Published in final edited form as:

Mol Cell Endocrinol. 2007 January 2; 260-262: 172–182. doi:10.1016/j.mce.2006.02.020.

Patterns of LH β cf among women in health and disease

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Abstract

Urine based gonadotropin assays provide a practical means of analyzing hormone secretion patterns. While research protocols have revealed pulsatile patterns of gonadotropins such as LH in the blood, these assays are of limited clinical use since daily venipuncture sampling is not feasible outside of a research environment. However, collection of several urine samples provides a method to achieve the same visualization of gonadotropin patterns in patients using a convenient and generally applicable technique based on analysis of the highly stable hLH β cf for monitoring LH and hCG β cf for monitoring pituitary hCG. We demonstrated that two different sampling techniques for analyzing these gonadotropin metabolites yielded the same information on their excretory patterns, either sampling of spot urines or collecting first morning void urines for several days. Next, we studied the core excretory patterns in several populations: menstruating and postmenopausal women from the general population, and two populations of women from a fertility center, one of which had polycystic ovaries (PCO). The PCO population was also subdivided into those with and without insulin resistance (IR). It was found that our hLH β cf assay did not measure the form of the LH core (v-hLH β cf) produced in subjects who were homozygous for a variant form of LH (v-LH). None of our patients tested were homozygous for the variant form of LH. It was also found that in most non-PCO (NPCO) patients, the hLH β cf peak lasted for 7–9 days while among the PCO patients this peak frequently lasted for less than 7 days and an erratic pattern tended to appear. The overall differences in patterns between the PCO and NPCO patients were confirmed by spectral statistical methods. The prevalence of certain characteristic hLH β cf patterns may be higher among women with PCO with a more severe clinical presentation. Use of urinary analysis of gonadotropin metabolites, especially hLH β cf, may supplement subjective ultrasound studies with more sensitive biochemical measurements.

Introduction

With the increasing concerns for the contribution of ovulatory disorders in reproduction, new information is needed on the role of the gonadotropins in syndromes such as metabolic and polycystic ovaries (PCO) and other menstrual dysfunctions to female fertility problems. (Alliende, 2002; Birken *et al.*, 1999; Huhtaniemi *et al.*, 1999) (Ramanujam *et al.*, 1999; Tulppala *et al.*, 1998) (Matt *et al.*, 1998; Ropelato *et al.*, 1999; Takahashi *et al.*, 2001; Van Dam *et al.*, 2002; Veldhuis and Pincus, 1998). Virtually all studies of these glycoprotein hormones have

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been conducted using blood sampling which allows detailed hormone monitoring only for research subjects (Clark *et al.*, 1997; Veldhuis *et al.*, 1993; Veldhuis and Pincus, 1998) (Clark *et al.*, 1997; Veldhuis, 1997). Urine sampling permits detailed hormone profiling as a routine diagnostic tool for any patient since collections can be done conveniently at home. Such studies open a new window on the secretion of gonadotropins in normal and aberrant clinical states and provide new methods to examine problems related to fertility. We have developed unique measurement tools to identify and quantify the metabolites of LH and pituitary hCG (hLH β cf for monitoring LH and hCG β cf for monitoring pituitary hCG) (Birken *et al.*, 1993; Birken *et al.*, 1996; O'Connor *et al.*, 1998). In this study we apply these tools to compare hLH β cf patterns in apparently healthy young women sampled from the general population and then extend these studies to two populations of female patients in a fertility center, one NPCO and one with PCO appearance of the ovaries.

We had earlier reported a preliminary study of the hLH β cf excretion patterns in the urine of women during reproductive aging which showed a relationship between aging and increased numbers and randomness of hLH β cf peaks in urine (Birken *et al.*, 1999). This report extends such studies to different sampling techniques, namely the hLH β cf excretion patterns determined by measuring sequential spot urines over the course of 1–2 days as compared to sequential first morning void urines. Young menstruating women who reported regular menstrual cycles and postmenopausal women were recruited from the general population. We present in this report a possible new avenue of laboratory investigation for evaluation of patients with PCO. In addition, we determined how our assay systems react with the metabolite of wild type LH as compared with that of the major variant form of LH which has two amino acid alterations in the beta subunit converting Trp at residue 8 to Arg and Ile at residue 15 to Thr. (Nilsson *et al.*, 2001b; Pettersson *et al.*, 1992; Rajkhowa *et al.*, 1995).

