# A factor analysis approach to examining relationships among ovarian steroid concentrations, gonadotrophin concentrations and menstrual cycle length characteristics in healthy, cycling women

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12 **A factor analysis approach to examining relationships among ovarian steroid**
13 **concentrations, gonadotropin concentrations, and menstrual cycle length characteristics in**
14 **healthy, cycling women.**
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16 Running title: Integration of menstrual function within cycles
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41   **Abstract**

42   Title: A factor analysis approach to examining relationships among ovarian steroid

43   concentrations, gonadotropin concentrations, and menstrual cycle length characteristics in

44   healthy, cycling women.

45   Study question: How are ovarian steroid concentrations, gonadotropins, and menstrual cycle

46   characteristics inter-related within normal menstrual cycles?

47   Summary answer: Within cycles, measures of estradiol production are highly related to one

48   another, as are measures of progesterone production, however the two hormones also show some

49   independence from one another, and measures of cycle length and gonadotropin concentrations

50   show even greater independence, indicating minimal integration within cycles.

51   What is known already: The menstrual cycle is typically conceptualized as a cohesive unit, with

52   hormone levels, follicular development, and ovulation all closely inter-related within a single

53   cycle. Empirical support for this idea is limited, however, and to our knowledge, no analysis has

54   examined the relationships among all of these components simultaneously.

55   Study design, size, duration: 206 healthy, cycling Norwegian women participated in a prospective

56   cohort study (EBBA-I) over the duration of a single menstrual cycle. Of these, 192 contributed

57   hormonal and cycle data used in the current analysis.

58   Participants/materials, setting, methods: Subjects provided daily saliva samples throughout the

59   menstrual cycle from which estradiol and progesterone concentrations were measured.  FSH and

60   LH concentrations were measured in serum samples from three points in the same menstrual

61   cycle and cycle length characteristics were calculated based on hormonal data and menstrual

62   records. A factor analysis was conducted to examine the underlying relationships among 22

63   variables derived from the hormonal data and menstrual cycle characteristics.

64   Main results and the role of chance: Six rotated factors emerged, explaining 80% of the variance

65   in the data. Of these, factors representing estradiol and progesterone concentrations accounted for

66   37% and 13% of the variance, respectively. There was some association between measures of

67    estradiol and progesterone production within cycles, however cycle length characteristics and

68    gonadotropin concentrations showed little association with any measure of ovarian hormone

69    concentrations.

70    Limitations, reasons for caution: Our summary measures of ovarian hormones may be imprecise

71    in women with extremely long or short cycles, which could affect the patterns emerging in the

72    factor analysis.  Given that we only had data from one cycle on each woman, furthermore, we

73    cannot how address cycle characteristics may covary within individual women across multiple

74    cycles.

75    Wider implications of the findings: Our findings are generalizable to other healthy populations

76    with typical cycles, however may not be applicable to cycles that are anovulatory, extreme in

77    length, or otherwise atypical. The results support previous findings that measures of estradiol

78    production are highly correlated across the cycle, as are measures of progesterone production.

79    Estradiol and progesterone concentrations are associated with one another, furthermore. However

80    factor analysis also revealed more complex underlying patterns in the menstrual cycle,

81    highlighting the fact that gonadotropin concentrations and cycle length characteristics are

82    virtually independent of ovarian hormones. These results suggest that despite integration of

83    follicular and luteal ovarian steroid production across the cycle, cycle quality is a multi-faceted

84    construct, rather than a single dimension.

92

93 **Introduction**

94

95 The menstrual cycle is typically characterized as a single, cohesive unit in which hormone levels,

96 follicular development, and ovulation are all closely inter-related. From this perspective, high

97 quality cycles are not only ovulatory, but have high estradiol and progesterone concentrations,

98 characteristic mid-cycle LH and FSH peaks, and are approximately 28 days in length. At the

99 other end of the spectrum, low quality cycles are not only anovulatory, but may have low

100 estradiol and progesterone concentrations, lack discernible LH and FSH peaks, and be atypical in

101 length.  In other words, the classical view of the menstrual cycle implies that the quality of a

102 given cycle is consistent across multiple measures, including follicular development, ovarian

103 steroid and gonadotropin concentrations, endometrial development, and cycle length

104 characteristics. This concept of consistent quality across the cycle is often implicitly accepted,

105 however few studies have directly examined this question. Certainly at the extreme end of the

106 spectrum of impaired ovarian function, multiple aspects of hormone production and cycle

107 characteristics can all be compromised, with possible cessation of menses and hormone cycling

108 (Ellison, 1990). Within the range of typical, healthy cycling, however, the degree of cohesiveness

109 or integration of cycle quality remains unclear. Empirically, if the quality of a cycle really is a

110 single dimension, the various measureable components should show a high degree of covariance

111 within cycles. The goal of the current analysis is to examine patterns of association among

112 component parts of the menstrual cycle (estradiol concentrations, progesterone concentrations,

113 gonadotropin concentrations, and cycle length variables) in order to better understand the normal

114 menstrual cycle.

