC and ENDO (G). (H) Multiple logistic regression modelling was used to fit a predictive model using androgen concentrations, age and BMI as dependent variables, and diagnosed endometriosis as the outcome variable. (I) ROC curve analysis of model demonstrated excellent discrimination between HC and ENDO with AUC of 0.99 ($P<0.0001$, CI 0.9794-1.000). (J) The predictive power of the model was calculated based on observed vs predicted outcomes. The negative predictive power of the model was 92.86%, and the positive predictive power was 96.84%. Statistical comparison was performed by Mann–Whitney *U* test.

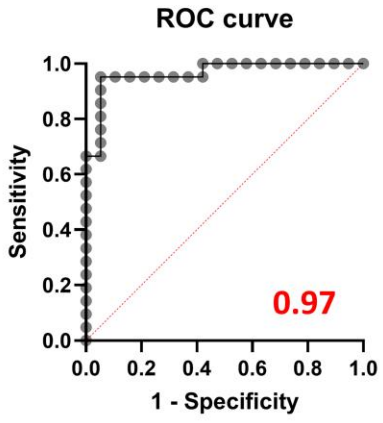
A

Area under the ROC curve				
Area	0.9649			
Std. Error	0.02682			
95% confidence interval	0.9124 to 1.000			
P value	<0.0001			
Classification table	Predicted 0	Predicted 1	Total	% Correctly classified
Observed 0	18	1	19	94.74
Observed 1	1	20	21	95.24
Total	19	21	40	95.00
Negative predictive power (%)	94.74			
Positive predictive power (%)	95.24			
Classification cutoff	0.5			

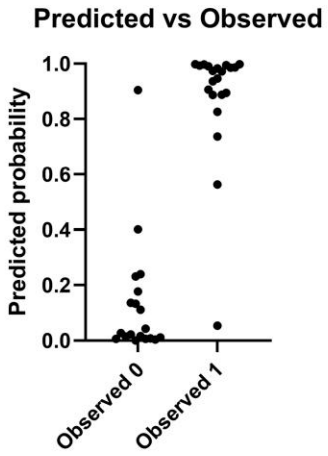
B

Model				
Parameter estimates	Variable	Estimate	Standard error	95% CI (profile likelihood)
β0	Intercept	-3.346	3.761	-12.24 to 3.981
β1	BMI	0.2497	0.1616	-0.03097 to 0.6403
β2	11OHA4	-0.01984	0.1548	-0.3135 to 0.3269
β3	11KA4	-2.181	0.7849	-4.154 to -0.9095
β4	11KT	3.071	1.432	0.4949 to 6.470
Odds ratios	Variable	Estimate	95% CI (profile likelihood)	
β0	Intercept	0.03522	4.811e-006 to 53.56	
β1	BMI	1.284	0.9695 to 1.897	
β2	11OHA4	0.9804	0.7309 to 1.387	
β3	11KA4	0.1129	0.01570 to 0.4027	
β4	11KT	21.56	1.640 to 645.3	

C



D



E

	N	Sensitivity (%)
All	138	95.65
Stage I & II	100	94.00
Stage III & IV	58	96.55
Stage I	66	95.46
Stage II	34	91.18
Stage III	26	100
Stage IV	32	93.75

Figure 4. A refined predictive model accurately identifies endometriosis in a blinded dataset. The whole data set was partitioned into train and validation groups. The training set contained dependent and outcome variables for HC (n=21) and ENDO (n=21), and the validation set contained dependent variables but was blinded to outcome variable (n=138). Multiple logistic regression was used to fit a refined predictive model using BMI, 11OHA4, 11KA4, and 11KT as dependent variables and diagnosed endometriosis as the outcome variable (A, B). Samples with missing data were excluded as part of the regression analysis. (C) ROC curve analysis of the model demonstrated excellent discrimination between HC and ENDO with AUC of 0.965 ($P<0.0001$, CI 0.9124-1.000). The estimated negative predictive power of the model was 94.74% with a positive predictive power of 95.24%. (A, D) The actual performance of the model was assessed using the remaining data with outcome blinded (validation set). (E) The sensitivity for ENDO was 95.65% with accurate prediction observed in all disease stage stratifications; including grouped minimal/mild (Stage I + II), moderate/severe (Stage III + IV), or by individual rASRM Stage (I-IV).