Materials and Methods

Subjects

Subjects from the general population—We recruited young menstruating women and postmenopausal women by advertisement. Women were asked to fill out a questionnaire to detail their menstrual cycle history, hormone-related medicines and other relevant information before admission to the study. The subset of menstruating participants for whom data was included in the present analysis, were drawn from a sample of 36 menstruating women, ranging in age from 18–30 years, and who all reported regular menstrual cycles. The study was approved by the Institutional Review Board (IRB) of Columbia University for human investigations.

Patients recruited from a fertility center—The sample of patients that agreed to participate in this study was drawn from approximately 700 new patients that initiated evaluation for infertility problems. Prior to any treatment, those patients underwent a baseline infertility workup for diagnostic purposes. This workup included monitoring of the ovulatory processes with sequential transvaginal ultrasounds (tvUS), hormonal profile (PRL, TSH, T₄, T₃ uptake, FTI, DHEA-S, testosterone, LH, FSH, estradiol, and progesterone). Besides the patient demographics, several clinical parameters were examined: the regularity of the menses, BMI, ovarian volume, and number of antral follicles on the second or third day of the menstrual cycle. The ovarian volume was calculated by applying the formula $\frac{4}{3} \pi R/2 (r + \hat{r} / 4)^2$, where R is the largest axis of the ovary and r and $\hat{r}$ are the two smaller ones.

The revised criteria of the 2004 consensus were used to select patients with PCO (Consensus Group (2004). Patients were classified to have polycystic ovaries when they had 12 or more follicles in either or both ovaries, and/or if the ovarian volume was more than 10 ml. In some instances, both criteria were present in the same patient. The patients that had PCO and were

included in the study were termed the study group. Any patient that did not have polycystic appearance of the ovaries and had regular ovulatory cycles was included in the NPCO group of patients. Since these patients suffered from infertility, they could not be considered as normal controls.

Since insulin resistance, diabetes, and obesity have been associated with PCO, we investigated the presence of metabolic syndrome in our patient population that was diagnosed with having polycystic appearance of the ovaries. A three hour oral glucose tolerance test (3-h OGTT) was performed using 100 gram glucose load and obtaining glucose and insulin serum levels.

Methods of Specimen Collection

Subjects from the General Population recruited by advertisement—Each subject received the appropriate collection kit, verbal and written instructions. The obtained samples were divided in aliquots and were kept frozen at -20°C until the time of their analysis. Each young menstruating woman was asked to complete a detailed questionnaire concerning her menstrual cycle, drugs used, and general relevant medical history. For sampling the entire menstrual cycle, young menstruating women were asked to collect first morning urine samples from the first or second day of menses to the first day of the next menses to cover one complete menstrual cycle. We also studied a population of postmenopausal women who were asked to start collecting urine on any random day and for the next 10 consecutive days.

For sampling “spot urines” over a two day time period, young menstruating women and postmenopausal participants were asked to serially collect samples of all urine passed for two consecutive days. Spot urine collection was started on the first or second day of menses by menstruating women. In the postmenopausal group, the starting collection day was selected arbitrarily. The date and time of each urine specimen collected was recorded.

For sampling the follicular phase of the cycle, young menstruating women were asked to collect samples of the first morning void urines from the first or second day of menses for 10 consecutive days.

Subjects from the Fertility Center—Urine collection procedures were the same as described above for the general population patients. Each patient received a urine collection kit, written and oral instructions, and all urine specimens were kept frozen until returned to the doctor’s office and then kept frozen in the laboratory until assayed for core content. Patients with irregular menses sometimes would need to wait until their next menses occurred before beginning collection. All human investigations studies were approved by both the Institutional Review Board (IRB) of Columbia Presbyterian Medical Center (CPMC) and by the IRB committee of the Virtua Health System in Voorhees, New Jersey. All the patients provided written informed consents prior to participating in the study.

Immunoassays: The structure of the sandwich immunoassays employed here have been described earlier (Birken *et al.*, 1999). Each urine specimen was measured at multiple dilutions to check for linearity of the assay results. Linear dilution results were averaged and presented in the graphic format after division by creatinine concentration (to correct concentrations differences caused by variations in urine volume).

Characterization of urinary metabolite assays

hCG β cf assay (B210 capture IRMA)—The specific hCG β cf assay which we developed based on capture antibody B210 and detection antibody B108 cross-reacts with hCG, hLH, hLH β or hCG β less than 1% and with the hLH β cf less than 2% (Birken *et al.*, 2003; Krichevsky *et al.*, 1991). This hCG β cf assay system detects both the hCG β cf which appears at high

concentrations in the urine of pregnant women and also detects the hCG β cf derived from hCG secreted by the pituitary. The least detectable dose is 0.6 fmole/ml. The interassay coefficient of variation was 10% and intra assay coefficients of variation was 4% (O'Connor *et al.*, 1998).

hLH β cf assay (B505 capture IRMA)—The assay developed to the hLH β cf is based on capture antibody B505 and detection antibody B503 and cross reacts with hCG less than 0.2%, with hLH and hLH β 1%, and with hCG β and hCG β cf less than 0.1%. The least detectable dose is 1 fmole/ml. The inter and intra assay coefficients of variation were both 9% (O'Connor *et al.*, 1998).

