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# Association of severity of menstrual dysfunction with hyperinsulinemia and dysglycemia in polycystic ovary syndrome

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**STUDY QUESTION:** Is the severity of menstrual cyclicity related to hyperinsulinemia and dysglycemia in women with hyperandrogenic polycystic ovary syndrome (PCOS)?

**SUMMARY ANSWER:** Hyperandrogenic PCOS women with amenorrhea, compared to those with oligomenorrhea or eumenorrhea, had a greater risk of post-challenge hyperinsulinemia, which may explain their higher prevalence of dysglycemia.

**WHAT IS KNOWN ALREADY:** PCOS is associated with metabolic dysregulation including insulin resistance (IR) and hyperinsulinemia, risk factors for type 2 diabetes mellitus (T2DM) and other vascular-metabolic morbidities. Although the severity of menstrual cyclicity is associated with IR in PCOS, it is unclear whether, and to what extent, it is related to hyperinsulinemia and glycemic abnormalities.

**STUDY DESIGN, SIZE, DURATION:** We prospectively compared the degree of menstrual cyclicity with the presence of dysglycemia (elevated 1-h plasma glucose ≥155 mg/dl; abnormal glucose tolerance [AGT], including prediabetes and T2DM; and AUC for glucose [G-AUC]) or dynamic state hyperinsulinemia (peak insulin levels either at 1 or 2 h of the oral glucose tolerance test (oGTT) and AUC for insulin [I-AUC]) in 333 hyperandrogenic PCOS women.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** In a tertiary care setting, hyperandrogenic PCOS participants with ovulatory eumenorrhea (Ov-Eumeno, n = 25), anovulatory eumenorrhea (Anov-Eumeno, n = 33), oligomenorrhea (Oligo, n = 150) and amenorrhea (Ameno, n = 125) underwent comprehensive phenotyping and a 2-h 75 g oGTT.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Mean BMI was greater among Ameno women than among Oligo, Anov-Eumeno or Ov-Eumeno women. Adjusting for BMI, the Ameno group demonstrated higher mean 1- and 2-h insulin and glucose, peak insulin and I-AUC and G-AUC, and either had a higher, or tended toward having a higher, prevalence of elevated 1-h glucose level and prevalence of AGT than the Oligo, Anov-Eumeno or Ov-Eumeno groups. In logistic regression, adjusting for BMI, Ameno women were more likely to have: AGT than Oligo women (odds ratio [OR]: 2.3; 95% CI: 1.3 to 4.2); elevated 1-h glucose (OR: 10.2; CI: 1.3–79.7) than those with Ov-Eumeno; and both AGT (OR: 1.7; CI: 1.1–2.6) and elevated 1-h glucose (OR: 1.8; CI: 1.1–2.8) than those with Anov-Eumeno or Ov-Eumeno when combined. Race/ethnicity, age, waist-to-hip ratio, fasting insulin and glucose, and biochemical or clinical measures of hyperandrogenism were similar across the four menstrual categories.

**LIMITATIONS, REASONS FOR CAUTION:** Our study was limited by its cross-sectional nature and by studying women affected by PCOS as defined by the Androgen Excess & PCOS Society criteria (i.e. Rotterdam Phenotypes A, B and C) who were identified in the clinical setting. Consequently, extrapolation of the present data to other PCOS phenotypes (e.g. PCOS Phenotype D) should be made with caution.

**WIDER IMPLICATIONS OF THE FINDINGS:** In hyperandrogenic PCOS phenotypes, a history of amenorrhea, compared to oligomenorrhea or eumenorrhea, suggests a more severe cardiometabolic risk, including a higher degree of hyperinsulinemia and greater prevalence of glycemic abnormalities. These findings may assist in refining the treatment and screening guidelines for glycemic abnormalities in PCOS.

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**Key words:** menstrual dysfunction / metabolic dysfunction / glucose intolerance / hyperinsulinemia / diabetes, 1-h glucose / PCOS

# Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine-medical disorder in females, occurring in 10–15% of reproductive-aged women (Bozdag *et al.*, 2016; Lizneva *et al.*, 2016) and is associated with enhanced risk for metabolic and vascular dysfunction (Ezeh *et al.*, 2018, 2019; Berni *et al.*, 2021; Joham *et al.*, 2021). Approximately 65–95% of PCOS women demonstrate insulin resistance (IR) and compensatory hyperinsulinemia (Dunaif *et al.*, 1989; Ezeh *et al.*, 2020b, 2021), which increase their risk of glycemic abnormalities, including prediabetes, type 2 diabetes mellitus (T2DM) (Legro *et al.*, 1999; Ryu *et al.*, 2021) and metabolic syndrome (Meyer *et al.*, 2020), which confer increased risks for cardiovascular disease (Berni *et al.*, 2021; Joham *et al.*, 2021). Overall, PCOS has substantial health and economic costs (Riestenberg *et al.*, 2021).

Women with PCOS have a 4-fold increased risk of T2DM, a population attributable risk of 19.4–28% (Rodgers *et al.*, 2019), a younger disease onset and a more rapid conversion from prediabetes to T2DM (Ehrmann *et al.*, 1999; Celik *et al.*, 2014) compared to individuals without PCOS, buttressing the concept of PCOS as a prediabetic state (Dunaif *et al.*, 1989; Ehrmann *et al.*, 1999; Legro *et al.*, 1999; American Diabetes Association, 2017). Compelling evidence indicates that early detection and intervention can delay, if not prevent, the transition from prediabetes to T2DM and reduce the diabetes-related disease burden (Tabák *et al.*, 2012; American Diabetes Association, 2017). Thus, it is important to identify PCOS women at the greatest risk of glycemic abnormalities in order to optimize preventive and therapeutic strategies.

Compensatory hyperinsulinemia, characterized by high circulating insulin levels stemming from peripheral IR, is a key feature of PCOS (Barbieri *et al.*, 1986; Dunaif *et al.*, 1989; Moghetti *et al.*, 1996; Ezeh *et al.*, 2018, 2020b). In fact, the degree of hyperinsulinemia in PCOS is greater than that found in other IR disorders (e.g. T2DM), as pancreatic islet β-cell function is generally still robust, albeit often not fully normal (e.g. distemporal and delayed) (Dunaif and Finegood, 1996; Ehrmann *et al.*, 2005). There is increasing evidence that postprandial hyperinsulinemia may be the root cause of many diseases, including obesity, coronary heart disease, T2DM and others (Rizza *et al.*, 1985; Després *et al.*, 1996; Abdul-Ghani and DeFronzo, 2021). Although the relationship of hyperinsulinemia to other morbidities in PCOS has been less well studied, it is well established that hyperandrogenism in PCOS is partly underpinned by hyperinsulinemia (Barbieri *et al.*, 1986; Moghetti *et al.*, 1996).