115

116 Theoretical support for close correlation of estradiol and progesterone production comes from the

117 fact that both ovarian steroids derive from the same underlying structures.  That is, the very cells

118    that produce estradiol in the follicular phase- the theca and granulosa cells of the preovulatory

119    follicle- are those that go on to comprise the progesterone-producing cells of the corpus luteum

120    after ovulation (Strauss and Williams, 2004). Based purely on the underlying cellular physiology,

121    therefore, we might predict consistency of ovarian steroid production across the menstrual cycle

122    (e.g. robust follicular estradiol production associated with robust luteal estradiol and progesterone

123    production). On the other hand, estrogen and progesterone play different physiological roles in

124    reproduction, and epidemiological evidence indicates that they can vary independently of one

125    another (Lipson and Ellison, 1996, Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al.,

126    2007, Venners, et al., 2006).  For instance, one study measuring daily ovarian hormone profiles in

127    premenopausal women found very low correlations between urinary estrogen and progesterone

128    metabolite concentrations within a cycle (r=-0.003 to 0.13) (Windham, et al., 2002).  Thus,

129    although a high degree of consistency between estradiol and progesterone indices might be

130    expected, empirical support for that prediction is mixed.

131

132    Much as we might expect consistency of ovarian steroid hormone production within a cycle, so

133    might we predict that ovarian steroid production is closely associated with production of pituitary

134    gonadotropins. Ovarian steroid hormone production and release comes from coordination of

135    ovarian theca and granulosa cell activity and is dependent upon gonadotropin input from the

136    pituitary gland, with luteinizing hormone (LH) stimulating theca cell function, while follicle

137    stimulating hormone (FSH) influences estradiol production by the granulosa cells (Strauss and

138    Williams, 2004). Although some follicular development can proceed in the absence of FSH

139    stimulation suggesting there is limited ovarian hormone activity independent of pituitary input

140    (Oktay, et al., 1998), gonadotropin stimulation is essential for advancing further to the steroid-

141    producing antral phase (Irving-Rodgers, et al., 2001). The complex feedback interactions between

142    gonadotropins and ovarian steroids continue throughout mid-cycle, when rising follicular

143    estradiol levels drive preovulatory surges in FSH and LH (Richards, et al., 2002). The apparent

144  interdependence of gonadotropin and ovarian steroid activity characteristic of normal ovarian

145  function suggests that the two may be closely associated throughout the cycle, at least during

146  certain periods. For instance, in one study of cycling women, there were weak correlations

147  between estradiol and FSH early in the follicular phase, but higher (inverse) correlations in the

148  mid-follicular phase (Robertson, et al., 2009) and other studies have found that estradiol has

149  inhibitory effects on FSH secretion in the luteal phase (de Ziegler, et al., 1992, Lahlou, et al.,

150  1999, Lasley, et al., 1975). In the luteal phase, moreover, both LH and FSH show weak to

151  moderate negative correlations with progesterone concentrations, while LH and estradiol

152  concentrations show a weak positive correlation (Robertson, et al., 2009). Thus there is evidence

153  to suggest some coordination of pituitary gonadotropin and ovarian steroid production within the

154  cycle, although the strength and direction of the relationship may vary at different points in the

155  cycle.

156

157  That cycle length variables should be linked to hormone concentrations and follicular

158  development is less obvious, but this prediction follows from the physiology nonetheless. The

159  length of the follicular phase reflects the speed at which the antral follicle is recruited and

160  develops, and thus by extension, follicular phase length should be related to gonadotropin and

161  ovarian steroid concentrations as well (Cabral and de Medeiros, 2007, Harlow, et al., 2000).

162  Experimental evidence in primates suggests that if the antral follicle is destroyed, the

163  characteristic preovulatory gonadotropin surge is delayed, extending both the length of follicular

164  phase and that of the total cycle (Goodman, et al., 1977). In humans, a limited body of work

165  suggests associations between ovarian steroid concentrations and cycle length parameters,

166  including total cycle length, follicular phase length, and luteal phase length, arguing further for

167  consistency of cycle quality across multiple domains (Harlow, et al., 2000, Landgren, et al., 1980,

168  Windham, et al., 2002). In particular, short follicular phases and short cycles may be associated

169  with relatively high estrogen and progesterone concentrations, whereas longer follicular phases

170   may be characterized by lower average estrogen concentrations (Harlow, et al., 2000, Landgren,

171   et al., 1980). Other studies have also observed positive correlations between progesterone levels

172   and luteal phase length (Landgren, et al., 1980, Windham, et al., 2002).

173

174   To date, it has been difficult to study associations among different measures of ovarian function

175   across the menstrual cycle because of the difficulty of obtaining repeated measures of hormonal

176   variables across the entire cycle.  Only a handful of studies have measured complete, daily

177   estradiol and progesterone profiles over the course of one or more cycles (De Souza, et al., 1998,

178   Liu, et al., 2004, Matthews, et al., 2006, Santoro, et al., 2004, Windham, et al., 2002). The

179   convenience and non-invasiveness of saliva collection compared to blood or urine makes it an

180   ideal medium for measuring daily ovarian steroid profiles.  However the extremely low

181   concentrations of estradiol in saliva made these analyses prohibitively difficult until relatively

182   recently (O'Rourke and Ellison, 1993). In this analysis using data from healthy, cycling women

183   participating in the Norwegian Energy Balance and Breast Cancer Aspects-I (EBBA-I) study, we

184   adopt a factor analysis approach to examine whether the menstrual cycle truly is a cohesive unit.

185   In particular, we focus on the relationships among four aspects of the menstrual cycle (estradiol

186   concentrations, progesterone concentrations, gonadotropin concentrations, and measures of cycle

187   length), looking at the strength of the associations between these components within cycles.