Methods of Statistical Analyses

The dependent variables, i.e., the concentrations of hLH β cf and hCG β cf, were treated as continuous variables for all analyses. Means, standard deviations, and number of observations were presented for each variable. The experimental unit was each individual subject.

The experiment used a repeated measures design with each subject evaluated at multiple days during a 30 day period. The null hypothesis was that there would be no difference between NPCO and PCO at any time point or within groups. Prior to analysis, all data were tested for normality using the Shapiro-Wilk test. The data were significantly non-normal for all variables. In order to apply ANOVA methods, a 'normalized-rank' transformation was applied to the data. The rank-transformed data was analyzed using a mixed-model ANOVA for repeated measures followed by multiple comparisons to detect significant mean differences between groups and time points. Multiple pair-wise comparisons used the Bonferroni adjustment to maintain an experiment-wise type I error of 0.05 or less. Differences between means (rejection of the null hypothesis) were considered significant if the probability of chance occurrence was ≤ 0.05 using two-tailed tests.

The area under the curve was calculated for each metabolite for each patient (AUC₈₋₃₀) for the day 8 to day 30 period. Between-group comparisons were carried out using an independent t-test.

A spectral analysis was carried out on the urine concentrations of each metabolite using SAS PROC SPECTRA (SAS Institute, Cary, NC). The idea of spectral analysis is to characterize highly variable data from a time series. Spectral analysis has been successfully used to characterize highly variable ECGs over time and to derive dominant frequencies in patterns of atrial fibrillation. The method uses a Fourier transform to derive a power spectral density (an estimate of the variability of the data) transformed to a frequency domain. Since conventional statistical analysis yielded little in the form of secretion patterns of the metabolites, spectral analysis offered an alternative approach to determine characteristics in different patient groups.

Graphs were drawn using Graphpad Prism 4 which was used for AUC calculations.

RESULTS

Characterization of hLH β cf assay (B505) for measurement of variant hLH β cf

Since it is speculated that up to 10–15% of the U.S. population may carry one or both alleles for a common variant form of LH which is not recognized by some LH immunoassays (Nilsson *et al.*, 1997; Nilsson *et al.*, 2001a; Nilsson *et al.*, 2001b; Pettersson *et al.*, 1992; Pettersson and Soderholm, 1991; Rajkhowa *et al.*, 1995), it was important to determine how our metabolite assays reacted with variant LH core molecules. This variant form of LH has two mutations in its β subunit that convert Trp at residue 8 to Arg and Ile at residue 15 to Thr which makes it more closely resemble hCG, including the second glycosylation site at Asn 13 that is absent

in wild type LH (Elter *et al.*, 1999; Pettersson *et al.*, 1992; Suganuma *et al.*, 1996). Table 1 shows the results of studies of 16 urine specimens from subjects whose LH genotype was known. We found that our specific immunoassay to hLH β cf, did not recognize the variant LH core metabolite. This is shown by the results for the patients homozygous for the variant form of LH (v-LH) who had no measurable hLH β cf although they still had low but measurable pituitary hCG β cf. The hLH β cf measurement in non-pregnant healthy individuals is always significantly higher than that of hCG β cf which is derived from the low circulating concentration of pituitary hCG. The specific hCG β cf assay does not recognize the variant molecule hLH β cf either as shown in table 1. The genotyping of LH was performed by an immunological screening technique which requires blood specimens. It appears that none of the patients we have studied is homozygous for the variant form of LH since LH core was measurable in all the patients.

