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Circadian Rhythms in Acute Intermittent Porphyria—a Pilot Study

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Abstract

Acute intermittent porphyria (AIP) is an inherited disorder of heme synthesis wherein a partial deficiency of porphobilinogen [PBG] deaminase [PBGD], with other factors may give rise to biochemical and clinical manifestations of disease. The biochemical hallmarks of active AIP are relative hepatic heme deficiency and uncontrolled up-regulation of hepatic 5-aminolevulinic acid [ALA] synthase-1 [ALAS1] with overproduction of ALA and PBG. The treatment of choice is intravenous heme, which restores the deficient regulatory heme pool of the liver and represses ALAS1. Recently, heme has been shown to influence circadian rhythms by controlling their negative feedback loops. We evaluated whether subjects with AIP exhibited an altered circadian profile. Over a 21 h period, we measured levels of serum cortisol, melatonin, ALA, PBG, and mRNA levels [in peripheral blood mononuclear cells] of selected clock-controlled genes and genes involved in heme synthesis in 10 Caucasian [European-American] women who were either post-menopausal or had been receiving female hormone therapy, 6 of whom have AIP and 4 do not and are considered controls. Four AIP subjects with biochemical activity exhibited higher levels of PBG and lower levels and dampened oscillation of serum cortisol, and a trend for lower levels of serum melatonin, than controls or AIP subjects without biochemical activity. Levels of clock-controlled gene mRNAs showed significant increases over baseline in all subjects at 5 am and 11 pm, whereas mRNA levels of ALAS1, ALAS2, and PBGD were increased only at 11 pm in subjects with active AIP. This pilot study provides evidence for disturbances of circadian markers in women with active AIP that may trigger or sustain some common clinical features of AIP.

Keywords

5-aminolevulinic acid; acute intermittent porphyria; circadian rhythms; clock-controlled genes; cortisol; heme; melatonin; porphobilinogen; porphyria

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INTRODUCTION

Acute Intermittent Porphyria (AIP) is an inborn error of heme synthesis caused by autosomal dominant mutations in PBGD, the third enzyme of the heme biosynthetic pathway, which carries out the polymerization of the monopyrrole porphobilinogen (PBG) into hydroxymethylbilane (HMB) (1–6). These defects, acting in concert with other genetic, environmental, and nutritional factors, may give rise to relative hepatic heme deficiency and uncontrolled up-regulation of hepatic ALA synthase-1, normally the rate-controlling enzyme for hepatic heme synthesis. Such induction leads to overproduction and over excretion of ALA and PBG in the urine, both of which are hallmarks of active AIP. Elevated levels of ALA, or a non-PBG product derived therefrom, are believed to be responsible for many of the symptoms common in biochemically active-AIP (1–7). Elevations of urinary and/or plasma levels of ALA and PBG are key criteria clinicians use to make a diagnosis of an acute porphyria. Along with resolution of symptoms, decreases in levels of ALA and PBG are helpful for gauging efficacy of therapy of acute porphyric attacks. Concentrations of these precursors to heme in plasma and urine have been shown to normalize quickly, albeit transiently, following intravenous heme therapy. They rapidly and permanently fall to normal following successful liver transplantation accompanied by complete and permanent remission of clinical presentation, suggesting an hepatic origin of the disease (8–10). Other evidence for the key role of ALA, as against PBG, in causing symptoms comes from observations of similar clinical features (severe bouts of abdominal or other pain, etc.) in subjects with acute lead poisoning or hereditary tyrosinemia, type I, in which marked inhibition of ALA dehydratase [also known as PBG synthase], the second enzyme of the heme synthetic pathway, gives rise to marked over-production and over-excretion of ALA without increased PBG (11, 12). Similar biochemical changes also are seen in the rare form of acute porphyria due to severe homozygous or compound heterozygous deficiency of ALA dehydratase (1–6).

