Assessing the androgenic and metabolic heterogeneity in Polycystic ovary syndrome (PCOS) using cluster analysis

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Abstract

Introduction: Some but not all women with polycystic ovary syndrome (PCOS) develop metabolic syndrome (MS). The objective of this study was to determine if a subset of women with PCOS had higher androgen levels predisposing them to MS, and whether routinely measured hormonal parameters impacted on the Metabolic syndrome score (siMS).

Methods: We included data from a discovery (PCOS clinic data) and a replication cohort (Hull PCOS Biobank) and utilized eight routinely measured hormonal parameters in our clinics (free androgen index [FAI], sex hormone-binding globulin, dehydroepiandrosterone sulphate, androstenedione, luteinizing hormone [LH], follicular stimulating hormone, Anti-Müllerian Hormone and 17 hydroxy-progesterone[17-OHP]) to perform a K-means clustering (an unsupervised machine learning algorithm). We used NbClust Package in R to determine the best number of clusters. We estimated the siMS in each cluster and used regression analysis to evaluate the effect of hormonal parameters on SiMS.

Results The study consisted of 310 women with PCOS (discovery cohort: n=199, replication cohort: n=111). The cluster analysis identified two clusters in both the discovery and replication cohort. The discovery cohort identified a larger cluster (n=137) and a smaller cluster (n=62), with 31% of the study participants. Similarly, the replication cohort identified a larger cluster (n=74) and a smaller cluster (n=37) with 33% of the study participants. The smaller cluster in the discovery cohort had significantly higher levels of LH (7.26 vs 16.1 IU/L, P<0.001), FAI (5.21 vs 9.22, P<0.001), androstenedione (3.93 vs. 7.56 nmol/l, P<0.001) and 17-OHP (1.59 vs 3.12 nmol/l, P<0.001). These findings were replicated in the replication cohort. The mean (±SD) SiMS score was higher in the smaller cluster, 3.1 (±1.1) vs 2.8(±0.8); however, this was not statistically significant (P=0.20). In the regression analysis, higher FAI (Beta=0.05, P=0.003) and androstenedione (Beta=0.03, P=0.02) were independently associated

with a higher risk of SiMS score, while higher DHEAS levels were associated with a lower SiMS score (Beta=-0.07, P=0.03)

Conclusion

We identified a subset of women in our PCOS cohort with significantly higher LH, FAI and androstenedione levels. We show that higher levels of androstenedione and FAI are associated with a higher siMS, while higher DHEAS levels were associated with lower siMS.

Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine condition in women of reproductive age [1-3]. The prevalence of metabolic syndrome in women with PCOS is high, and up to 33% of women with PCOS will develop metabolic syndrome[4-6]. Metabolic syndrome increases the risk for cardiovascular disease (CVD), type II diabetes, certain cancers, sleep apnoea and psychological problems[4-6]. Women with PCOS demonstrate clinical and biochemical heterogeneity and there are no clear predictors of metabolic syndrome in this population. The previous work[7] on identifying sub- phenotypes of women with PCOS identified reproducible reproductive and metabolic subtypes of PCOS; however, this study used both the hormonal parameters and metabolic outcomes in the clustering process.. More importantly, they did not include important markers such as androstenedione and the emerging marker for PCOS Anti-Müllerian Hormone (AMH) in the clustering algorithm.. The objective of this study was to understand if the routinely measured hormonal parameters in PCOS can identify a subset of women with PCOS who have a higher Metabolic syndrome score (siMS). To investigate this we use cluster analysis to identify subgroups of PCOS based on routine hormonal parameters; next, we if any of those subgroups are associated with a higher siMS and, finally, we look at the association of individual hormonal parameters clustered within those subgroups with the siMS

Methods

Study sample

The study consisted of: 1) a discovery cohort of women diagnosed with PCOS in a clinic from the PCOS audit conducted at the Hull University Teaching Hospitals NHS Trust and 2) a replication cohort which consisted of women with PCOS who participated in the Hull PCOS