stage stratifications (Figure 4E), including grouped minimal/mild ENDO (Stage I + II; 94% sensitivity), moderate/severe ENDO (Stage III + IV; 96.55% sensitivity), or by individual rASRM stage (I-IV; range 91.2%-100% sensitivity). Consistent with univariate analysis, 11KT had a large odds ratio estimate, indicating the importance of this variable in determining diagnostic outcome. These data confirm the utility of using androgen concentrations to predict diagnostic outcome, with a refined model accurately identifying >95% of women with endometriosis in a blinded cohort.

Discussion

We conducted androgen profiling in women with endometriosis and discovered a disease-specific hormone signature characterized by altered adrenal androgen production. When used alone or in combination with multiple logistic regression, androgen concentrations effectively differentiated between healthy controls and women with endometriosis, establishing altered adrenal androgens as a key feature of endometriosis pathophysiology. When data were partitioned into training and validation

cohorts, a refined statistical model demonstrated over 95% sensitivity in a blinded sample set, highlighting the potential for androgen concentrations to be used for diagnostic purposes. These findings represent a significant breakthrough in the search for disease-relevant blood biomarkers, positioning 11-oxygenated androgens at the core of future diagnostic approaches for endometriosis.

We found that 11-oxygenated androgens are increased in endometriosis but whether they are key drivers of disease is yet to be determined. 11KT can activate androgen-dependent processes with a similar potency to T[12] and in our study was more abundant than T in women with endometriosis. Androgens can regulate processes associated with the formation of endometriosis lesions and the androgen receptor has been identified as an endometriosis-associated transcription factor.[16] We propose that androgen excess, described herein, may drive endometriosis pathophysiology through direct regulation of androgen-dependent processes (via T and 11KT) and by acting as obligate substrates for aromatase (via A4 and T) to fuel estrogenic processes in lesions. Changes in androgen metabolism may also have systemic effects that increase the risk of lesion formation over the life course, including changes in innate and adaptive immune cell function and inflammation,[8] which has been reported in other contexts of androgen excess such as PCOS.[17] This may explain how retrograde menstruation can lead to lesion formation in some women but not others.

While our findings demonstrate a positive correlation between circulating androgens and endometriosis, a recent study utilizing genetic methods to examine the causal relationship between androgens and risk of endometriosis showed an inverse relationship between the two.[18] Specifically, Gjorgoska et al. performed two-sample Mendelian randomization analysis to investigate the causal effects of the androgen precursor, DHEAS, T, and the androgen metabolite, androsterone sulfate, on different subphenotypes of endometriosis and showed that genetically predicted DHEAS and T levels are causally associated with decreased risk of endometriosis, while genetically predicted total T levels are inversely associated with risk of peritoneal endometriosis. The potential discrepancy between the current study and that reported by Gjorgoska et al. likely reflects differences in the biological exposure captured by each approach. While mass spectrometry provides direct measurement of hormones at point of sampling, Mendelian randomization estimates the effect of lifelong genetically predicted hormone exposure and risk of endometriosis. Further work integrating observational and genetic approaches will be essential to delineate causality and determine how this may change across the life course and natural history of disease.

If 11-oxygenated androgens are driving pathophysiology then targeting their synthesis or action could be an effective therapeutic approach. Notably, the concentrations of 11KT are not diminished by oral contraceptives or gonadotropin-releasing hormone analog-mediated ovarian suppression.[14] Although widely used, a recent Cochrane review suggested the overall evidence for gonadotropin-releasing hormone analogs reducing pain in endometriosis was low to very low quality.[19] Thus, if 11KT drives endometriosis pathogenesis, this may explain why gonadotropin-releasing hormone analogs are ineffective in some women.