188

189   **Methods**

190

191   *Subject population, participants, and study design*

192   Women were recruited for the EBBA-I study, based in Tromsø, Norway, between 2000 and 2002.

193   The study's goal was to examine the role of energetics and other lifestyle variables on known

194   breast cancer risk factors in healthy, premenopausal women. To participate, women had to be age

195   25-35 with regular menstrual cycles and could not have been pregnant, lactated, or used hormonal

196     contraception in the previous six months. Women with known histories of infertility,

197     gynecological disorders, and chronic illnesses (such as type II diabetes) were excluded as well.

198     In total, 206 women participated in EBBA-I, and the subject population, recruitment methods,

199     and study design have been described elsewhere in detail (Furberg, et al., 2005). Subjects

200     received 1000 Norwegian kroner (approximately $160 US dollars at the time) to cover

201     transportation and other expenses related to their participation.

202

203     *Ethical approval*

204     Participating women signed informed consent and the study was approved by the Regional

205     Committee for Medical Research Ethics and the Norwegian Data Inspectorate as well as the

206     human subjects review boards at all participating institutions.

207

208     *Salivary steroid assay*

209     As part of the study, subjects collected daily waking saliva samples over an entire menstrual

210     cycle according to protocols developed by the Reproductive Ecology Laboratory at Harvard

211     University (Lipson and Ellison, 1989).  Free estradiol concentrations were assayed in samples

212     from 20 cycle days (reverse cycle days -5 to -24), and progesterone concentrations were assayed

213     for the last 14 days of each cycle (reverse cycle days -1 to -14).  Levels of both hormones were

214     measured using I-125-based radioimmunoassay (RIA) kits (Diagnostics Systems Laboratory,

215     Webster, TX, USA) using methods reported elsewhere (Furberg, et al., 2005). The sensitivity of

216     the estradiol assay was 4 pmol/L (1.1 pg/mL), the average intra-assay variability was 9%, and the

217     interassay variability ranged from 23% to 13% for the low and high pools, respectively.  The

218     sensitivity of the progesterone assay was 13 pmol/L (4.1 pg/mL). Based on our assayed samples,

219     the average intra-assay variability was 10%, and the inter-assay variability ranged from 19% to

220     12% for the low and high pools, respectively.

221

222 Once estradiol assays had been completed, the daily concentrations across each cycle were

223 examined in order to identify the day of the greatest mid-cycle drop in estradiol using methods

224 described elsewhere (Lipson and Ellison, 1996). For each cycle, a mid-cycle estradiol "drop day"

225 was first determined. The drop day was defined as the second of the two consecutive days in a

226 mid-cycle window during which the greatest decrease in estradiol occurred.  The mid-cycle

227 window for identifying peak estradiol was days -18 to -12, thus the drop day was constrained to

228 fall between days -17 to -11.  This estradiol drop provides a good marker for the timing of

229 ovulation, and the drop day was subsequently designated as day '0'.  Thus days in the follicular

230 phase have negative prefixes (e.g. day -1, day -2), whereas days in the luteal phase have positive

231 prefixes (e.g. day +1, day +2). A drop day could not be assigned for 14 subjects. Eight of the 14

232 had missing hormone data for at least one day during the interval between reverse cycle days -18

233 to -12. The remaining six subjects had no discernable rise or drop in estradiol during the critical

234 time window and their mid-cycle LH levels were low as well, suggesting that the cycles were

235 anovulatory.  Because determination of drop day is needed to calculate hormonal indices and

236 separate cycles into follicular and luteal phases, these 14 women were excluded and only the 192

237 women with aligned cycles were included in analyses.

238

239 *Creation of ovarian hormone indices*

240 From the daily estradiol and progesterone concentrations we were able to calculate a number of

241 different indices of hormone levels, representing different components or periods of ovarian

242 function.  Each index was calculated as the mean hormone concentration across samples collected

243 during a particular period of the cycle relative to ovulation (day 0). Seven estradiol indices were

244 calculated for each cycle: *total estradiol* (mean estradiol for all cycle days measured) reflects

245 average estradiol exposure across the cycle; *follicular estradiol* (mean estradiol, days -10 to -1)

246 reflects average estradiol prior to ovulation; *mid-follicular estradiol* (mean estradiol, days -10 to -

247 6) reflects estradiol production around the time of the emergence of the dominant follicle; *late*

248     *follicular estradiol* (mean estradiol, days -5 to -1) reflects the secretory activity of the dominant

249     follicle prior to ovulation; *maximum follicular estradiol* (highest estradiol concentration

250     measured between days -10 to -1), *maximum estradiol* (highest estradiol at any point in the

251     cycle), and *magnitude of the midcycle estradiol drop* (maximum estradiol minus estradiol on day

252     0) reflect midcycle estradiol secretion as well as the decrease in circulating estradiol

253     accompanying ovulation of the dominant follicle. If follicular phase length was shorter than 10

254     days, follicular estradiol and mid-follicular estradiol calculations were adjusted accordingly (e.g.

255     if the follicular phase was only 9 days, was calculated as the mean of days -9 through -1, rather

256     than -10 through -1).  Similarly, if data were missing (for instance, due to estradiol concentrations

257     below the sensitivity limit at the beginning of the cycle), indices were calculated as the mean

258     across the days with measurable concentrations in that interval.