Biobank study[8,9] and presented sequentially at the Department of Academic Diabetes, Endocrinology and Metabolism. The PCOS audit was approved by the Clinical Audit and Effectiveness Team at the Hull University Teaching Hospital NHS Trust and the Hull PCOS Biobank study was approved by the Newcastle & North Tyneside Ethics committee (ISRCTN70196169). All patients gave written informed consent and all study procedure were conducted in accordance with the Declaration of Helsinki and local regulations. The diagnosis of PCOS was based on at least two out of three of the diagnostic criteria of the Rotterdam consensus, namely clinical and biochemical evidence of hyperandrogenism (Ferriman-Gallwey score >8; free androgen index >4, total testosterone >1.5 nmol/l), oligomenorrhea (Oligomenorrhea is the medical term for infrequent menstrual periods (fewer than six) or very light menstural periods.) or amenorrhea and polycystic ovaries on transvaginal ultrasound[1]. We excluded conditions that mimic PCOS, such as non-classical 21-hydroxylase deficiency, hyperprolactinemia, Cushing's disease and androgen-secreting tumours by using appropriate tests. We used 17-alpha hydroxyprogesterone, prolactin levels, 24-hour urinary cortisol if clinical suspicion of Cushing's disease and free testosterone levels to androgen-secreting tumours. The study measurements have been described in detail previously[8,9]. In summary, we measured body mass index (BMI) (kg/m2), waist circumference (cm), hip circumference (cm), AMH (pmol/l), salivary testosterone (pmol/l), total testosterone (nmol/L), salivary androstenedione (pmol/l), serum androstenedione (nmol/L), SHBG (nmol/L), FAI (%), follicle-stimulating hormone (FSH) (IU/L), luteinizing hormone (LH) (IU/L), fasting glucose (mmol/L), 2-hour glucose (mmol/L) (following an oral glucose tolerance test) and insulin (μIU/ml) levels according to established protocols. We also ascertained oral contraceptive use in both cohorts.

Statistical methods

Univariate comparative analyses were performed using the non-parametric Mann-Whitney tests to evaluate differences in baseline demographics, clinical characteristics, and androgen

levels between PCOS clusters. Means (standard deviations) were used to summarize continuous variables, while proportions and frequencies were used for categorical variables. We imputed missing values using an iterative imputation method, missForest[10]. In brief, this is a non-parametric imputation method which builds a random forest model for each variable and subsequently uses the model to predict missing values in the variable with the help of observed values. There was no missing androgen data in the validation cohort, as they belonged to all the participants of the Hull-PCOS Biobank, which is a very well-characterized cohort. In the PCOS audit cohort, no participant had a missing free-androgen index, and 6% of the participants had missing Androstenedione levels, while 5% of participants had missing DHEAS level.

Clustering

We used K-means clustering[11], an unsupervised machine learning algorithm for partitioning the two PCOS datasets set into a set of k groups (i.e. k clusters). This algorithm classifies objects in multiple clusters such that objects within the same cluster are as similar as possible (with regards to the parameters used in the clustering process), whereas objects from different clusters are as dissimilar as possible (i.e., low inter-class similarity). We used eight routinely measured parameters; Free androgen index (FAI), Sex hormone-binding globulin (SHBG), Dehydroepiandrosterone sulphate (DHEAS), Androstenedione, Luteinizing hormone (LH), Follicular stimulating hormone (FSH), Anti-Müllerian Hormone (AMH) and 17 hydroxy-progesterone (17-OHP) for clustering in the replication and the discovery cohort. We used NbClust package to determine the optimal number of clusters. This package provides 30 indices for assessing the number of clusters and suggests the best clustering scheme from the different results obtained by varying all combinations of number of clusters, distance measures, and clustering methods. To understand the cluster adequacy, we have used the Jaccard coefficient. The Jaccard coefficient measures how frequently pairs of items are joined together in two clustering data sets and how often pairs are observed only in one set. The Jaccard coefficient of more than 0.60

indicates moderate cluster adequacy, while more than 0.75 indicates excellent cluster adequacy. In the validation cohort, we observed a Jaccard coefficient of 0.63, indicating moderate cluster stability. All the statistical analyses were done in R 4.2.1. (https://www.r-project.org/)

siMS score calculation

We used siMS Score[12], a simple method for quantifying metabolic syndrome. SiMS score was calculated by using the formula: [2*waist circumference (cm) /height (cm)) + (baseline glucose [in nmol.L]/5.6) + (triglycerides [in mmol/L]/1.7) + (systolic BP/130) - (HDL [in mmol/L]/1.28)].

Biochemical analyses

Serum Testosterone and Androstenedione were measured by LC/MS/MS on an Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Manchester, UK). SHBG was measured by an immunometric assay with fluorescence detection on the DPC Immulite 2000 analyzer using the manufacturer's recommended protocol (upper limit of the reference range 2.0 nmol/l). The free androgen index (FAI) was calculated as the total testosterone × 100/SHBG. Plasma glucose was measured using a Synchron LX 20 analyzer (Beckman-Coulter), using the recommended protocol. The coefficient of variation for the assay was 1.2% at a mean glucose value of 5.3 mmol/litre. AMH was measured using a Beckman Coulter Access automated immunoassay. 17-OHP was measured in the early morning sample, and if on the higher side of the nomogram, congenital adrenal hyperplasia was excluded with the ACTH stimulation test.