Measurement of hLH β cf in the general population of women

We first compared sampling techniques: Subjects were asked either to collect urine specimens for various time periods by the first morning void sampling technique or to collect urine specimens for 1–2 days by spot urine samples in which the patient would collect samples of each urination during this time period. Typical core fragment patterns were selected from all the patients examined for inclusion in the figures. Postmenopausal women started to collect urine specimens on any random day (figure 1a) while menstruating women collected urine starting the first or second day of menses (figure 1b). Figure 1 spot urine patterns are plotted with the X-axis indicating the time of day of collection and all collections were aligned to a 6pm arbitrary start axis point and the actual time of each collection is plotted. Figure 1 shows that spot urine collections all exhibited the very wide amplitude variations observed with the first morning void sampling technique. The young menstruating women (figure 1b), sampled during the early follicular phase (menses days 1–2 or 2–3), display the same variations that appear as pulses of hLH β cf in urine as did the postmenopausal group (figure 1a). Only the Y-axis scale is different since the concentrations of hLH β cf are 10–100 fold greater in the postmenopausal population as compared to the young menstruating population (area under the curve range, in fmole mg min/creatinine, for postmenopausal women was 52–2133 while for menstruating women the range was 3.6–59.2). The similarity of the spot urine sampling technique with the first morning void technique is further documented in figure 2 which shows the two techniques applied to the same two subjects, a young menstruating woman and a postmenopausal woman. Further analysis of the data indicate that the area under the peak for the spot sampling technique as compared to the first morning void technique is statistically indistinguishable in the same woman (four postmenopausal women were examined whose spot urine AUC range was 282–2133 and whose 10 day first morning void (FMV) AUC range was 251–6832), see table 2. This indicates that the concentration of hLH β cf excreted over time is constant in postmenopausal women despite the very wide fluctuations in amplitude and the difference in sampling techniques. The young menstruating group studied led to the same conclusion, namely that either sampling method indicated approximately the same concentration of hLH β cf was excreted over time. Stability tests performed on hLH β cf in urine indicated no change in measured concentrations after multiple freeze-thaw cycles showing that the wide fluctuations observed in these studies were not related to instability of the analyte in urine.

Young menstruating women (figure 3) were studied for one full menstrual cycle and usually displayed a single hLH β cf peak that presumably corresponds to the mid-cycle LH surge (figure 3). A number of these subjects displayed atypical patterns with multiple main peaks or late mid-cycle surge hLH β cf although the majority displayed the typical patterns shown in the figure. The women who participated in this study considered their menstrual cycles to be normal or regular.

Studies of women at a fertility Center—A summary of the classification characteristics of our fertility center patient population appears in Table 3. The table enumerates the patients placed in each of one of five diagnostic categories along with their age distribution, menstrual cycle character, LH-genotype and whether they became pregnant subsequent to treatment. In addition, the distribution of hLH β cf patterns and BMI are also shown.

There were 11 patients in the NPCO group and 25 in the study group. All our patients were in reproductive age, 24–39 years old, and 53% became pregnant following treatment. One patient in the NPCO group had a marginal elevation of serum testosterone, while there were 14 patients in the study group that had elevated total serum testosterone levels (>50 ng/dL) representing 52% of the study patient population. Eighty per cent of the patients that had PCO and IR had elevated serum testosterone and most of them (70%) had enlarged ovaries (>10 ml). By contrast 33% of the patients with PCO, without IR, had an elevated serum testosterone and only 40% had enlarged ovaries, possibly indicating a disease process. Two patients in each group, the NPCO and the study group, had marginal elevation in the DHEA-S levels (>250 μ g/dL).

Although menstrual dysfunction is the most commonly found clinical symptom in PCO patients, 11 of the patients with PCO had regular menses and one patient in the NPCO group had irregular menses (Table 3) during her initial evaluation. This last patient had elevated FSH serum levels on the third day of the menstrual cycle and repeatedly during subsequent treatment cycles and she did not become pregnant. Three patients had markedly elevated LH serum levels on day 3 of their cycle. All of them had a rather large number of antral follicles on each ovary. All of those patients became pregnant with treatment. Three patients had elevated baseline level FSH serum levels on the 3rd day of their cycle (>11 mIU/ml). The patient that had a baseline FSH of 11.1 mIU/ml became pregnant while the other two did not become pregnant, baseline FSH 13.1 and 13.2 mIU/ml (Yanushpolsky et al., 2003).

The different categories of the body mass index (BMI), were evenly distributed in the NPCO group. By contrast, in the study group, obesity was observed with a higher prevalence at the expense of lower incidence of the normal weight, obese (52%), overweight (33%) and normal weight (15%). Forty four percent of the patients with PCO (11/25) had IR. With one exception, none of the patients had clinical hypothyroidism. Nine patients were diagnosed with subclinical hypothyroidism and were treated for the potential effects on this condition on the fetus.