Previously, the synthetic androgen danazol was found to reduce endometriosis-associated pain,[20] but its use was stopped due to unacceptable masculinizing side effects. The mechanism of action of danazol in endometriosis has not been fully characterized, but in other contexts, it has been shown to inhibit adrenal androgen synthesis via suppression of androgen metabolizing enzyme activity.[21] We have found that enhanced adrenal androgen synthesis was a key feature of endometriosis; as characterized by altered activities of the metabolizing enzymes HSD11B2, HSD11B1, and AKR1C3 and the enhanced biosynthesis of 11KT. Thus, the efficacy of danazol in endometriosis may be due to its action on androgen metabolism, which reframes our perception of the pharmacodynamics of this drug. Although danazol was poorly tolerated, our results support revisiting danazol (eg, administered by a local delivery system) or a more selective derivative as a therapeutic approach in endometriosis. The findings in the current study provide a platform for further research into the role of adrenal hormones in endometriosis pathophysiology, with the potential to develop new therapeutic approaches that can transform endometriosis care.

Proposed blood biomarkers for endometriosis include the glycoprotein CA-125, inflammatory cytokines (eg, IL-6, IL-8, TNF-α, and MCP-1 [CCL2]), and the pro-angiogenic factor VEGF.[22,23] However, these markers lack specificity and have not been validated in larger studies. Emerging results from a proteomic screen of serum samples from a large cohort of 805 patients showed promise using a combination of 10 serum protein biomarkers with strong predictive accuracy for the diagnosis of endometriosis, particularly those with severe endometriosis (Stage IV).[24] Similarly, an undisclosed panel of salivary miRNAs has been reported to detect endometriosis with high sensitivity but whether any of these markers vary across different populations, endometriosis subtypes or menstrual cycle stage is still unknown.[25,26]

Finding biomarkers for endometriosis is challenging because they must accurately reflect endometriosis pathophysiology, be consistent across subtypes and not vary with menstrual cycle stage or with hormone treatment. 11-Oxygenated androgens show significant promise in this regard because they meet many of these criteria. However, larger and more representative cohorts will be needed to understand if this hormone signature is generalizable across different demographics and endometriosis subtypes. Our study primarily included participants of European ethnicity, and future research should ensure broader representation to reduce bias. Comparisons to other endocrine disorders will also be important to understand distinct features of endometriosis and enable differential diagnosis from other conditions. Notably, the androgen profile in endometriosis, as described in our study, is different from that reported for PCOS.[13] In PCOS, median concentrations of 11OHA4, and 11KA4, as reported by O'Reilly et al., are 31.7 nmol/L and 13.4 nmol/L, respectively, and are increased relative to healthy controls. In contrast, these analytes were decreased compared to healthy controls in endometriosis in our study with lower median concentrations of 11OHA4 and 11KA4 in endometriosis; 5.67 and 1.64 nmol/L, respectively. An integrated analysis of androgen profiles from both disorders is warranted as this could provide resolution on their distinct hormone features with the potential to support differential diagnoses. In the context of endometrial cancer, Gjorgoska et al. evaluated the diagnostic and prognostic utility of preoperative serum steroid profiles, including both classic and 11-oxygenated androgens, alongside established biomarkers.[27] They found that steroid measurements alone had modest diagnostic and prognostic performance, but their incorporation into multivariable models enhanced overall

diagnostic accuracy. A similar integrative approach as outlined in the present study may also prove valuable in the context of endometriosis diagnosis.

One of the limitations of this study is the inference of enzymatic activities from ratios of products/substrates instead of measuring enzymatic activities directly. This caveat needs to be considered for data interpretation, especially because the products of the reactions can be metabolized further. Measuring enzymatic activities directly is challenging in the current paradigm since the cells contributing to the altered androgen metabolism are not known, necessitating further investigation. Potential sources of altered androgen metabolism in endometriosis could be the lesions themselves, as endometrial cells have been shown to have the capacity to express the full complement of the steroidogenic genes involved in androgen and estrogen synthesis.[28] Adipose tissue also impacts the bioavailability of 11-oxygenated androgens through AKR1C3-dependent activation of 11KA4 to 11KT. General indicators of adiposity such as BMI may therefore influence the interpretation of 11-oxygenated androgen concentrations in different cohorts. The BMI of the endometriosis cohort was greater than healthy controls in the current study, but neither cohort was in the obese range. To limit bias in interpretation of direct comparisons between cohorts, we included BMI in our statistical models to account for any independent effects of this variable. Notably, 11-oxygenated androgens robustly distinguished between healthy controls and women with endometriosis when BMI was included in predictive models, consistent with a distinct association between endometriosis and 11-oxygenated androgen concentrations.