259

260     Six indices of progesterone concentrations were calculated as well. *Total progesterone* (mean

261     progesterone, days 0 to +14) reflects average progesterone exposure during the luteal phase;

262     *early-mid luteal progesterone* (mean progesterone, days 0 to +9) represents the average

263     circulating progesterone concentrations during the beginning and middle of the luteal phase; *mid-*

264     *luteal progesterone* (mean progesterone, days +5 to +9) reflects the level of progesterone

265     secretion at the peak of the luteal phase; *very early luteal progesterone* (mean progesterone, days

266     0 to +2) and *early luteal progesterone* (mean progesterone, days +3 to +5) together reflect the

267     early luteal progesterone rise, before any possible effects of human chorionic gonadotropin (hCG)

268     from a potential conceptus; and *late luteal progesterone* (mean progesterone, days +10 to +14)

269     reflects post-peak secretion of progesterone during the regression of the corpus luteum prior to

270     menstruation. If luteal phase length was shorter than 14 days, total progesterone and late luteal

271     progesterone calculations were adjusted accordingly (e.g. if the luteal phase was 12 days, total

272     progesterone was calculated as the mean of days 0 through +12, rather than 0  through +14).

273     When progesterone values were missing for individual days, the hormone indices were calculated

274    as the means of those days with data. Summary statistics for the hormonal variables are provided

275    in Table 2.

276

277    *Calculation of menstrual cycle phase lengths*

278    Three cycle length variables were measured using the hormone data and self-reported dates of

279    menses. *Overall cycle length* was the number of days from menstrual onset to menstrual onset, as

280    determined by self-reported menses. *Follicular phase length* was the number of days from

281    menstrual onset to the mid-cycle estradiol drop day. Finally, *luteal phase length* was the number

282    of days from the day after the mid-cycle estradiol drop day to onset of subsequent self-reported

283    menses.

284

285    *Serum sample collection and gonadotropin assay*

286    At three points in the cycle, fasting serum samples were taken by trained nurses at the University

287    Hospital of Northern Norway, Tromsø. These collections were done between days 1-2, 7-12, and

288    21, reflecting the early follicular, pre-ovulatory, and luteal phases of the cycle. Luteinizing

289    hormone (LH) and follicular stimulating hormone (FSH) were measured in serum samples from

290    all three time points using Techicon Immuno1 immunometric assays (Bayer Corp, Tarrytown,

291    NY). Both assays were standardized against the WHO 2nd International Standard (for FSH: IRP

292    78/549 and for LH: IRP 68/40). The sensitivity of the FSH assay was 0.1 IU/L and the coefficient

293    of variation was less than 7 percent.  For LH, the assay sensitivity was 0.3 IU/L and the

294    coefficient of variation was 5-10 percent.

295

296    *Statistical analyses*

297    All statistical analyses were carried out in SAS Enterprise 4.3 (SAS Corporation, Cary, NC).

298    Because hormone values typically follow non-normal distributions, all hormone indices were first

299    log transformed to normalize variances. We first examined bivariate correlations between the

300    ovarian hormone indices in our analysis.  We then conducted a factor analysis by principal

301    components extraction, with and without orthogonal varimax rotation of axes on the correlation

302    matrix of the study variables. The goal of factor analysis is to condense a large number of

303    correlated variables into a smaller number of factors and in doing so, reveal underlying

304    relationships among the variables. Orthogonal varimax rotation then rotates these factors so that

305    they are uncorrelated with one another, creating factors for which one or more variables have

306    high loadings, while loadings for the other variables are close to zero (Manly, 2005). Each factor

307    has an eigenvalue, which indicates the amount of variance in all of the variables that is accounted

308    for by that factor, and following the conventionally used Kaiser criterion, only factors with

309    eigenvalues greater than 1 (i.e. explaining more than 1 percent of the total variance) are retained

310    in the analysis (Kaiser, 1958).  Thus factors with large eigenvalues explain a large amount of

311    variance in the overall data, whereas factors with small eigenvalues explain little of the variance.

312    A Scree plot (which helps to visually discriminate between those factors explaining a large

313    fraction of the variance and those which are relatively unimportant) was made to confirm the

314    number of factors that should be included in the analysis.  For each factor with an eigenvalue

315    greater than 1, we examined the loading of each menstrual cycle variable, which is similar to a

316    standardized regression coefficient when the factor is regressed on the variables (DeCoster,

317    1998). Loadings of $\geq 0.7$ were considered strong loadings, while those $<0.7$, but $\geq 0.35$ were

318    considered moderate loadings. Loadings $<0.35$ were considered weak to negligible.

319

320    One of the useful aspects of principal component extraction is the collapsing of highly correlated

321    variables into a smaller number of axes representing linear functions of those correlated variables.

322    Here, for instance, although high correlations might be expected among the different indices of

323    each steroid (particularly those that overlap), it is not necessarily the case that seemingly related

324    indices would all cluster on the same rotated axes resulting from factor analysis.  Luteal and

325    follicular estradiol secretion, for example, might be governed by different patterns of

326     gonadotropin secretion and hence manifest significant independence. Similarly mid-follicular

327     estradiol might reflect the combined secretory activity of a recruited cohort of follicles under FSH

328     stimulation, whereas late follicular and maximum follicular estradiol presumably reflect secretion

329     by the dominant follicle alone. The degree to which these aspects of estradiol production are

330     independent will affect the degree to which they individually correlate with average estradiol

331     levels over the entire follicular phase or the entire cycle as well. Thus the current analyses allow

332     us to examine the relationships among the specified variables without making any *a priori*

333     assumptions about the independence (or multicollinearity) of different indices of ovarian steroid

334     levels and other cycle characteristics. Instead, factor analysis allows us to identify those clusters

335     of variables that are highly redundant and thus reduce the number of indices studied.