Some PCO patients who had irregular menstrual cycles waited more than a month to initiate their urine collection. Each first morning urine was measured using the specific hCG β cf and hLH β cf assays. Most patients were also genotyped to determinant whether they carried the variant LH and none of those patients was found to be homozygous for the v-hLH. Since we detected hLH β cf in all patients which would be undetectable if v-LH β cf was the only LH core molecule in the urine, we infer that none of our patients was homozygous for v-hLH β cf.

In all the NPCO patients (figure 4) with only three exceptions, the hLH β cf peak occurred prior to day 16 of the cycle. In the other three cases, it occurred on days 17, 19 and 22 of the cycle. When the patterns of hLH β cf urinary excretion were examined, it was noted that this group had a single peak, occurring and no smaller hLH β cf peaks were seen during the follicular phase with three exceptions (3/11, 27%). By contrast seven patients in the PCO group (figure 5) had small peaks of hLH β cf observed during follicular phase (as PCO 2 and PCO 3) and eight patients (15/25, 60%) had a completely disordered pattern without a distinct hLH β cf main peak as shown by patient PCO 5–6.

Overall, a mixed pattern of hLH β cf excretion in the urine was observed. Most patients, especially those that belonged in the NPCO group, had a single peak, although additional patterns were noted mostly in the PCO group. Some of the hLH β cf patterns had a second peak

noted either preceding or following the hLH β cf peak approximately by one or two days and achieving a comparable concentration of beta core fragment (PCO 3). Although only two patients (18%) in the NPCO group had an hLH β cf pattern of two peaks, 38% of the patients in the PCO group, that did not have a disordered pattern, had two hLH β cf peaks. Among the PCO patients, three different patterns of hLH β cf urinary secretion predominated. One that had a single peak occurring later in the cycle (11/25, 48%, PCO 2), another one that had smaller hLH β cf surges early in the menstrual cycle followed by a dominant hLH β cf surge later in the cycle (7/25, 28%, PCO 4), and finally, a disordered pattern of hLH β cf throughout the cycle (7/25, 28%, PCO 5–6). Among patients that had PCO and IR (figure 6), seven had a disordered peak pattern (7/10, 70%) one of which are shown in the figure (PCO-IR 2).

Measurements of peaks of hCG β cf were much lower than hLH β cf (usually 2–10 fmoles/mg creatinine for hCG β cf) as would be expected from the much lower level of circulating hCG as compared to hLH, both derived from the pituitary. It was observed that some PCO-IR patients have high pulses of hCG β cf, as high as 88–138 fmoles/mg creatinine, (PCO-IR 1 and PCO-IR 2 in Figure 5). This was an uncommon observation among the patients and the meaning is not clear. Statistical analysis (see section below) did not indicate that hCG β cf measurement was a useful discriminant for the PCO as compared to the NPCO patient populations.

Statistical Analyses

Fig 7 shows a spectral analysis of hLH β cf and hCG β cf concentration patterns from 11 NPCO as compared to 25 PCO patients. Spectral analysis characterizes variability of a time-varying process. The power spectral density indicates relative amount of variability at particular frequencies of the data set. Fig 7a shows a clear difference in “dominant frequency” between hLH β cf levels from NPCO (.2478 Hz) and PCO patients (.3478 Hz). This suggests different patterns of secretion with different variability. Different patterns were not observed with hCG β cf levels. Similar results were obtained when the metabolite levels were normalized to creatinine levels.

Discussion

Measurement of the stable urinary core metabolites of LH and hCG opens a new window on examination of events occurring in the blood. Multiple sampling and pattern differentiation can be accomplished with ease for a patient without repeated blood sampling. Although little data exists on the metabolism of LH to the hLH β cf, there are several publications on the hCG urinary metabolite, hCG β cf, which is very similar in structure and may be generated in a similar fashion (Birken *et al.*, 1988; Birken *et al.*, 1996; Blithe *et al.*, 1989; Wehmann *et al.*, 1984; Wehmann *et al.*, 1989; Wehmann *et al.*, 1990; Wehmann and Nisula, 1980). A series of papers attest to the stability of this terminal metabolite of hCG which is excreted as a remarkably uniform molecule, composed of the hCG β peptide chain structure of 6–40 disulfide-bridged to 55–92 (Birken *et al.*, 1988; Birken *et al.*, 1996). A series of metabolic studies including human studies by the groups of Nisula and that of Norman both presented data which indicated the hCG β cf was generated predominately by the kidney from hCG presumably absorbed and degraded within the renal tubule structures (de Medeiros *et al.*, 1992a; de Medeiros *et al.*, 1992b; Norman *et al.*, 2000; Wehmann *et al.*, 1984; Wehmann *et al.*, 1989; Wehmann *et al.*, 1990; Wehmann and Nisula, 1980). While authentic hCG β cf has only been isolated from urine and thus, is thought to be solely or mainly a kidney product derived from proteolytic digestion of hCG absorbed from the blood, hLH β cf has been purified both from pituitary tissue as well as from urine and the structures are the same. Therefore, hLH β cf may be produced both from LH extracted from the blood during passage through the kidney and also directly by secretion by pituitary tissue into the blood and be very rapidly excreted in urine. (Birken *et al.*, 1993; Birken *et al.*, 1996; Birken *et al.*, 1999; Birken *et al.*, 2001).