In summary, we found that women with endometriosis had a distinct hormone signature characterized by systemic differences in adrenal androgen concentrations. Together, these findings redefine endometriosis as an androgen-dependent disorder and highlight the potential for 11-oxygenated androgens to be used as diagnostic biomarkers and therapeutic targets.

Materials and methods

Study design

Serum was collected from women with laparoscopically confirmed endometriosis (ENDO) and healthy controls (HC). Samples were analyzed using LC–MS/MS to comprehensively measure serum androgen hormones in endometriosis. Steroid measurements were compared between groups and used to generate statistical models to predict diagnostic outcome. Research was conducted in compliance with the Declaration of Helsinki.

Study approvals

Written informed consent was obtained prior to study participation from healthy volunteers identified through advertisement. Ethical approval was granted by the Science, Technology, Engineering and Mathematics Ethical Review Committees of the University of Birmingham, UK (ERN_17-0494, ERN_17-0494B).

Written informed consent was obtained from all endometriosis study participants prior to surgery. Ethical approval was granted by the Lothian Research Ethics Committee (LREC 11/AL/0376), South Central-Hampshire A research ethics committee (IRAS:237815 REC reference 19/SC/0449) and Wales REC 6 A research ethics committee (IRAS 268806; REC ref: 19/WA/0271). Methods were carried out in accordance with the local Tissue Governance guidelines and international EPHect guidelines (https://endometriosisfoundation.org/ephect/).

Patient cohorts

Healthy controls

Inclusion criteria for healthy controls were pre-menopausal women aged 18 years or above. A standardized questionnaire was used to record demographic data including age and BMI; the use of hormonal contraceptives and menopausal status were recorded. Healthy control data were selected from a broader cohort of participants as detailed in Schiffer et al. to match the age range of the patient cohort in the current study.[14]

Endometriosis

Eligible participants were women with chronic pelvic pain (aged 18-50 years) of >3 months duration who were undergoing diagnostic laparoscopy for suspected endometriosis in NHS Lothian. Pelvic pain was defined as pain located within the true pelvis (between and below the anterior iliac crests). Participants at the Liverpool Women's Hospital were those undergoing diagnostic laparoscopy for suspected endometriosis who were recruited from endometriosis or general gynecology clinics. Diagnostic outcome, age, BMI, menstrual cycle stage, and hormone status were obtained and recorded along with other key clinical data. Diagnosis of endometriosis was confirmed macroscopically at laparoscopy. Endometriosis was subsequently classified according to the revised scoring system of the American Society for Reproductive Medicine (rASRM) which ranges from Stage I to Stage IV based on the location and extent of lesions, as well as adhesions, visualized at surgery.[29]

Menstrual cycle phase

Menstrual cycle phase was determined based on the day of the start of the last menstrual bleed (defined as day 1) and samples categorized as either proliferative (days 1-14) or secretory phase (days 14 or greater) based on the day of sample collection relative to the day of last menstrual bleed. In the endometriosis cohort, the menstrual cycle phase was further corroborated by histological staging of endometrial tissue by an expert pathologist.

Exclusion criteria

Exclusion criteria for both cohorts were any acute or chronic disease affecting steroid biosynthesis or metabolism (including PCOS) and the intake of any medication known to interfere with steroid biosynthesis or metabolism. Participants using hormonal contraceptives (combined oral contraceptives, contraceptive depot injection, or implant) were excluded.

Serum steroid analysis

All venous blood samples were collected in the morning and, in the case of women with suspected endometriosis, prior to anesthesia on the morning of surgery. All blood samples were collected in serum-separating tubes. After centrifugation, aspirated serum was aliquoted and stored at −80 °C. ENDO

($n = 159$) and HC ($n = 57$) serum samples were profiled using a sensitive LC–MS/MS assay to simultaneously measure androgen hormones/metabolites.