336

337     **Results**

338

339     General characteristics of the study subjects are provided in Table 1. The study population was

340     predominantly Caucasian and highly educated with a mean age of 30 years. 61% of subjects were

341     married and half had at least one child. The average cycle length was 28 days (range: 20-47), of

342     which 15 were spent in the follicular phase and 13 in the luteal phase. Bivariate analyses indicate

343     moderate to strong positive correlations between the estradiol and progesterone indices and are

344     presented in Table 2. Most of the estradiol indices have correlation coefficients with each other

345     in the range 0.7 to 0.95. The exception is the magnitude of the midcycle estradiol drop, which

346     correlates very weakly, and typically negatively, with the other estradiol variables. Similarly, the

347     progesterone indices have correlation coefficients with each other in the range of 0.57 to 0.97.

348     Except for magnitude of the estradiol drop, the correlations between the estradiol and

349     progesterone indices are moderate, ranging from 0.38 to 0.60.

350

351    The unrotated factor matrix (not shown) generated six factors with eigenvalues greater than 1.

352    Typically, the first unrotated factor represents the single vector that captures the greatest amount

353    of the multivariate variance and in this case, all of the estradiol and progesterone measures (aside

354    from the magnitude of the estradiol drop) had loadings of 0.70 or greater on Factor 1.  Of the

355    remaining variables, seven had loadings between 0.05 and 0.16, with the remaining three having

356    loadings less than 0.05. Factor 1 of the unrotated matrix accounted for only 38% of the total

357    multivariate variance in the sample, however, indicating that the majority of the multivariate

358    variance could not be captured by a single axis.

359

360    Subsequent orthogonal varimax rotation of the axes obtained from the factor analysis maximized

361    the separation of the factor loadings of the original variables onto different axes and generated six

362    rotated factors with eigenvalues greater than 1, which together explained 80 percent of the

363    variation in the data.  Factor loadings for the six rotated factors are presented in Table 3. The

364    varimax rotation largely succeeded in separating the original variables onto different axes, each of

365    which was orthogonal to, or independent of, the others.   All variables loaded on at least one

366    factor, but no variable had a strong loading ($\geq 0.7$) on more than one factor.  Two variables, mid-

367    cycle LH and mid-cycle FSH, showed split moderate loadings on more than one factor.  Mid-

368    cycle LH loaded strongly on Factor 5 and moderately (and negatively) on Factor 3, while mid-

369    cycle FSH showed a moderate, negative loading on Factor 3, and moderate, positive loadings on

370    Factors 4 and 5. Only two original variables, mid-cycle FSH and magnitude of the mid-cycle

371    estradiol drop, did not have a strong loading on any of the six rotated factors.  Mid-cycle FSH

372    instead had moderate loadings on three factors, while the magnitude of the mid-cycle estradiol

373    drop had a moderate loading on one factor.

374

375    The first rotated factor explained 37% of the variance and included all of the measures of

376    estradiol except for the magnitude of the mid-cycle estradiol drop.  The second rotated factor

377     included the six progesterone indices and explained 13% of the variance. The third rotated factor

378     accounted for 11% of the variance and included cycle length and follicular phase length with

379     minor loadings on mid-cycle gonadotropin concentrations. The fourth rotated factor explained

380     9% of the variance and included luteal gonadotropin concentrations, with a minor loading on

381     mid-cycle FSH concentrations and the magnitude of the estradiol drop. The fifth rotated factor,

382     explaining 5% of the variance, had major loadings on early follicular and mid-cycle LH

383     concentrations and minor loadings on early follicular and mid-cycle FSH loadings. Finally, only

384     luteal phase length was included in the sixth rotated factor, which accounted for 5% of the

385     variance in the data set. Sensitivity analyses (not shown) using only subjects with complete daily

386     hormone data did not change the basic relationships among variables and factors.

387

388     **Discussion**

389

390     The purpose of this analysis is to examine the extent to which the menstrual cycle is a cohesive

391     unit in healthy, reproductive-age women, as measured by the strength of the relationships among

392     hormonal measurements and cycle characteristics. Or, phrased as a question, to what extent is any

393     one measure of menstrual function predictive of or independent of others within the same cycle?

394     Of particular interest is the extent to which there is coordination of ovarian steroid production

395     across the follicular and luteal phases of the cycle. In our study population, the relationship

396     between follicular phase estradiol and luteal phase progesterone is significantly positive, as

397     reflected in the bivariate correlations. These correlations are much higher than reported in at least

398     one other study in cycling women, in which correlations between urinary estradiol and

399     progesterone metabolite concentrations were 0.13 or lower (Windham, et al., 2002). Because

400     urinary assays measure conjugated metabolite concentrations and are thus one or more steps

401     removed from circulating free hormone concentrations, such assays may introduce additional

402     noise related to inter-individual metabolic variation (Gann, et al., 2001). For that reason, the

403    stronger correlations found in the current study based on free (bioactive) salivary steroid

404    concentrations may be a more accurate reflection of the true relationship between estradiol and

405    progesterone concentrations in healthy, cycling women.