We reported preliminary results using this technique for examination of LH core excretion in a small set of women during reproductive aging (Birken *et al.*, 1999). In the current report, we have expanded the patient set for these studies as well as examined different urinary sampling techniques such as spot urines as compared to the more routinely used first morning void urine series. We found that the pulsatile variation displayed with the first morning void sampling appears similar or identical to that observed by spot urine sampling method during a 1–2 day interval. The areas under the curves in the same group of subject (menstruating or postmenopausal) were also statistically the same indicating a constant concentration of hLH β cf appears in the urine over time despite the very widely variant amplitudes. An explanation for the wide variance in amplitude during urine sampling of the hLH β cf is still difficult to develop since the core is apparently produced 24–48hrs after LH passes through the kidney and a portion is absorbed into the kidney tissue (O'Connor *et al.*, 1998). It is as if the kidney is acting as a pulsatile endocrine organ and delivering pulses of core production to the urine excreted into the bladder. The observation of a pulsatile release of hLH β cf by the kidney remains an unexpected and unexplained observation since one would presume the extended processing time of 24–48hrs to generate the core fragment after renal cell absorption of hLH would be variable and not directly related to the pulsatile pattern of hLH in blood. In addition, the amplitude of the hLH β cf pulses are much greater than the amplitude of the pulses of hLH in the blood. Although over time, the same total concentration of hLH β cf is excreted, the time course of its release varies widely. The hLH β cf is as stable as the hCG β cf in urine and stability plays no role in the observations of its wide fluctuations in urine (O'Connor *et al.*, 1998).

About a decade ago, it was found that a variant form of LH was present in many patient populations and was not recognized by a number of assay systems for LH based on monoclonal antibodies (Pettersson *et al.*, 1992). We explored recognition of the urinary core metabolite of this variant by our hLH β cf and hCG β cf assays. The hLH β cf immunometric assay did not react with the v-hLH β cf and neither did the hCG β cf assay system. None of the patients we examined were homozygous for the variant LH molecule since hLH β cf was measurable in every one of our patients.

This is the first report on the metabolism of LH and pituitary hCG in patients classified as PCO as compared to NPCO patients within the population of a fertility center. Little information exists on LH metabolism since no assay for its urinary metabolites existed before the assay system was developed in this laboratory. The goal of these studies was to determine if the pattern of excretion of the LH metabolite, hLH β cf, as well as the pituitary hCG metabolite, hCG β cf, was different between these two populations and whether those differences may be used as part of the diagnostic criteria for placing a patient within the PCO patient group. It appears that more of the PCO patients displayed “abnormal” hLH β cf patterns as compared to the control population of women but how best to apply this information requires further study. It has been reported that healthy women exhibit variations from the model mid-cycle LH surge peak and may exhibit split peaks and multiple peaks as well as delayed peaks (Alliende, 2002). This is consistent with what we have observed with hLH β cf patterns from the group of young menstruating women selected from the general population as well as the group of NPCO patients studied within the fertility center.

Small LH peaks that occurred prior to or after the mid cycle LH peak have been observed by others. Alliende found approximately 20% of women of proven fertility with regular menses and ovulatory cycles had small LH peaks, although no reference was made as to the number of small LH peaks and if they occurred prior to or after the LH surge (Alliende, 2002). The high frequency in which those small peaks occurred in the PCO and PCO with IR groups, along with the disordered pattern, may be responsible for luteinization of developing follicles, anovulation, poor ovulatory outcome, and possible miscarriage.