IR (Martin *et al.*, 1992) and hyperinsulinemia (Haffner *et al.*, 1986) predict future development of glycemic abnormalities. Interestingly, we have previously demonstrated an association between the severity of menstrual cyclicity and IR, estimated either at baseline (Brower *et al.*, 2013) or dynamically (Ezeh *et al.*, 2021), in PCOS. Furthermore, many epidemiological studies have associated women with a history of irregular menstruation with greater risks of T2DM, metabolic syndrome, coronary heart disease and premature mortality, compared to healthy women with regular menstrual cycles (Solomon *et al.*, 2001; Wang *et al.*, 2011, 2020a,b; Polotsky *et al.*, 2012).

We hypothesize that the severity of menstrual dysfunction is associated with hyperinsulinemia and glycemic abnormalities and could potentially be used as a proxy for metabolic dysfunction in women with PCOS. To test this hypothesis, we studied a population of 333 hyperandrogenic PCOS women, recruited prospectively, who underwent a 75-g oral glucose tolerance test (oGTT), assessing both plasma insulin and glucose levels.

# Materials and methods

## Study population

Research participants included women with PCOS prospectively and consecutively recruited through advertisements, and the clinical and research practices of the University of Alabama at Birmingham (UAB) and the Center for Androgen-Related Disorders at Cedars Sinai Medical Center (CSMC), Los Angeles. Because we were interested in studying metabolic dysfunction, only adult PCOS female participants with hyperandrogenic PCOS diagnosed by the Androgen Excess & PCOS Society criteria (Azziz *et al.*, 2006), equivalent to Phenotypes A, B and C of the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004), were included. Study subjects demonstrated clinical and/or biochemical hyperandrogenism, including a modified Ferriman-Gallwey (mF-G) hirsutism score $\geq$6 and/or hyperandrogenemia (i.e. total testosterone [T], free T or dehydroepiandrosterone sulfate [DHEAS] above normal) while other known related endocrinopathies were excluded, as previously described (Knochenhauer *et al.*, 1998; Azziz *et al.*, 2004a,b). Only PCOS participants of African American, Hispanic White and non-Hispanic White ancestry were enrolled in this prospective cohort study, because of the small number of other ethnicities/races.

Study exclusion criteria included pregnancy or lactation state, other endocrine disorders including known pre-existing T2DM, which precluded the performance of an oGTT, inability to assess menstruation or ovulation status (e.g. prior hysterectomy, bilateral oophorectomy, vaginal agenesis, or postmenopausal or premenarcheal state), or use of any hormonal medication (including oral contraceptives, insulin-sensitizing agents, anti-diabetic medications, antiandrogens or glucocorticoids) within 3 months preceding the evaluation. All subjects had normal thyroid-stimulating hormone, 17-hydroxyprogesterone and prolactin levels, as previously described (Knochenhauer *et al.*, 1998; Azziz *et al.*, 2004a,b). Screening for Cushing's syndrome and androgen-secreting neoplasms was performed if clinically indicated.

## Ethical approval

The study was reviewed and approved by the Institutional Review Boards of CSMC and UAB. All subjects were fully informed about the study and provided written informed consent before study entry.

## Protocol

All subjects completed a uniform questionnaire providing information regarding age, race and menstrual history, which were further reviewed at consultation. Participants with PCOS were grouped according to the interval between episodes of vaginal bleeding (Treloar *et al.*, 1967; Brower *et al.*, 2013; Ezeh *et al.*, 2021). As previously described, women with cycle lengths <26 days were considered to have polymenorrhea (Poly); those with 26–34 days bleeding intervals were considered eumenorrheic (Eumeno) and their ovulatory function assessed by measuring a menstrual cycle Days 22–24 progesterone (P4) level; eumenorrheic women with a P4 <4 ng/ml were considered anovulatory (i.e. Anov-Eumeno), whereas the remainder were considered to be ovulatory (i.e. Ov-Eumeno) (Wathen *et al.*, 1984; Brower *et al.*, 2013; Ezeh *et al.*, 2021). PCOS women with 35 days to 3 months bleeding intervals were classified as oligomenorrheic (i.e. 35 days to 6 weeks [Early-Oligo] and 6 weeks to 3 months [Late-Oligo]), and those with cycles >3 months were classed as amenorrheic (Ameno) (Brower *et al.*, 2013; Ezeh *et al.*, 2021).

We should note that continuing controversy has surrounded the definition of normal versus abnormal menstruation, with some investigators defining normal menstrual length as a 21–35 days interval (Steiner *et al.*, 2001), while others define it as 26–31 days (Solomon *et al.*, 2001; Wang *et al.*, 2020a,b) or 25–45 days (Gaete *et al.*, 2010). In turn, the FIGO Menstrual Disorders Committee has standardized the classification of Abnormal Uterine Bleeding and set the definition of normal menstrual length/frequency as a 24–38 days interval (Munro *et al.*, 2018). Considering these controversies, in the present study, as well as in our prior studies (Brower *et al.*, 2013; Ezeh *et al.*, 2021), we have defined a normal menstrual interval as being 26–34 days and defined polymenorrhea as a menstrual cycle <26 days in length in order to provide continuity to our definition of menstrual cyclicity.

All subjects underwent a history and physical examination, as previously described (Knochenhauer *et al.*, 1998; Azziz *et al.*, 2004a,b). In addition to height, weight and mF-G score, waist circumference was measured at the narrowest portion of the torso approximately midway between the lower costal margin and the iliac crest, and the hip circumference was measured over the widest portion of the gluteal and greater trochanteric region. The BMI and waist-to-hip ratio (WHR) were then calculated. Polycystic ovarian morphology was assessed by either transvaginal ultrasonography or abdominal ultrasonography for those patients not tolerating or nondesirous of transvaginal ultrasonography (Philips EnVisor Ultrasound System, with 6.26 MHz endovaginal transducer) and was defined per 2003 Rotterdam criteria contemporary with recruitment, including at least one ovary containing 12 or more ovarian follicles measuring 2–9 mm and/or an ovarian volume of more than 10 $cm^3$ (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004; Brower *et al.*, 2013).

## Metabolic assessment

After an overnight fast, blood samples were obtained on Days 3–8 of a spontaneous or a Prometrium® (Solvay Pharmaceuticals, Marietta, GA, USA)-induced withdrawal bleed (i.e. the follicular phase) for measurement of circulating total T, free T and DHEAS, as well as insulin and glucose levels (Woods *et al.*, 2002). All subjects also underwent a standard 2-h 75-g oGTT (American Diabetes Association, 2017) and plasma insulin and glucose levels were determined at 0 min, 1 and 2 h. In accordance with the American Diabetes Association guidelines, glucose tolerance was classified as follows: (i) normal glucose tolerance, by a fasting glucose of <100 mg/dl and/or a 2-h glucose <140 mg/dl; (ii) prediabetes, by a fasting glucose between 100 and 125 mg/dl (impaired fasting glucose) or a 2-h glucose between 140 and 200 mg/dl (impaired glucose tolerance); and (iii) T2DM, by a fasting glucose ≥126 mg/dl or a 2-h glucose ≥200 mg/dl (American Diabetes Association, 2017). An elevated 1-h glucose was defined as ≥155 mg/dl during the oGTT, reported to be a robust predictor of future risk for T2DM (Cubeddu and Hoffmann, 2010; Bianchi *et al.*, 2013; Peddinti *et al.*, 2019; Jagannathan *et al.*, 2020).