Statistical methods

To evaluate differences in baseline demographics, clinical characteristics and androgen levels between PCOS clusters, univariate comparative analyses were performed using the non-parametric Mann-Whitney tests. Means (standard deviations) were used to summarize continuous variables, while proportions and frequencies were used for categorical variables. We imputed missing values using an iterative imputation method, missForest[10]. In brief, this is a non-parametric imputation method which builds a random forest model for each variable and subsequently uses the model to predict missing values in the variable with the help of observed values. There was no missing androgen data in the validation cohort, as they belonged to all the participants of the Hull-PCOS Biobank, which is a very well-characterized cohort. In the PCOS audit cohort, no participant had a missing free-androgen index, and 6% of the participants had missing Androstenedione levels, while 5% of participants had missing DHEAS level.

Results

Baseline demographic and clinical characteristics

Table 1 shows the demographic and clinical characteristics of the participants in the discovery (n=199) and replication (n=111) cohorts. The mean age of the study participants in the discovery cohort was 28.7 (±6.4) years, with a mean BMI of 37.2 (±8.2) kg/m2 and waist circumferences of 110 (±17.9) cm. The mean FAI of the participants in the discovery cohort was 6.46 (±3.74) with mean levels of SHBG of 28.4 (±19.0) nmol/l, DHEAS of 7.77 (±3.67) µmol/l and androstenedione of 5.06 (±2.28) nmol/l. The mean age of the study participants in the replication cohort was 27.7 (±6.0) years, with a mean BMI of 33.9 (±7.5) kg/m2 and waist circumferences of 101 (±16.0) cm. The mean FAI in the replication cohort was 6.46 (±3.74) with an SHBG of 33.3 (±20.2) nmol/L, DHEAS of 6.11 (±2.83) µmol/l and androstenedione levels of 11.4 (±6.10) nmol/l.

Clustering

Table 2 shows the results of the cluster analysis. The cluster analysis identified two clusters in both the discovery and replication cohort. The discovery cohort identified a larger cluster (n=137) and a smaller cluster (n=62) comprising of 31% of the study participants. Similarly, the replication cohort identified a larger cluster (n=74) and a smaller cluster (n=37) comprising of 33% of the study participants. **Figures 1a and 1b** show the schematic representation of the two clusters. The smaller cluster in the discovery cohort had significantly higher levels of LH (7.26 IU/L vs 16.1 IU/L, P<0.001), FAI (5.21 vs 9.22, P<0.001), androstenedione (3.93 vs 7.56 nmol/l, P<0.001) and 17-OHP (1.59 nmol/l vs 3.12 nmol/l P<0.001). These were replicated in the smaller cluster in the replication cohort with statistically significantly higher levels of LH (6.03 IU/L vs 10.5 IU/L P<0.001), FAI (4.21 vs 10.1, P<0.001), androstenedione (9.2 nmol/l vs 15.8 nmol/l P<0.001) and 17-OHP (5.2 nmol/l vs 6.58 nmol/l P<0.001) (supplementary figure 1).

Association of siMS with clusters and androgens

The mean siMS score was higher in the smaller cluster, 3.1 (±1.1) vs 2.8(±0.8); however, this was not statistically significant (P=0.2). Table 3 shows the results of regression analysis showing the association of the siMS score with the seven hormonal parameters used in the clustering analysis. SHBG was not used in the regression analysis as it is correlated with FAI. In these analyses, higher FAI (Beta=0.05 P=0.003) and androstenedione (Beta=0.03, P=0.02) were independently associated with a higher risk of siMS score, while higher DHEAS levels were associated with a lower siMS score (Beta=-0.07, P=0.03). Supplementary figure 2a shows the mean BMI in four quartiles of DHEAS in the PCOS audit cohort. The BMI was highest in the first quartile (38.87(±9.2)) and incrementally lower in the second (37.3(±8.2)) third (36.7(±7.8)) and fourth (34.8(±7.2)) (P-Anova =0.01) quantile of DHEAS. A similar trend was

seen in the waist circumference with (Supplementary figure 2b) with highest waist circumference seen in the first DHEAS quartile (112.4(±19.7)) and incrementally lower in second (109.6(±16.8)), third (107.5(±15.6)) and fourth quantile (106.1(±17.9)) of DHEAS (P-Anova =0.06) .

Association of clusters with use of oral-contraceptive pills, metformin and spironolactone

In the PCOS audit data, 163 (89%) women were on metformin, 50 (25%) were on spironolactone and 59 (29%) were on the oral contraceptive pills. We looked at the use of oral-contraceptive use, metformin and spironolactone in each of the clusters in the discovery cohort. Since most of these patients were included from first clinic visit to the hospital a small proportion of them were on medications. The use of oral-contraceptive pills (P=0.42), metformin (P=0.39) and spironolactone (P=0.41) was not significantly different across the three cohorts.