Prolonged LH peaks and two LH peaks have been observed by others and have been associated with new follicular development or atresia. (Garcia et al., 1981; Lemay et al., 1982; Martinez et al., 1991; Moghissi et al., 1972; (Lemay et al., 1982; Martinez et al., 1991). The hLH β cf peaks in our studied population lasted for four to nine days, but there was a shift in the observed pattern. For most of the patients (2/3) in the NPCO group, the hLH β cf peak lasted for seven to nine days. By contrast, in the PCO and PCO with IR groups, when a single hLH β cf peak occurred, it lasted for less than seven days. In these two groups, a disordered pattern was more prevalent. If PCO with IR are considered a more advanced stage of the same condition, then the disordered pattern was also more prevalent than in any other group of patients. Different hLH β cf excretory patterns were observed in young menstruating women that had regular menstrual cycles, patients that suffered from infertility but did not have polycystic appearance of the ovaries, patients that suffered from PCO, and those that suffer from IR and had or did not have polycystic appearance of the ovaries. Although no systematic serum data were obtained to corroborate the urinary hLH β cf peaks among patients attending the fertility center, in those cases that limited information was available, the serum LH levels corresponded temporally to the hLH β cf peaks.

In our study we noted a delay in the occurrence of a single hLH β cf peak in the urine (expected 24–48 hrs after the mid cycle LH surge in blood), possibly indicating delayed ovulation. We also noted small hLH β cf peaks during the follicular phase followed by a delayed hLH β cf peak possibly indicating luteinization of the developing follicles. Finally, we observed a completely disorganized hLH β cf peak pattern. This progression in hLH β cf urinary excretion pattern was associated with higher prevalence of elevated testosterone, overweight or obesity and IR.

No clinical information other than the one obtained through a questionnaire was available for the young menstruating women and they might represent a mixture of patients, as we demonstrated by studying the infertility center patient population. The menstrual pattern did not prove to be predictive of the PCO appearance of the ovaries since half of patients with regular menses had polycystic ovaries. By the same token the PCO appearance of the ovary did not have any predictive value for IR. Again, approximately half of the patients with PCO had IR. If one assumes that irregularity of menstrual cycles, increased body weight, and PCO are part of a progressive condition, then those patients that have most if not all of those indicators, especially if they have them for a protracted period of time, would display a disorganized pattern of LH secretion from the pituitary and, therefore, a completely disorganized urinary hLH β cf excretion pattern.

It would, therefore, be useful to further investigate use of the urinary excretion pattern of hLH β cf to identify those patients that may have an ovulatory aberration, but also are at high risk to have underlying metabolic abnormalities.

It is apparent from this study that normal populations of women of reproductive age with intact reproductive capacity should ideally be characterized very carefully over an observation period of several menstrual cycles. Even in that population of women there might be alternative patterns of serum LH secretions and/or urinary hLH β cf excretory patterns.

The prevalence of certain patterns in the PCO, and PCO with IR groups might point to a disease process whereupon worsening of the clinical picture or protracted illness can alter the excretory pattern of hLH β cf in the urine. Determination of these patterns and their significance might help in the diagnostic processes, the need assessment of clinical intervention, and monitoring of therapeutic outcomes.

Finally, these patterns or parameters, if used as the diagnostic criteria, might assist the interpretation of subjective ultrasonographic appearance of the ovaries with more sensitive and reproducible biochemical measurements. The spectral analysis results suggest differing

patterns of excretion of the metabolite of LH, hLH β cf, between NPCO and PCO patients. The different dominant frequencies that emerged from the PCO and NPCO groups provides a way to characterize the widely variable patterns of metabolism and excretion of hLH metabolites in the urine.

Acknowledgements

Chomee Yoon is acknowledged for help with data handling and Jessica Macdonald is acknowledged for technical support. This work was supported in part by R01AG13783 and R21CA98350.

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Spot Urines From Postmenopausal Women (PM)

Time of Day of Spot Urine Sample Collection

Figure 1. Typical spot urine concentration profiles of hLH β cf excretion by postmenopausal (PM) (a) as compared to young, menstruating women (YMW) (b)

Serial urine collections were made of all urine excreted over a 2 day time interval. The time of day the collection is indicated on the X-axis although the X axis is plotted on a minute scale to permit calculation of area under the curve/min for comparison with samples collected once per day (see figure 2). All X-axes begin at 6pm and end at 12pm of second day of collection. The menstruating women (b) collected urine on days 1–2 or 2–3 of menses. Note the Y-axis scale for the postmenopausal women (a) is 10 to 100 times that of the young women (b) but the overall patterns are quite similar.

Comparison of Daily versus Spot Urine Collections on Same Patients

Figure 2. Comparison of the profiles of hLH β cf concentrations in spot urine collections versus the once-a-day urine sampling technique

A young menstruating woman (one of several is shown) collected urines by the once a day technique as compared to the spot urine method and a similar study was done with postmenopausal subjects. Each subject was asked to either serially collect each urination for two days or the first morning void urine for 10 consecutive days. The menstruating woman started on day 1 or 2 of menses for both sampling studies. It was found that the area/minute under the curves for both techniques were similar (see table 2).