406

407    In the current study, the positive association between follicular and luteal steroid profiles is

408    further illustrated by sorting the study subjects into quartiles on the basis of the indices of one

409    steroid and comparing the full daily profiles of the other. Sorting the subjects into quartiles by

410    mean follicular estradiol concentrations shows that women with high mean follicular estradiol

411    concentrations also tend to have high progesterone concentrations throughout the luteal phase

412    (Figure 1).  Similarly, when subjects are sorted by mean luteal progesterone concentrations, those

413    with the highest quartile of luteal progesterone concentrations tend to have high follicular

414    estradiol concentrations as well (Figure 2). In both cases, the quartiles are clearly distinct from

415    one another. Thus crude analyses suggest that, across women, within a cycle, levels of one of

416    these hormones are indicative of levels of the other.

417

418    The subsequent factor analysis allowed simultaneous examination of the relationships between

419    the ovarian steroid concentrations and other measures of cycle quality to identify more complex

420    underlying patterns.  The factors obtained after varimax rotation represent the "sorting" of

421    variables into groups that are highly correlated among the group while being orthogonal, or

422    independent, of the groups represented by other factors. Factor 1 has very strong loadings (0.85 or

423    greater) for all the estradiol indices except the magnitude of the estradiol drop.  It reflects the high

424    consistency of estradiol production across the ovarian cycle and supports previous work finding

425    high correlations between estradiol measures at multiple points across the cycle (Windham, et al.,

426    2002).  The progesterone indices load weakly on this factor (0.20 to 0.30), with loadings being

427    highest for early luteal progesterone measures and lower for indices capturing the later part of the

428    luteal phase. This suggests that estradiol and progesterone production cannot be fully

429    disentangled, particularly in the early luteal phase. Factor 2 has very high loadings (0.73 or

430    greater) for all the progesterone indices, suggesting that progesterone production is highly

431    consistent across the luteal phase. In Factor 2 there are weak loadings (0.2 to 0.34) for most of the

432    estradiol indices, again indicating that there is some aspect of the relationship between

433    progesterone and estradiol production that cannot be disarticulated, as suggested in the crude

434    analyses.

435

436    Nevertheless, the degree to which indices of the two ovarian steroids separate onto different axes

437    in the factor analysis reflects the degree to which they are actually independent of each other. It is

438    noteworthy, that no other variables have loadings on the first two rotated factors, which we

439    therefore regard as the estradiol and progesterone factors, respectively.  In particular, the loadings

440    for both the gonadotropin variables and the cycle length measures are extremely low. This

441    suggests that a woman's circulating estradiol and progesterone concentrations are not a clear

442    function of her circulating gonadotropin concentrations, nor are they closely related to her cycle

443    length. Rather, other factors including gonadotropin receptor densities or sub-types, co-

444    gonadotropins such as insulin and IGF-1, or other unknown genetic, developmental, or

445    constitutional components may explain inter-individual variance in ovarian steroid

446    concentrations.  It remains possible that differences in gonadotropin concentrations may account

447    for more of the documented within-individual variance in ovarian steroid concentrations (between

448    multiple cycles in the same woman, for instance) (Lipson and Ellison, 1996, Venners, et al.,

449    2006).

450

451    Factor 3 has very high loadings (0.88 or greater) for total cycle length and follicular phase length,

452    which confirms the close association between follicular phase length and overall cycle length that

453    has been noted elsewhere (Fehring, et al., 2006, Waller, et al., 1998).  The moderate negative

454    loadings of the mid-cycle concentrations of LH and FSH on this factor are more surprising,

455   suggesting that factors associated with slow follicular growth (resulting in a longer follicular

456   phase and longer total cycle length) may later result in poor steroid response in the luteal phase.

457   Because this factor is independent of steroid concentrations themselves (Factors 1 & 2), even in

458   the luteal phase, it may indicate that higher gonadotropin levels are required to stimulate a given

459   amount of steroid production in cycles with longer follicular phases than in those with shorter

460   follicular phases.  This may again be consistent with variation in the ovarian responsiveness to

461   gonadotropin stimulation rather than the level of that stimulation itself, an effect that might be

462   moderated at the receptor level. Further study is needed to understand these unexpected

463   relationships.

464

465   Factor 4 has strong loadings (0.80 or greater) for luteal FSH and LH concentrations. It also has

466   moderate loadings for mid-cycle FSH (0.49) and the magnitude of the mid-cycle estradiol drop

467   (0.41) It is noteworthy that the magnitude of the mid-cycle estradiol drop only clusters with luteal

468   gonadotropins (albeit moderately) and not with any of the ovarian steroid measures. In fact,

469   magnitude of the mid-cycle estradiol drop is the only steroid index that correlates significantly

470   with levels of gonadotropin stimulation, although it is somewhat surprising that it clusters with

471   luteal, rather than mid-cycle gonadotropin concentrations.   Baseline gonadotropin concentrations

472   tend to be very low across the entire luteal phase and pulsatile release of gonadotropins occurs at

473   low frequency (Hall, 2004, Johnson and Everitt, 2000), so it is unclear why they should be

474   associated with the magnitude of the estradiol drop. Additional research is needed to confirm and

475   better understand this unexpected observation.