Insulin data from the oGTT were used to calculate peak insulin, defined as the highest insulin levels reached during the oGTT (either at 1 or 2 h) (Jagannathan *et al.*, 2020). The dynamic insulin and glucose responses were quantified by calculating the AUC for insulin (I-AUC) and glucose (G-AUC) during the oGTT, using the standard mathematical trapezoidal method (American Diabetes Association, 2017; Jagannathan *et al.*, 2020).

## Hormonal and biochemical analysis

Total T was measured using a high-turbulence liquid chromatography–tandem mass spectrometry (LC-MS/MS), and free T was determined by equilibrium dialysis (Quest Diagnostics, San Juan Capistrano, CA, USA), as previously described (Salameh *et al.*, 2014) in 282 participants. In 51 consecutive participants, total T was measured using a high-quality radioimmunoassay (RIA) method after serum extraction and chromatography, and sex hormone-binding globulin (SHBG) activity was measured by diffusion equilibrium dialysis, using Sephadex G-25 (Sigma-Aldrich Corp., St. Louis, MO, USA), and [3H]T as the ligand and the free T was calculated, as previously described (Azziz *et al.*, 1995; Boots *et al.*, 1998); the results were converted to LC-MS/MS values as previously reported, as these methods of assessing total T and free T are highly correlated (Salameh *et al.*, 2014; Ezeh *et al.*, 2019). Serum DHEAS and P4 were measured by direct RIA, using commercially available kits (DHEAS and P4 from Diagnostic Products Corp., Los Angeles, CA), as previously described (Knochenhauer *et al.*, 1998). Plasma insulin was assayed by chemiluminescence

(ADVIA Centaur chemiluminescent immunoassay system; Siemens Healthcare, Deerfield, IN, USA) and plasma glucose was measured using the hexokinase/glucose-6-phosphate dehydrogenase method (Roche Applied Sciences). Except for plasma glucose, samples were batched at regular intervals for analysis to minimize the impact of interassay variability. The intra-assay and interassay variations for total T, SHBG, DHEAS, A4, prolactin, thyroid-stimulating hormone, 17-hydroxyprogesterone and P4 have been previously reported and did not exceed 10% (Knochenhauer *et al.*, 1998; Salameh *et al.*, 2014).

## Statistical analysis

The primary outcome for hyperinsulinemia was the peak insulin level, and for dysglycemia, it was the prevalence of abnormal glucose tolerance (AGT), including prediabetes and T2DM. Secondary outcomes included mean I-AUC and G-AUC, and proportion of subjects with elevated 1-h glucose ≥155 mg/dl. The Shapiro–Wilks W-test was used to determine whether continuous variables were normally distributed. All continuous variables, except for the mF-G score, reasonably followed a parametric distribution on the original or log scales.

For the analysis of menstrual dysfunction, participants with PCOS were divided into four groups (Ov-Eumeno, Anov-Eumeno, Oligo and Ameno). Mean intergroup differences were evaluated using a one-way ANOVA (with Tukey's *post hoc* multiple-comparison test) for normally distributed continuous variables or the Kruskal–Wallis test for mF-G score. Differences in frequency were computed with the $\chi^2$ test with Yates correction or Fisher's exact test as appropriate. Differences in mean glucose and insulin levels, and prevalence of glycemic abnormalities were adjusted for BMI, using linear regression and logistic regression analysis, respectively. Because of the same study design, patient phenotyping and laboratory analysis by the same team, and similar populations in the cohorts, data from UAB and CSMC cohorts were pooled for analysis. Continuous variables were expressed as mean ± standard error or geometrical mean (range) if log transformed and categorical variables were expressed as count (percent) unless otherwise stated. A two-sided $P < 0.05$ was considered statistically significant. All statistical analyses were conducted using the Stats Direct statistics software package, version 3.2.10 2020 (Cheshire, UK).

To estimate power analysis, sample size was assessed for hyperinsulinemia using peak insulin levels as the endpoint. Based on our previous studies of the association of menstrual dysfunction and IR (Brower *et al.*, 2013; Ezeh *et al.*, 2021), a power analysis with a pooled standard deviation of 1.49, with an 80% power and an $\alpha = 0.05$, based on unpaired t testing, indicated that a sample size of 35 participants per group was sufficient to detect a mean difference of 35% change in mean peak insulin levels between Ameno and Oligo PCOS. Additionally, we used the prevalence of T2DM as the endpoint to estimate statistical power for assessing differences in dysglycemia. As there is no previous study comparing the prevalence of T2DM by menstrual categories, we considered a difference of 8% in the prevalence of T2DM between Ameno and Oligo PCOS as being clinically significant. This difference is similar or less than that observed when comparing the prevalence of T2DM between PCOS and control subjects (Ehrmann *et al.*, 1999). Using this assumption, we estimated that a sample size of 100 patients in each group would give the study 80% power and an $\alpha = 0.05$.

# Results

A total of 387 consecutive PCOS participants underwent screening for enrollment, of whom 44 (11.3%) participants were excluded because they did not meet the requirements of the study (26 were Asian Americans, 6 Native Hawaiian/Pacific Islander/other Americans and 5 multiracial; and 7 subjects had unclear menstrual history as the only problem), while 10 (2.6%) participants with polymenorrhea (Poly; cycle length <26 days) were also excluded due to the small size of this subgroup, leaving 333 (86.1%) PCOS participants for study. Of the 333 participants finally enrolled during this prospective cross-sectional study, 25 (7.5%) were Ov-Eumeno, 33 (9.9%) were Anov-Eumeno, 150 (45.1%) were Oligo and 125 were (37.5%) Ameno.

## Baseline features

The basic demographic, anthropometric and endocrine characteristics of the 333 participants enrolled in the study are shown in Table I. The majority of the participants were of non-Hispanic White ethnicity. Mean age, biochemical and clinical measures of hyperandrogenism (i.e. mF-G score, and free and total T and DHEAS) were similar across the four menstrual categories, except for a slightly greater mean mF-G score among Anov-Eumeno compared to Oligo (Table I). The prevalence of hirsutism was also similar between groups. Based on current international guidelines for defining hirsutism (i.e. mF-G score ≥ 4) (Teede *et al.*, 2018), the percent of hirsutism was similar among the groups: Ameno (76.4%), Oligo (76.9%), Anov-Eumeno (87.9%) and Ov-Eumeno (73.9%). Similar results were obtained when the cutoff mF-G score ≥6 was used to determine the prevalence of hirsutism (as used for study recruitment criteria, as discussed in the Materials and methods section above): Ameno (61.8%), Oligo (60.8%), Anov-Eumeno (78.1%) and Ov-Eumeno (56.5%). Mean BMI was similar between Oligo and Eumeno but was greater among Ameno than Oligo or Ov-Eumeno and tended toward being greater than Anov-Eumeno (Table I). Therefore, in subsequent analyses, the outcome variables were adjusted for BMI.