Figure 3. Complete menstrual cycle profiles of hLH β cf excreted by young menstruating women collected from the general population

Subjects began collection at the start of menses (day 1 or 2) and continued collecting first morning void urines until the start of their next cycle. X-axis is plotted as days and Y-axis is hLH β cf fmoles/mg of creatinine.

Figure 4. Complete menstrual cycle profiles of hLH β cf and hCG β cf excreted by NPCO women collected at an infertility center

Collection method and plotting was similar to figure 3 except that both hLH β cf and hCG β cf were measured and plotted on the same graphs.

Figure 5. Complete menstrual cycle profiles of hLH β cf and hCG β cf women diagnosed with PCO at an infertility center

Collection and plotting techniques were as in figure 4.

Figure 6. Complete menstrual cycle profiles of hLHβcf and hCGβcf women diagnosed with PCO and Insulin Resistance an infertility center
Collection and plotting techniques were as in figure 4.

Figure 7. Spectral analysis of hLHβcf and hCGβcf urinary metabolite concentration patterns from NPCO as compared to PCO patients

The power spectral density indicates relative amount of variability at particular frequencies of the data set. Fig 7a shows a clear difference in “dominant frequency” between hLHβcf concentrations from NPCO (.2478 Hz) and PCO patients (.3478 Hz). This suggests different patterns of excretion with different variability. Different patterns were not observed with hCGβcf concentration levels.

Table 1

Determination of Immunoreactivity of the variant hLH β cf, v-hLH β cf, (Trp 8 to Arg8 and Ile15 to Thr15) using Two Immunometric Assays

Analyte	LH Genotype		
	Wildtype hLH n=4 Mean $\pm$ SEM (Range)	Heterozygous v-hLH n=9 Mean $\pm$ SEM (Range)	Homozygous v-hLH n=2
hLHβcf	264.3 $\pm$ 194.1 (54.6, 845.7)	244.7 $\pm$ 95.0 (6.5, 793.1)	Undetectable
hCGβcf	4.79 $\pm$ 2.24 (1.64, 11.43)	7.60 $\pm$ 3.07 (0.66, 24.75)	0.92, 1.08
Creatinine, mg/ml	0.88 $\pm$ 0.33 (0.65, 1.83)	1.17 $\pm$ 0.15 (0.54, 1.85)	1.09, 0.93

Values for the hLH β cf and hCG β cf are in fmoles/mg of creatinine for the means $\pm$ standard error of the mean with minimum and maximum presented as well. Results for 14 women are presented along with one male subject. There was one female and one male homozygous for the variant form of hLH (v-hLH) and each individual value in fmoles/mg creatinine is presented.

Table 2

Correlation of Area Under the curve (AUC) for 2 day versus 10 day sampling technique in postmenopausal women

Area/min 2 day Pearson correlation	2 day 1	10 day 0.987*
Sig. (2-tailed)		0.013
n	4	4
Area/min 10 day Pearson correlation	0.987	1
Sig. (2-tailed)	0.013	
n	4	4

* correlation is significant at 0.05 level (two-tailed)

Table 3**Characteristics of Patients from Fertility Clinic**

	NPCO (n=11)	PCO (n=10)	PCO&IR (n=2)	PCOS (n=5)	PCOS&IR (n=8)
Menses					
Regular	10	6	1	3	1
Irregular	1	4	1	2	7
LH Genotype					
Wild type	7	2	1	2	4
Heterozygous v-LH	0	1	0	1	1
Homozygous v-LH	0	0	0	0	0
Unknown	4	7	1	2	3
Pregnancy					
Yes	4	6	2	4	1
No	7	3	0	1	6
Unknown	0	1	0	0	1
hLHβcf peaks					
Normal	7	1	0	2	1
Delayed	2	2	0	1	3
Small & Delayed	2	4	0	2	2
Disorganized	0	3	2	0	2
BMI					
Underweight	0	0	0	0	0
Normal weight	3	2	0	1	1
Overweight	4	5	0	1	2
Obese	4	3	2	3	5

Classification of patients is: without polycystic ovaries, non-PCO (NPCO), with polycystic ovaries (PCO), with polycystic ovaries and elevated testosterone serum levels, PCO syndrome (PCOS), and patients in each of the last two groups that also had insulin resistance (PCO&IR) and (PCOS&IR). Values are numbers in each category.

Unknown information was due to unavailable blood samples for LH genotyping or for those patients that did not undergo fertility treatment.