Serum steroids were quantified using a previously published and validated approach for multi-steroid profiling using LC–MS/MS.[30] Briefly, 200 μL of serum was mixed with stable isotope-labeled internal standards, and following protein precipitation with 50 μL acetonitrile, the samples were extracted by liquid–liquid extraction with 1 mL methyl tert-butyl ether (MTBE). The MTBE organic phase was removed, dried, and reconstituted in 50/50 methanol/water. Steroids were chromatographically separated using a Phenomenex Luna Omega C18 column (1.6 μm, 100Å, 2.1 mm × 50 mm) and a water (0.1% formic acid)–methanol gradient. Ammonium fluoride was introduced by post-column infusion to aid ionization. Steroids were quantified relative to a calibration series ranging from 0.02 to 250 ng/mL with inclusion of a blank calibration point. Analysis was performed on a Waters Xevo TQ-XS mass spectrometer using electrospray ionization in positive ion mode.

Analyte key

Serum analytes were grouped as follows: classic androgens, DHEA, A4, T, and DHT; 11-oxygenated androgens, 11β-hydroxy androstenedione (11OHA4), 11β-hydroxytestosterone (11OHT), 11-ketoandrostenedione (11KA4), and 11-ketotestosterone (11KT).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10. Undetected steroid concentrations or data below the lower limit of quantification (LLOQ) were replaced by 0.5×LLOQ for statistical purposes. Data were summarized using mean ± SD, presented for HC ($N = 57$) and ENDO ($N = 159$) or as median ± interquartile range in tables, as indicated. T test was used to compare the difference in the means of the two groups. Two-way ANOVA was used to determine the significance between treatments in grouped data. Non-parametric testing was utilized where sample sizes were insufficient to confirm the normality of data distribution. The Mann–Whitney test was used to assess variance between two groups, and the Kruskal–Wallis test was used to assess differences between multiple groups. Criterion for significance was $P < 0.05$. Correlation between analytes was assessed using the Pearson matrix. Discrimination between cohorts using metabolite concentrations or ratios was assessed by ROC curve analysis. Predictive modeling for multiple analytes was performed using multiple logistic regression.

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Authors' contributions

Ioannis Simitsidellis (Formal analysis, Investigation, Methodology, Writing—original draft, Writing—review & editing [equal]), Rebecca J Ainslie (Formal analysis, Methodology, Writing—original draft, Writing—review & editing [equal]), Angela Taylor (Data curation, Formal analysis, Methodology, Writing—original draft, Writing—review & editing [equal]), Lorna C. Gilligan (Data curation, Formal analysis, Methodology [equal]), Fozia Shaheen (Data curation, Formal analysis, Methodology [equal]), Samira Blanke (Writing—original draft, Writing—review & editing [equal]), Craig Anderson (Data curation, Formal analysis [equal]), Dharani K. Hapangama (Methodology, Resources [equal]), Wiebke Arlt (Formal analysis, Funding acquisition, Writing—review & editing [equal]), Andrew W Horne (Resources, Writing—original draft, Writing—review & editing [equal]), Phillipa Saunders (Funding acquisition, Writing—original draft, Writing—review & editing [equal]), and Douglas A Gibson (Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing—original draft, Writing—review & editing [lead], Data curation, Investigation, Methodology, Resources [equal])

Supplementary material

Supplementary material is available at *European Journal of Endocrinology* online.

Conflicts of interest

A.W.H. reports receiving grants from the UK Research and Innovation, UK National Institute for Health and Care Research, Scotland's Chief Scientist's Office, Wellbeing of Women, and Roche Diagnostics; consultancy fees from Roche Diagnostics, Gesynta, and Thramex; lecture fees from Gedeon Richter; serving as the President of the World Endometriosis Society, Co-editor in chief of Reproduction and Fertility, Trustee and Medical Adviser to Endometriosis UK, and Specialty Adviser to the Scottish Government's Chief Medical Officer for Obstetrics and Gynaecology. P.T.K.S.'s institution (University of Edinburgh) receives payment for consultancy from Gesynta and Heranova. D.A.G., P.T.K.S., and A.W.H. are listed as co-inventors on UK Patent Application (No. 2217921·2).

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Data availability

All data are available in the main text or the supplementary materials.

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