476

477   Factor 5 has high loadings (greater than 0.70) for early follicular and mid-cycle LH, with

478   moderate loadings (0.5-0.7) for early follicular and mid-cycle FSH. This indicates that

479   gonadotropin concentrations in the first half of the cycle are closely associated even though the

480   two have distinct functional differences, with FSH stimulating further development of the antral

481    follicle while LH promotes ovarian steroid production and eventually, ovulation (Hall, 2004,

482    Strauss and Williams, 2004). Nevertheless, given that both are produced and secreted by a

483    common source (the pituitary gland) and that both are responsive to fluctuating ovarian steroid

484    concentrations (through negative feedback), it is not surprising that FSH and LH concentrations

485    would load on the same factor. More surprising, perhaps, is how weak the ovarian steroid

486    loadings are on this factor, which may indicate that across women, there is little relationship

487    between early follicular and mid-cycle gonadotropins and ovarian steroid concentrations.  Once

488    again, this suggests that it may be sensitivity to gonadotropin stimulation (for instance through

489    receptor densities or the effect of co-gonadotropins), and not absolute gonadotropin

490    concentrations that are most important for regulating ovarian steroid production.

491

492    Finally, only luteal phase length loads on Factor 6 (0.97), indicating that it is virtually

493    independent of the other hormone and cycle characteristics considered in this analysis.  It is not

494    surprising that luteal phase length did not cluster with the other cycle length variables given that

495    the literature suggests that while total cycle length and follicular phase length are tightly

496    correlated, luteal phase length tends to be less variable and show only moderate correlations with

497    both (Fehring, et al., 2006, Waller, et al., 1998).  In fact, one study found that only three percent

498    of the variance in total cycle length was attributable to variation in luteal phase length, whereas

499    follicular phase variation explained over 84 percent (Waller, et al., 1998).  At least one study has

500    identified differences in urinary ovarian steroid concentrations in relation to luteal phase length,

501    however those differences were in comparisons of cycles with short (≤10 days), average (11-14

502    days), and long (≥15 days) luteal phases and did not consider luteal phase lengths continuously

503    within the normal range (Windham, et al., 2002). In general, little is known about predictors or

504    determinants of luteal phase length and additional research is needed to understand the existing

505    variation and how it is related to other cycle indicators of ovarian function.

506

507  Overall, the results of this factor analysis suggest that although there is some consistency of

508  menstrual function across domains, the particular cycle measures considered here also show

509  considerable independence from one another across women. Perhaps of greatest interest are the

510  associations between estradiol and progesterone indices, which clustered onto two distinct

511  factors, but also showed minor loadings on each other's primary factors, suggesting some inter-

512  dependence between the two. This finding is interesting in light of previous work suggesting that

513  follicular estradiol concentrations are higher in conception cycles than non-conception cycles

514  (Lipson and Ellison, 1996). One explanation is that high estradiol concentrations better stimulate

515  the developing oocyte and prime the endometrium for proliferation, thus increasing the odds of a

516  successful conception. Our results suggest a second explanation should be considered as well,

517  namely that there may also be correlated luteal phase effects, including endometrial secretions

518  and support for implantation that is necessary for successful conception. The associations

519  between estradiol measures (which are primarily follicular) and progesterone measures (which

520  are luteal)  is clinically important, moreover, in that it further supports the idea that luteal phase

521  defects are actually product of problems with follicular development earlier in the cycle

522  (DiZerega and Hodgen, 1981).

523

524  At the same time, estradiol and progesterone measures also showed a degree of independence

525  from one another, and the fact that ovarian steroid concentrations were not associated with

526  variation in gonadotropin concentrations or in cycle and phase lengths, moreover, suggests that it

527  may be mediated by tissue sensitivity, perhaps reflecting differences in receptor expression or

528  variation, or other physiological, genetic, developmental, or constitutional factors.  Such a

529  mechanism would be consistent with findings of a study of adult Bangladeshi migrants to the UK,

530  that indicated that progesterone, but not estradiol, was related to individual developmental history

531  (Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al., 2007). Indeed, at least one study

532  found that progesterone levels tend to be predictable within individuals over intervals of as much

533    as one year, whereas estradiol levels may vary dramatically within individuals over the same time

534    period (Chatterton, et al., 2005). The dissociation of estradiol and progesterone profiles observed

535    in such studies suggests that developmental history may exert long-lasting influence on some

536    aspects of ovarian steroid production, whereas other aspects are more responsive to acute cues in

537    the immediate environment.

538

539    There are some limitations to the interpretation of results from this study. First, the current study

540    assessed only inter-individual effects, finding, for instance, that women who have high estradiol

541    levels tend to have high progesterone levels and vice versa.  Because hormone levels were only

542    measured for the duration of a single cycle in this study, we are unable to examine whether the

543    same trend holds between cycles within individual women. Additional research following women

544    longitudinally over time is needed to determine whether, within a given woman, high estradiol

545    cycles are likely to also feature high progesterone levels, while low estradiol cycles tend to have

546    low progesterone levels. Similarly, our results do not address whether other cycle characteristics

547    tend to covary within individual women across multiple cycles.

548

549    Because we measured estradiol concentrations only in samples collected on reverse cycle days -5

550    to -24 (i.e. from 5 to 24 days before the start of the next menstrual bleeding), our estradiol indices

551    may be inaccurate for any women with extremely long cycles.  Although the recruitment criteria

552    generally excluded women with atypical cycles, in practice, approximately 5 percent of subjects

553    had cycles longer than 35 days.  In a 35 day cycle, for instance, by assaying estradiol

554    concentrations only in days -5 to -24 of the cycle, our calculated estradiol indices are artificially

555    truncated, omitting concentrations in the early follicular phase. By contrast, in women with

556    shorter cycles, nearly the entire follicular phase would be captured in our estradiol indices.