Discussion

In this study, we identified two subtypes of PCOS based on routinely measured hormonal parameters. We show that a smaller subgroup of women with PCOS with higher LH, FAI and androstenedione have a higher siMS; however this was not statistically significant in this study. We also showed that higher androstenedione and FAI levels are independently associated with a higher risk of siMS, while higher levels of DHEAS level is associated with lower siMS.

In women with PCOS, the major circulating androgens and/or pro-androgens are DHEAS, Dehydroepiandrosterone (DHEA,) androstenedione, testosterone, and Dihydrotestosterone (DHT)[13]. The cells in the ovaries are stimulated to produce androgens via LH-mediated activation of a number of regulatory enzymes, such as StAR, P450scc, 3a-HSD-II, and

P450c17[13]. As there is no robust negative feedback mechanism[14] for androgens, the subset of women with PCOS with high LH levels can produce a phenotype of PCOS with elevated androgen levels. In this study, we identified a subgroup of women with PCOS in two independent cohorts with higher LH levels and, consequently, higher FAI and androstenedione levels. The cause of LH hypersecretion in this subgroup is unclear, but could be due to enhanced pituitary sensitivity to gonadotropin-releasing hormone (GnRH)[15,16].

In the next step, we assessed if this smaller subgroup of PCOS had higher LH androgens levels and had a higher risk for metabolic syndrome. It is difficult to assess variations in metabolic syndrome in women with PCOS as most of them have a high BMI and waist circumference and would be classified as having metabolic syndrome. Hence, we used a continuous measure of metabolic syndrome using the siMS[12]. We showed that the smaller subgroup of women with high LH and androgens were likely to have a higher siMS; however, this finding was not statistically significant.

Previous studies have shown that FAI and androstenedione are associated with metabolic syndrome in PCOS[17-21]. In this work, we showed that these two androgens are independently related to siMS in women with PCOS and thus, could contribute to metabolic syndrome in PCOS through different pathophysiological pathways. Women with PCOS are known to have higher levels of DHEAS[22]. Interestingly, we show that higher levels of DHEAS were associated with a lower siMS. DHEAS is known to be pro-androgen or androgen metabolite, depending on how it is metabolized. DHEAS can be converted to Dehydroepiandrosterone (DHEA) which is a metabolic intermediate in the biosynthesis of the androgen and estrogen sex steroids both in the gonads and in various other tissues[23,24]. The literature on PCOS suggests that the predominant source of androgen overproduction in women with PCOS is from the ovaries than the adrenals[25-28]. However, higher levels of DHEAS from the ovaries are also found in 25-50% of women with PCOS[25,27,28]. Studies have also shown women with PCOS who have a higher DHEAS level are younger,

have a lower BMI, and have more hirsutism than PCOS patients with normal DHEAS levels[26]. The relationship between adrenal production of DHEAS in PCOS is not well understood, however, it is possible that there is a subset of women with PCOS with predominant adrenal androgen contribution and reduced susceptibility to metabolic complications of PCOS. Large population-based studies and Mendelian randomization studies looking at the effect of genetic determinants of DHEAS levels on metabolic syndrome in PCOS can delineate the effects of DHEAS in PCOS. Several studies have shown the beneficial effect of DHEAS on obesity[29,30], lipid profile, glucose levels[31,32]and insulin resistance[33,34]. Supplementation of DHEAS in obese postmenopausal women has been shown to increase plasma DHEAS levels and improve anthropometric characteristics, leading to a better metabolic profile[35]. A similar trend was observed in our study with lower levels of DHEAS associated with higher BMI and DHEAS which improved with increase in the DHEAS levels. Higher levels of DHEAS may reflect attenuation of pathways which convert into DHEAS to DHEA, resulting in lower levels of androgens and thus offering protection against metabolic syndrome in this population. Nonetheless, these data call for randomized controlled trials looking at the effect of DHEAS supplementation on metabolic syndrome in PCOS.

Our study had several limitations. Although we did not collect data on the ethnicity of study participants, the majority were Caucasians due to the demographics of the recruitment area and hence limiting the generalizability of these results to other ethnic groups. Since this was a cross-sectional study, the causality of the findings cannot be established, and it is unclear if DHEAS levels cause improvement in the siMS. Despite these limitations, we have demonstrated the heterogeneity in PCOS using routinely measured hormonal parameters.

In summary, we showed that women with PCOS demonstrate an androgenic heterogeneity which can influence metabolic outcomes in this population. Specifically, a subset of women with PCOS had higher LH and androgen levels appear to be at higher risk for metabolic syndrome. We also show the beneficial effects of DHEAS on siMS in women with PCOS.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request pending approval from the Hull University teaching hospital NHS trusts