The clinical features of AIP typically include, diffuse gastrointestinal pain, vomiting and constipation, as well as tachycardia and hypertension (1–6). Severe attacks can include development of peripheral neuropathies, including pain, muscle weakness, and CNS features such as delirium, mental confusion, and seizures. Although the exact pathogenesis of acute porphyric attacks remains unclear, it is well known that women are more likely to develop porphyric attacks than men, especially during the luteal phase of their menstrual cycles (13). Moreover, gonadotropin releasing hormone analogues have been shown to reduce or eliminate cyclical attacks in some women (14). These observations, along with other complaints such as disorders of mood and sleep (insomnia), have led to speculation that AIP attacks may be related to hormonal imbalances (1, 2, 4–6). Anterior pituitary hormones are secreted as pulses in a rhythmic manner, and these irregularities in mood and sleep may partly be caused by alterations in gonadal hormones. As post-menopausal women have decreases gonadal hormones, fluctuations in sex hormones are but one of several possible reasons responsible for the altered mood states and sleep disorders. Another possible factor influencing irregularities in mood and sleep is alteration in the circadian release of melatonin (15, 16).

Disruption of circadian rhythms, the mechanisms that adjust our physiology to external environmental signals, has emerged as a new potential risk factor in the development of numerous diseases and disorders. These rhythms are deranged by shift work and jet-lag (17), and in disparate conditions such as insomnia, sleep syndromes (18), acute myocardial infarction, and depression; and such disruption is an important factor that contributes to cancer development and progression (19, 20). Circadian oscillations respond to three components: an input signal (*e.g.*, light, temperature) that entrains the body rhythms to environmental cues, the suprachiasmatic nucleus that integrates signals, and a third

component known as the output pathway that results in, for example, hormone secretion or a locomotor activity. The levels of cortisol and melatonin in serum are accepted as standards of intrinsic clock activity (21, 22).

Cellular heme levels are controlled in part by circadian expression of the heme-degrading enzyme, heme oxygenase-1, activity of which peaks at night (23, 24). Heme, in turn, controls the activities of a variety of signal transducers and transcriptional regulators, including the circadian transcription factors Npas2, Rev-erb-alpha, and Per2, which are responsible for driving the positive and negative feedback loop of the intracellular clock and which accumulate at night (25–27). The Npas2 factor forms heterodimers with its counterpart Bmal1 to regulate the expression of target genes; thus, cellular heme status controls the binding of Npas2 to DNA (28). Kaasik and Lee showed that heme-Npas2 regulates ALAS1 expression, and that heme also differentially modulates the expression of *P*er*1 and 2* genes (24). Moreover, *Per2*, a transcription factor that accumulates at night, enhances *Npas2/Bmal1* activity towards the *Alas1* gene, whereas partial ablation of both *P*er genes in mice results in circadian disruption of *ALAS1* and *2* expression (29). Binding of heme occurs in two distinct regions of *hPER2*, including a novel heme regulatory motif located near the carboxyl terminus of the protein. Interestingly, proteasome-mediated degradation of *hPER2* is exclusively associated with heme-mediated oxidation and ligand binding to the carboxyl-terminus domain. In agreement, heme synthesis directly controls *hPER2* levels *in vivo,* and its specific binding to the heme regulatory motif influences the period length and phase-shifting properties of the clock in synchronized cells (27). The current model suggests the existence of reciprocal regulatory loops between heme biosynthesis and circadian protein expression. Thus, dys-regulation of any of these pathways will impact cell homeostasis and could be a determining factor for disease initiation and progression.

These observations prompted us to hypothesize that subjects with AIP, especially those with biochemical activity, indicative of relative hepatic heme deficiency, may demonstrate abnormalities in normal circadian rhythms and that these may help to account for some of the common complaints of subjects with AIP, such as difficulties with sleep and alterations of mood and affect. We present here results of a pilot study comparing the circadian rhythmicity of serum cortisol and melatonin, heme precursor concentrations, and selected clock-controlled gene expression in control and AIP subjects.

METHODS

Chemicals

ALA, (MW: 167.59) and PBG (MW: 244.24) were purchased from Frontier Scientific (Logan, UT). Heavy-isotope labeled versions, namely, 5-amino-2,2-levulinic-2,2-d2 acid HCl (MW: 169.59) and carbon-13 labeled porphobilinogen (MW: 246.24) were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada) and Frontier Scientific (Logan, UT), respectively. A porphyrin standard mixture (porphyrin acids chromatographic marker) was purchased from Frontier Scientific (Logan, UT). Creatinine was purchased from Sigma-Aldrich (St. Louis, MO). Oasis HLB solid phase extraction cartridge (1 cc, 30 mg), used in sample clean-up, was purchased from Waters Corp. (Milford, MA). Water, acetonitrile and formic acid (FA) of mass spectrometry grade were purchased from EMD Chemicals, Inc. (Darmstadt, Germany). Unless stated otherwise, all chemicals were of HPLC or analytical grade.