## Insulin response to glucose challenge

Table II and Fig. 1 depict the insulin response among all the PCOS participants according to the four categories of menstrual dysfunction. At baseline, mean fasting insulin levels were similar across the menstrual groups. The degree of hyperinsulinemia in response to the glucose challenge (i.e. elevated mean 1- and 2-h insulin, peak insulin and I-AUC) was higher in Ameno vs. Oligo and Ov-Eumeno subjects, but not Anov-Eumeno (Table II and Fig. 1). These values were also higher for Anov-Eumeno versus Ov-Eumeno, but not Oligo patients. The 2 h and peak insulin levels were higher in Oligo versus Ov-Eumeno (Table II and Fig. 1). Separately, we also assessed the insulin response among all the PCOS participants, including PCOS women with Poly or those with Oligo categorized into Early-Oligo (bleeding intervals 35 days to 6 weeks) and Late-Oligo (bleeding intervals 6 weeks to 3 months) (Brower *et al.*, 2013), as depicted in Supplementary Fig. S1 and Table SI.

**Table I Differences in anthropometric and endocrine features by menstrual cyclicity for hyperandrogenic polycystic ovary syndrome (PCOS).**

| Variables | Menstrual cyclicity | | | | *P*-value between groups (adjusted for BMI) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ovulatory PCOS | Ovulatory dysfunction PCOS | | | Ovulatory dysfunction PCOS | | | Ovulatory PCOS vs Ovulatory dysfunction PCOS | | |
| | Ov-Eumeno (n = 25) | Anov-Eumeno (n = 33) | Oligo (n = 150) | Ameno (n = 125) | Anov-Eumeno vs Oligo | Oligo. vs Ameno | Anov-Eumeno vs Ameno | Ov-Eumeno vs Anov-Eumeno | Ov-Eumeno vs Oligo | Ov-Eumeno vs Ameno |
| **Demographics** | | | | | | | | | | |
| Age (years) | 29.4 ± 1.2 | 26.2 ± 1.4 | 28.0 ± 1.4 | 27.5 ± 0.7 | 0.125 | 0.926 | 0.926 | 0.123 | 0.530 | 0.126 |
| BMI (kg/m$^2$)* | 30.2 ± 1.2 | 29.9 ± 1.8 | 30.7 ± 0.8 | 34.2 ± 1.0 | NA | ***0.005*** | 0.082 | NA | NA | ***0.011*** |
| WHR | 0.83 ± 0.02 | 0.84 ± 0.02 | 0.83 ± 0.01 | 0.86 ± 0.01 | 0.071 | 0.810 | 0.810 | 0.671 | 0.899 | 0.461 |
| **Race/ethnicity—no./total no. (%)** | | | | | | | | | | |
| African American | 3 (12) | 5.0 (15.2) | 12 (8.0) | 15 (12.0) | NA | NA | NA | NA | NA | NA |
| Hispanic White | 1 (4) | 6.0 (18.2) | 29 (19.3) | 22 (17.6) | NA | NA | NA | NA | NA | NA |
| Non-Hispanic White | 21 (84) | 22 (66.7) | 108 (72.0) | 88 (70.4) | NA | NA | NA | NA | NA | NA |
| **Androgen measures** | | | | | | | | | | |
| mF-G (Hirsutism) score | 7.5 ± 1.1 | 9.8 ± 1.0 | 7.0 ± 0.37 | 7.7 ± 0.5 | ***0.006*** | 0.517 | 0.075 | 0.147 | 0.565 | 0.762 |
| Free T (pmol/l) | 13.64 ± 2.67 | 14.9 ± 1.8 | 22.4 ± 2.9 | 23.0 ± 4.3 | 0.374 | 0.414 | 0.211 | 0.743 | 0.180 | 0.164 |
| Total T (nmol/L) | 1.92 ± 2.0.31 | 1.5 ± 0.2 | 1.6 ± 0.1 | 1.4 ± 0.1 | 0.556 | 0.854 | 0.624 | 0.158 | 0.390 | 0.241 |
| DHEAS (mol/l) | 7.10 ± 0.76 | 6.63 ± 0.60 | 6.97 ± 0.32 | 6.3 ± 0.4 | 0.592 | 0.235 | 0.949 | 0.994 | 0.850 | 0.847 |

Ameno, amenorrhea; Anov-Eumeno, anovulatory eumenorrhea; DHEAS, dehydroepiandrosterone sulfate; mF-G, modified Ferriman-Gallwey hirsutism score; N/A, not applicable; Oligo, oligomenorrhea; Ov-Eumeno, ovulatory eumenorrhea; PCOS, polycystic ovary syndrome; T, testosterone; WHR, waist to hip ratio. P values in italics and bold are considered statistically significant.
*Denotes that the p-value of BMI between groups is not adjusted.

## Glucose response to glucose challenge and prevalence of glycemic abnormalities

Post-challenge glucose responses according to the severity of their menstrual dysfunction are also depicted in Table II. At baseline, mean fasting glucose levels were similar across the four menstrual groups. The G-AUC was higher for Ameno versus Oligo or Ov-Eumeno subjects and higher for Oligo versus Ov-Eumeno. The mean 1-h glucose was higher in Ameno or Oligo versus Ov-Eumeno, and the 2-h glucose was higher for Ameno vs Oligo (Table II).

The prevalence of glycemic abnormalities according to the severity of menstrual dysfunction are also depicted in Table II and Fig. 2. Of the 333 participants analyzed, 265 (79.6%) had normal glucose tolerance, 86 (25.8%) had an elevated 1-h glucose (i.e. ≥155 mg/dl) and 68 (20.4%) had AGT, including 44 (13.2%) with prediabetes and 24 (7.2%) with T2DM. A significant trend was detected for hyperglycemia at 1 h, which was observed in 36.8% of PCOS participants with Ameno, 22.0% with Oligo, 18.2% with Anov-Eumeno and 4.0% with Ov-Eumeno ($P = 0.001$) (Fig. 2A). No significant difference in trend were detected in the prevalence of prediabetes (18.4%, 10.0%, 12.1% and 8.0% of Ameno, Oligo, Anov-Eumeno and Ov-Eumeno, respectively, $P = 0.079$) (Fig. 2B), although T2DM was detected in significantly more Ameno PCOS women (12.0%, 5.3%, 3.0% and 0% in Ameno, Oligo, Anov-Eumeno and Ov-Eumeno, respectively, $P = 0.008$) (Fig. 2C). Overall, the proportion of PCOS participants with any AGT was highest for Ameno and lowest for Ov-Eumeno patients (30.4%, 15.3%, 15.2% and 8.0% for Ameno, Oligo, Anov-Eumeno and Ov-Eumeno, respectively, $P = 0.008$) (Fig. 2D). In logistic regression, adjusting for BMI, PCOS Ameno patients were more likely to have: (i) an AGT than Oligo patients (odds ratio [OR]: 2.3; 95% CI: 1.3 to 4.2); (ii) an elevated 1-h glucose (OR: 10.2; CI: 1.3–79.7) than those with Ov-Eumeno; and (iii) both a higher frequency of AGT (OR: 1.7; CI: 1.1–2.6) and an elevated 1-h glucose (OR: 1.8; CI: 1.1–2.8) than those with Anov-Eumeno or Ov-Eumeno combined (Table III).