557    Similarly, given that progesterone concentrations were only assayed in sample from reverse cycle

558    days -1 to -14 (i.e. from 1 to 14 days before the start of the next menstrual bleeding), in women

559  with extremely long luteal phases, the progesterone indices might not capture the earliest days of

560  the luteal phase. For several reasons, however, we believe that this potential error is unlikely to

561  affect our results.  First, we conducted a sensitivity analysis (not shown) restricting the analyses

562  to subjects with cycle lengths ranging from 24-34 days and found that although there were slight

563  differences in the exact factor loadings, the patterns and relationships that emerged were

564  unchanged from those found using the whole cohort. Second, the fact that ovarian hormones and

565  cycle phase lengths load on different factors in our analysis suggests the two are largely

566  independent of one another. If there were significant confounding of these variables, there would

567  have been strong loadings of ovarian hormone and cycle length characteristics on the same

568  factors, which there was not. Ultimately, if there were bias due to improper calculation of

569  hormone indices in long cycles, it would be for cycles with longer follicular phases to have higher

570  average estradiol levels (since it would be the early follicular levels, which are typically low, that

571  were omitted from the calculated indices), and we do not see any evidence of that.  Any bias in

572  progesterone levels due to cycle length would be similar, but there is strong evidence from many

573  studies, including these data, that variation in luteal phase length is minimal (Matsumoto, et al.,

574  1962, Vollman, 1977).  Thus we suggest that any bias in this regard is negligible.

575

576  Another limitation is our subject population, which was specifically recruited to be ages 25-35

577  and self-identifying as having regular cycles.  Our population's cycle length and cycle phase

578  lengths were typical of healthy women in this age range, however we cannot necessarily

579  extrapolate our findings to address this question in other groups of women (Chiazze, et al., 1968,

580  Treloar, et al., 1967, Vollman, 1977).  In particular, women with less typical cycles (who would

581  have been excluded from participation in the current study) might show different patterns of cycle

582  hormones and characteristics, as might the 14 subjects whose hormonal profiles did not allow us

583  to readily identify an estradiol drop day (and hence were excluded from analysis). Whether these

584  results also apply to younger and older women (whose cycles may tend to be more erratic and

585    have lower hormone levels) remains unknown (Chiazze, et al., 1968, Lipson and Ellison, 1992,

586    Treloar, et al., 1967).  Further research is also needed to determine whether these patterns hold

587    true in non-Western populations in which the level of ovarian function (as evidenced by estradiol

588    and progesterone concentrations) is typically lower (Ellison, et al., 1993).  Given the results of

589    migrant studies (Nunez-De La Mora, et al., 2008, Nunez-de la Mora, et al., 2007), it may be of

590    particular interest to examine women whose current environment differs radically from the

591    environment in which they were born and raised.  Perhaps under such conditions, there will be

592    even weaker relationships across domains, with different cycle components reflecting

593    developmental and current conditions.

594

595    Finally, our findings on the relative independence of gonadotropin concentrations from other

596    measures of cycle quality should be interpreted with caution.  As discussed, one possibility is that

597    although serum gonadotropin concentrations may not be directly associated with ovarian steroid

598    concentrations or cycle length characteristics, other indicators of gonadotropin activity (such as

599    ovarian receptor densities) may be.  It is also possible, however, that because gonadotropins are

600    released in approximately hourly pulses (Kazer, et al., 1987, Moret, et al., 2009), our

601    measurement techniques (based on single serum samples at three points in the cycle) may have

602    been too imprecise to capture circulating concentrations and have resulted in additional "noise" in

603    our data.  Gonadotropin concentrations would be better quantified by repeated blood sampling at

604    short intervals (approximately five minutes) during an extended time period followed by pulse

605    detection analysis (Moret, et al., 2009). Even with that improved methodology, however, we

606    would not be able to address whether the serum gonadotropin concentrations reflected the

607    concentrations in the follicle, which are ultimately of greatest relevance and interest.

608

609    In conclusion, this study is the first to directly address the extent to which multiple components of

610    the menstrual cycle and ovarian function are inter-related in healthy, cycling, Western women.

611     We have found that there is a significant degree of coordination of ovarian steroid production

612     across the cycle, however estradiol and progesterone production also show considerable

613     independence from one another. We have determined, furthermore, that across women,

614     circulating gonadotropin concentrations and cycle length characteristics are almost entirely

615     unrelated to ovarian steroid concentrations, suggesting that these aspects of cycle quality are

616     independent of one another. Contrary to the textbook depiction of the menstrual cycle, cycle

617     quality is not uniform across measures. Even in healthy, cycling women, different components of

618     the cycle (ovarian steroids, gonadotropins, and cycle phase lengths) do not necessarily covary in a

619     straight-forward, predictable manner.  Future research may look at not only how these measures

620     of ovarian function are related to (or independent of) one another within women, but also how

621     additional aspects of ovarian function, such as follicular development, follicular gonadotropin

622     levels, or endometrial proliferation fit into this complex system.

623
624     **Authors' Roles:**
625

646
647    **Conflicts of interest:**
648
649    The authors have no conflicts of interest to declare.

650
651    References:
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