# Discussion

We previously reported that in oligo-ovulatory women with PCOS, the severity of menstrual dysfunction was associated with the presence of and degree of IR (Brower *et al.*, 2013; Ezeh *et al.*, 2021). In this prospective cohort study, we provide novel evidence demonstrating that the severity of menstrual cyclicity is also linked to hyperinsulinemia and glycemic abnormalities in women with hyperandrogenic PCOS (i.e. Rotterdam A, B and C phenotypes), a population shown to have a four-fold higher prevalence of T2DM and a more rapid onset of T2DM than PCOS women with a normoandrogenic phenotype (Persson *et al.*, 2021).

Amenorrheic women with PCOS had greater degrees of hyperinsulinemia compared to those who were oligomenorrheic or eumenorrheic. Furthermore, and despite their higher degree of hyperinsulinemia, PCOS women with amenorrhea demonstrated a higher degree of dysglycemia, as indicated by their higher 1- and 2-h glucose levels, and G-AUC than those with oligomenorrhea or

**Table II** Differences in metabolic characteristics by menstrual cyclicity for hyperandrogenic PCOS.

| Variables | Menstrual cyclicity | | | | *P*-value between groups (adjusted for BMI) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ovulatory PCOS | Ovulatory dysfunction PCOS | | | Ovulatory dysfunction PCOS | | | Ovulatory PCOS vs Ovulatory dysfunction PCOS | | |
| | Ov-Eumeno (n = 25) | Anov-Eumeno (n = 33) | Oligo (n = 150) | Ameno (n = 125) | Anov-Eumeno vs Oligo | Oligo vs Ameno | Anov-Eumeno vs Ameno | Ov-Eumeno vs Anov-Eumeno | Ov-Eumen vs Oligo | Ov-Eumeno vs Ameno |
| **Plasma glucose levels (mg/dl)** | | | | | | | | | | |
| Fasting glucose | 85.5 ± 2.7 | 87.8 ± 2.4 | 86.8 ± 1.1 | 88.4 ± 1.3 | 0.626 | 0.811 | 0.711 | 0.554 | 0.766 | 0.786 |
| 1-hour glucose | 104.7 ± 5.5 | 123.1 ± 8.2 | 123.8 ± 3.6 | 143.7 ± 5.2 | 0.948 | 0.072 | 0.389 | 0.074 | ***0.033*** | ***0.018*** |
| 2-hour glucose | 88.7 ± 2.8 | 106.5 ± 7.3 | 103.7 ± 3.0 | 129.2 ± 7.7 | 0.602 | ***0.010*** | 0.272 | 0.052 | 0.052 | 0.055 |
| G-AUC (mg/dl/120 min) | 11 136.3 ± 528.2 | 13 136.4 ± 756.4 | 13 121.4 ± 312.2 | 15, 135.4 ± 501. | 0.815 | ***0.016*** | 0.267 | 0.079 | ***0.043*** | ***0.015*** |
| **Plasma insulin levels (μIU/ml)** | | | | | | | | | | |
| Fasting insulin | 11.3 ± 2.6 | 11.3 ± 1.3 | 14.9 ± 1.4 | 26.5 ± 6.1 | 0.399 | 0.133 | 0.371 | 0.403 | 0.158 | 0.068 |
| 1-h insulin | 80.9 ± 25.7 | 102.7 ± 15.2 | 92.0 ± 7.2 | 167.3 ± 14.4 | 0.309 | ***<0.001*** | 0.256 | ***0.031*** | 0.172 | ***0.007*** |
| 2-h insulin | 49.1 ± 12.3 | 76.2 ± 13.4 | 66.6 ± 5.6 | 138.6 ± 13.4 | 0.260 | ***<0.001*** | 0.225 | ***0.005*** | ***0.027*** | ***<0.001*** |
| Peak insulin | 85.2 ± 25.9 | 110.1 ± 16.1 | 102.3 ± 7. | 181.4 ± 14.5 | 0.341 | ***0.001*** | 0.230 | ***0.009*** | ***0.012*** | ***0.037*** |
| I-AUC (mg/dl/120 min) | 6654.0 ± 1875.9 | 8575.2 ± 1251.8 | 7964.6 ± 578.5 | 14 899.6 ± 1261.0 | 0.342 | ***<0.001*** | 0.184 | ***0.018*** | 0.404 | ***0.033*** |
| **Glycemic abnormalities—no. (%)** | | | | | | | | | | |
| Abnormal glucose tolerance | 2 (8.0) | 5 (15.2) | 23 (15.3) | 38 (30.4) | 0.896 | ***0.007*** | 0.143 | 0.448 | 0.453 | 0.066 |
| Prediabetes | 2 (8.0) | 4 (12.1) | 15 (10.0) | 23 (18.4) | 0.736 | 0.089 | 0.494 | 0.620 | 0.767 | 0.303 |
| Type 2 Diabetes | 0 (0) | 1 (3.0) | 8 (5.3) | 15 (12.0) | 0.626 | 0.092 | 0.210 | 0.995 | 0.987 | 0.986 |
| Elevated 1-hour PG* | 1 (4.0) | 6 (18.2) | 33 (22.0) | 46 (36.8) | 0.697 | 0.139 | 0.185 | 0.150 | 0.080 | ***0.027*** |

*1-h plasma glucose (PG) ≥155 mg/dl during oGTT.
Ameno, amenorrhea; Anov-Eumeno, anovulatory eumenorrhea; G-AUC, glucose AUC; I-AUC, AUC for insulin; Oligo, oligomenorrhea; Ov-Eumeno, ovulatory eumenorrhea; PCOS, polycystic ovary syndrome.
P values in italics and bold are considered statistically significant.

eumenorrhea. Hyperandrogenic PCOS women with amenorrhea, compared to those with oligomenorrhea or eumenorrhea, were also more likely to develop an elevated 1-h glucose (i.e. ≥ 155 mg/dl), which has been suggested to be a more robust predictor than fasting or 2-h glucose for the future development of metabolic syndrome, β-cell dysfunction, T2DM and coronary vascular disease (Cubeddu and Hoffmann, 2010; Bianchi *et al.*, 2013; Peddinti *et al.*, 2019; Jagannathan *et al.*, 2020). These observations occurred regardless of age, racial/ethnic composition, WHR, degree of hyperandrogenism and even after adjustment for BMI, suggesting that the elevated cardiometabolic risk profile in PCOS participants with amenorrhea is more than can be explained by these cofounding factors. Overall, these data highlight the value of amenorrhea as a marker for the increased risk of hyperinsulinemia and dysglycemia in PCOS.

Notably, hyperandrogenic PCOS women with ovulatory eumenorrhea (Phenotype C) had less post-challenge hyperinsulinemia than anovulatory PCOS women (Phenotypes A/B), consistent with other studies demonstrating that PCOS women with the latter phenotype have higher risk of IR, fasting hyperinsulinemia and metabolic syndrome than those with other types of PCOS phenotypes (Moghetti *et al.*, 2013). These data also affirm the need to evaluate ovulatory function in PCOS women with apparent eumenorrhea.

Our findings also indicate that PCOS women with the most severe form of oligo-ovulation (i.e. amenorrhea), and to some extent those with oligomenorrhea or anovulatory eumenorrhea, had higher degrees of hyperinsulinemia than those with ovulatory eumenorrhea, despite no significant differences in hyperandrogenism. Therefore, it appears that ovulation status (and secondarily, the severity of menstrual cyclicity) are linked more closely to the degree of hyperinsulinemia rather than hyperandrogenism. This hypothesis is supported by studies showing that chronic hyperinsulinemia disrupts ovarian follicular development (Thong *et al.*, 2020), reports indicating that increased prevalence of PCOS in women with Type 1 diabetes is related to the ovarian impact of supraphysiologic doses of exogenous insulin not exposed to hepatic degradation (Thong *et al.*, 2020; Łebkowska *et al.*, 2021), and studies showing that reduction in hyperinsulinemia with lifestyle changes, bariatric surgery or insulin sensitizers enhance ovulation and restore menstrual cyclicity (Morley *et al.*, 2017).

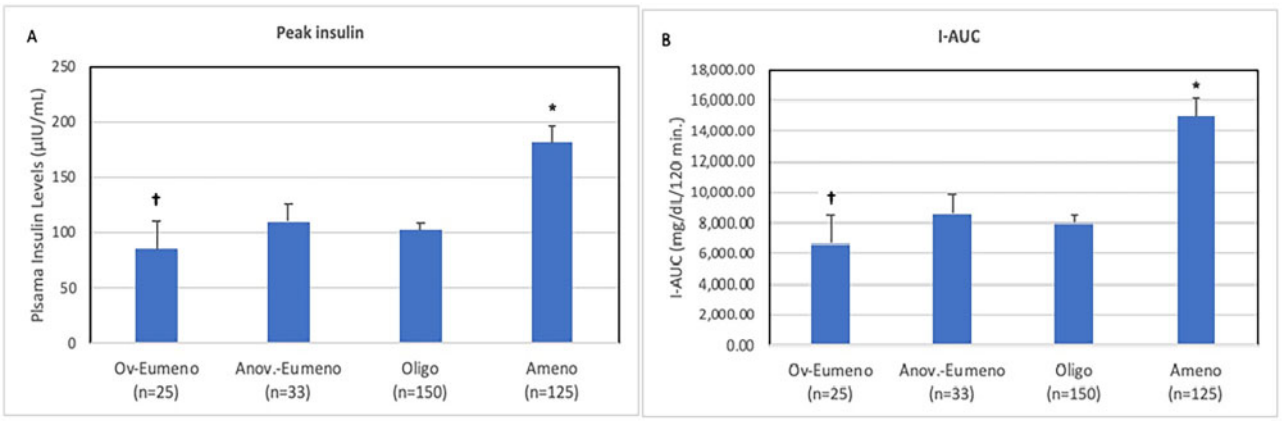


**Figure 1. Differences in the degree of hyperinsulinemia in hyperandrogenic PCOS women according to severity of menstrual cyclicity.** The degree of hyperinsulinemia, reflected by post-challenge peak insulin levels (**A**) and AUC for insulin [I-AUC] (**B**) according to four categories of menstrual cyclicity described as ovulatory eumenorrhea (Ov-Eumeno; bleeding intervals 26- to 34-day with ovulation confirmed by a menstrual cycle Days 22–24 progesterone [P4] level), anovulatory eumenorrhea (Anov-Eumeno; bleeding intervals 26- to 34-day with anovulation confirmed by a menstrual cycle Days 22–24 P4 level); oligomenorrhea (Oligo, bleeding intervals 35 days to 3 months) and amenorrhea (Ameno; bleeding intervals >3 months). Error bars represent SEM. Values have not been adjusted for BMI. *Denotes significantly higher degree of hyperinsulinemia in Ameno than in other menstrual categories. †Denotes significantly lower degree of hyperinsulinemia in Ov-Eumeno than in Anov-Eumeno, or Oligo. PCOS, polycystic ovary syndrome.

The potential mechanisms linking amenorrhea to glycemic abnormalities are not fully understood, and may reflect the pathogenic sequalae of hyperinsulinemia, given that hyperinsulinemia has been implicated in the development of IR, metabolic syndrome and many diseases including obesity, T2DM, coronary vascular disease and cancer (Rizza *et al.*, 1985; Haffner *et al.*, 1986; Martin *et al.*, 1992; Després *et al.*, 1996; Abdul-Ghani and DeFronzo, 2021), instead of hyperinsulinemia just representing mere compensation for IR (Kahn *et al.*, 1993). Furthermore, studies in rodents indicate that chronic hyperinsulinemia uncouples insulin-mediated regulation of glucose transporter-4 and the Forkhead box protein O1 (FoXO1) transcription factor (Gonzalez *et al.*, 2011) and impairs insulin-mediated suppression of circulating non-esterified free fatty acid levels (Koopmans *et al.*, 1999) in adipocytes, leading to adipocyte IR. Furthermore, pharmacological suppression of hyperinsulinemia results in improvements in metabolic syndrome and increased insulin sensitivity, reduction in hyperglycemia (Templeman *et al.*, 2017; Loves *et al.*, 2018) and extension of lifespan (Templeman *et al.*, 2017).

Additionally, hyperandrogenism is known to be underpinned, at least in part, by hyperinsulinemia, via its synergy with LH as a co-gonadotropin to augment ovarian androgen biosynthesis, increased adrenocorticotropic hormone-induced adrenal androgen production, and increased testosterone bioavailability through suppression of hepatic SHBG biosynthesis (Barbieri *et al.*, 1986; Moghetti *et al.*, 1996). This raises the possibility that further increases in hyperandrogenism may exacerbate IR and increase the risk of glycemic abnormalities. Indeed, several studies have suggested that hyperandrogenism contributes to IR in PCOS via adipocyte dysfunction including altered body composition (Ezeh *et al.*, 2014), altered adipocyte morphology (O'Reilly *et al.*, 2017; Dumesic *et al.*, 2019) and non-esterified free fatty acids kinetics (Ezeh *et al.*, 2019), and through inhibition of adipogenesis or/and lipolysis, and promotion of lipogenesis (Dumesic *et al.*, 2019), increased visceral adiposity (O'Reilly *et al.*, 2017; Dumesic *et al.*, 2019) or an increase in adipose tissue IR (Ezeh *et al.*, 2020a). However, our findings indicating that the degree of hyperinsulinemia, but not hyperandrogenism, tracked with the severity of menstrual dysfunction do not support the concept of hyperandrogenism-induced glycemic abnormalities. It should be noted that the evidence that hyperandrogenism contributes to glycemic abnormalities or metabolic risks of PCOS remains overall unresolved. Some longitudinal studies have observed no associations between hyperandrogenism and metabolic syndrome, T2DM, coronary vascular disease or stroke (Calderon-Margalit *et al.*, 2010; Polotsky *et al.*, 2014; LeBlanc *et al.*, 2017; Kim *et al.*, 2018).

The 75-g oGTT is the gold standard for the screening, diagnosis and management of glycemic abnormalities, including prediabetes and T2DM (American Diabetes Association, 2017; Jagannathan *et al.*, 2020). Indeed, many expert organizations recommend routine screening of women with PCOS using a 2-h oGTT (e.g. Endocrine Society, American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and PCOS Society, and the International PCOS Guidelines) (Legro *et al.*, 2013; Teede *et al.*, 2018; Goodman *et al.*, 2015), recognizing the limitations of simpler screening tests such as fasting glucose or HbA1C levels in PCOS (Legro *et al.*, 1999; Lerchbaum *et al.*, 2013). Nonetheless, for many regions of the world and for many patients with PCOS, obtaining an oGTT is not as easy as these guidelines would suggest. Identification of PCOS women at greatest risk of glycemic abnormalities, potentially using a readily obtainable marker, such as the degree of clinically evident menstrual dysfunction, may help refine the indications for oGTT in PCOS and maximize the efficiency of prevention strategies.

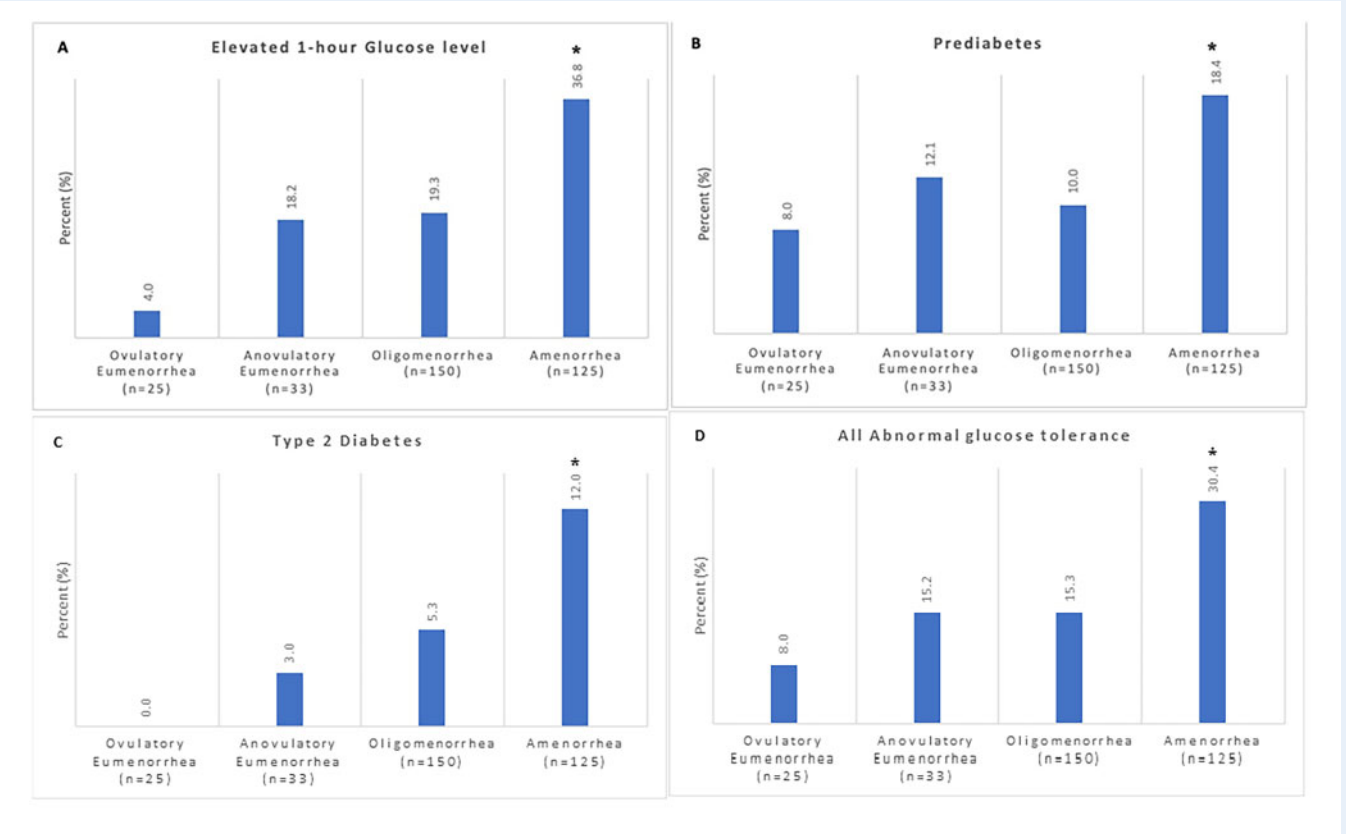


**Figure 2. Differences in the prevalence of glycemic abnormalities in hyperandrogenic PCOS women according to severity of menstrual cyclicity.** The prevalence of glycemic abnormalities in PCOS subjects identified as having an elevated 1-h glucose level (i.e. ≥155 mg/dl) (**A**), prediabetes (**B**), Type 2 diabetes (**C**) and all abnormal glucose tolerance (AGT) combined (**D**) in Ov-Eumeno (n = 25), Anov-Eumeno (n = 33), Oligo (n = 150) and Ameno (n = 125) PCOS women is depicted. Analyses have been adjusted for BMI. *Denotes significantly higher or trend toward higher prevalence of glycemic abnormalities in Ameno than in other menstrual categories. PCOS, polycystic ovary syndrome.

The strengths of our study include the use of well-phenotyped PCOS participants, its prospective design, and the unique classification of PCOS phenotypes according to the degree of menstrual dysfunction. Our study was limited by its cross-sectional nature, and concerns that the number of subjects in some phenotypic subsets were small (e.g. those with ovulatory or anovulatory eumenorrhea). We also excluded non-hyperandrogenic PCOS (i.e. Rotterdam phenotype D) from our study. The use of a 5- or 9-point 3-h oGTT instead of a 3-point 2-h oGTT could also have enabled simultaneous measurements of plasma C-peptide levels to potentially obtain a more robust assessment of β-cell function and determination of insulin clearance and insulin sensitivity. There is also the potential for measurement bias, since in 51 participants total T was measured using a high-quality RIA method, while in the remaining total T was assessed using LC-MS/MS, and the number of subjects assessed using LC-MS/MS was significantly greater in Oligo and lower in Ameno subjects (data not shown). However, our previous studies indicate a very good correlation between circulating total and free T levels determined by either method (Salameh *et al.*, 2014; Ezeh *et al.*, 2019). Furthermore, in the current study, the distribution of demographic parameters and androgen measures were similar across the four menstrual categories for subjects recruited at either at UAB or CSMC (data not shown). Finally, while our data support the use of the degree of menstrual dysfunction as a marker of metabolic dysfunction, we should note that this observation does not indicate causality.

In conclusion, a history of amenorrhea is associated with greater degrees of post-challenge hyperinsulinemia in all ethnic/racial groups studied (women of African American, White Hispanic and non-White Hispanic ancestry), compared to those with oligomenorrhea or eumenorrhea (either ovulatory or anovulatory), which may explain their greater prevalence of glycemic abnormalities. Our data suggest that menstrual history, relatively non-invasive and easy to obtain information, can be a simple but clinically important marker of metabolic dysfunction risk. Furthermore, our data also suggests that if a screening oGTT is to be done in a PCOS patient, then insulin levels should also be assessed during the test (e.g. at 0, 1 and 2 h) to determine her degree of hyperinsulinemia. These findings can potentially be used to refine the guidelines for the screening of PCOS women for metabolic dysfunction and risk.

# Supplementary data

Supplementary data are available at *Human Reproduction* online.

**Table III** Odds ratio for association of menstrual cyclicity and glycemic abnormalities in hyperandrogenic PCOS.

| Ov-Eumeno vs Oligo or Ameno | | | | | | |
|---|---|---|---|---|---|---|
| **Glycemic abnormalities** | **Ov-Eumeno vs Oligo** | | | **Ov-Eumeno vs Ameno** | | |
| | **OR** | **95% CI** | **Adjusted *P*-value** | **OR** | **95% CI** | **Adjusted *P*-value** |
| Abnormal glucose tolerance | 0.75 | 0.35–1.60 | 0.453 | 0.24 | 0.05–1.10 | 0.066 |
| Prediabetes | 0.75 | 0.35–1.60 | 0.767 | 0.44 | 0.09–2.08 | 0.303 |
| Type 2 Diabetes | 0.001 | – | 0.987 | 4.85 | – | 0.986 |
| Elevated 1-h PG* | 0.40 | 0.14–1.11 | 0.080 | 10.20 | 1.31–79.74 | ***0.027*** |
| **Anov-Eumeno vs Ov-Eumeno or Oligo** | | | | | | |
| **Glycemic abnormalities** | **Anov-Eumeno vs Ov-Eumeno** | | | **Anov-Eumeno vs Oligo** | | |
| | **OR** | **95% CI** | **Adjusted *P*-value** | **OR** | **95% CI** | **Adjusted *P*-value** |
| Abnormal glucose tolerance | 0.80 | 0.45–1.43 | 0.448 | 0.93 | 0.32–2.72 | 0.896 |
| Prediabetes | 1.86 | 1.47–1.56 | 0.620 | 0.82 | 0.25–2.67 | 0.736 |
| Type 2 Diabetes | 0.006 | – | 0.995 | 1.70 | 0.20–14.46 | 0.626 |
| Elevated 1-h PG* | 0.58 | 0.28–1.22 | 0.150 | 1.23 | 0.43–3.55 | 0.697 |
| **Ameno vs Anov-Eumeno or Oligo** | | | | | | |
| **Glycemic abnormalities** | **Ameno vs Anov-Eumeno** | | | **Ameno vs Oligo** | | |
| | **OR** | **95% CI** | **Adjusted *P*-value** | **OR** | **95% CI** | **Adjusted *P*-value** |
| Abnormal glucose tolerance | 1.48 | 0.88–2.50 | 0.143 | 2.31 | 1.26–4.24 | ***0.007*** |
| Prediabetes | 1.23 | 0.68–2.21 | 0.494 | 1.88 | 0.91–3.87 | 0.089 |
| Type 2 Diabetes | 1.95 | 0.69–5.64 | 0.210 | 2.22 | 0.88–5.62 | 0.092 |
| Elevated 1-h PG* | 1.99 | 0.72–5.52 | 0.185 | 1.56 | 0.87–2.81 | 0.139 |
| **Eumeno combined (Ov-Eumono and Anov-Eumeno) vs Ameno or Oligo** | | | | | | |
| **Glycemic abnormalities** | **Eumeno (Combined) vs Oligo** | | | **Eumeno (Combined) vs Oligo** | | |
| | **OR** | **95% CI** | **Adjusted *P*-value** | **OR** | **95% CI** | **Adjusted *P*-value** |
| Abnormal glucose tolerance | 1.25 | 0.50–3.13 | 0.638 | 1.66 | 1.06–2.59 | ***0.028*** |
| Prediabetes | 0.97 | 0.35–2.66 | 0.949 | 1.33 | 0.81–2.19 | 0.259 |
| Type 2 Diabetes | 0.31 | 0.37–25.77 | 0.295 | 2.64 | 0.94–7.45 | 0.067 |
| Elevated 1-h PG* | 0.14 | 0.19–1.27 | 0.143 | 1.79 | 1.14–2.83 | ***0.012*** |

*1-h plasma glucose (PG) ≥155 mg/dl during oGTT.
Ameno, amenorrhea; Anov-Eumeno, anovulatory eumenorrhea; Oligo, oligomenorrhea; OR, odds ratio; Ov-Eumeno, ovulatory eumenorrhea; PCOS, polycystic ovary syndrome.
P values in italics and bold are considered statistically significant.

## Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

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## Authors' roles

U.E. and R.A. designed the study, identified and phenotyped the participants, researched the data, contributed to the discussion, wrote the manuscript, and reviewed and edited the manuscript. M.D.P. assisted in phenotyping the participants, obtained study samples, researched the data and reviewed the manuscript. U.E. also performed additional statistical analyses. R.A. is the guarantor of this work and, as such, takes responsibility for the data integrity and accuracy of the data analysis.

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## Conflict of interest

M.D.P. has no competing interests to declare. U.E. is an investor in Concentric Analgesics, Inc. R.A. serves as a consultant for Spruce Biosciences and Fortress Biotech, and Aurora Forge; serves on the Medical/Scientific Advisory Board of PCOS Challenge, and CARES Foundation; and received honoraria from Virtual Int'l Congress on the future of Women's Health-PCOS and King Abdulaziz University in 2021.

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