Study subjects

Post-menopausal Caucasian subjects, six with AIP and four sex-matched controls, were enrolled in this study. Post-menopausal women were intentionally selected to avoid the effect of variations in gonadotropic and sex hormones during the menstrual cycle in the circadian release of cortisol and melatonin, thus, simplifying data collection and analysis. Additionally, subject C1 did not report having a period for more than one year and was thus considered peri-menopausal. Due to poor peripheral venous access, one control subject had blood obtained at only the first two time points (11 am, 2 pm). All AIP subjects were asymptomatic in regards to typical symptoms of acute porphyric attacks, were post-menopausal, and had a well- documented, stable clinical history of AIP with confirmatory genetic testing [Table 1], not needing hospitalization or therapy for AIP in the past three months, and did not have other major chronic diseases of any organ or system. On the day of study, two of the subjects with AIP were judged to have no detectable biochemical activity, defined as normal urinary [< 4 uM] and plasma [< 1 uM] levels of PBG, whereas four, although without symptoms, had biochemical activity with mild to moderate increases in urinary and plasma PBG [Table 1, Fig 3]. Control subjects were women without documented AIP or any other forms of porphyria, and documented single-void or 24-h urinary ALA and PBG levels within the normal range. All subjects resided in the adjacent eastern or central time zones of the USA and had not traveled to other time zones within two months of the study days.

Exclusion criteria for control and AIP subjects were acute psychiatric illness, including but not limited to major depression and seasonal affective disorders (SAD) (30) within six months of the study, as well as those requiring psychoactive medications within this period. Those with a history of psychiatric disease in first degree family members were also excluded. Subjects were screened using the self-report Beck Depression Inventory II (BDI-II), and subjects with scores greater than 19 and thus suggesting moderate depression were excluded. Other exclusion criteria were advanced chronic disease other than porphyria, those currently using oral contraceptive pills currently or within the past six months, blood hemoglobin less than 10 g/dL, blood transfusion within six months of study, those regularly taking non-steroidal anti-inflammatory (NSAIDs), beta-blockers (31), or alpha-blockers, acute illness in the past two months, travel to another time zone within the preceding two months, history of alcohol abuse, treatment with PanhematinR within three months of study, and inability or unwillingness to give informed consent.

Study protocol and sample collection

All human studies were carried out in a sleep center in Charlotte, NC, in accordance with the principles of the Declaration of Helsinki and adhered to a protocol approved by the Institutional Review Board of Carolinas Medical Center. Subjects who volunteered for the study were instructed to eat balanced meals and abstain from alcohol for one week before the day of the study. Subjects were asked not to exercise vigorously during the study day but were free to move about the sleep center throughout the day as they would normally. Subjects underwent 15 mL blood draws beginning at 11am and every three hours thereafter (1400, 1700, 2000, 2300, 0200, 0500 and 0800), using indwelling IV catheters located in a suitable peripheral vein for the duration of the study period. A solution of heparin in 0.154 M NaCl was used to keep the catheter patent, and ~20 mL of blood were removed prior to each sample and replaced into the subjects, followed by ~ 10 mL additional heparinized saline. Samples collected between the hours of midnight and 8am were collected and processed under dim light of <30 lux (32, 33), using a F8T5 fluorescent red 12′ lamp (Antares Enterprises, Inc., Santa Fe, CA) that has been previously shown to minimally affect the human circadian clock (34). All sample tubes were wrapped in aluminum foil to prevent any light influence on hormonal or mRNA profiles. Subjects were asked to sleep with eye

masks and ear plugs in the dark during the time of their usual bed rest, but were instructed to get out of bed if they felt they had been awake for more than 15 minutes prior to their expected time of waking. The ambient temperature of the bed room was kept at 23±2°C via a thermostat.

Whole blood samples were apportioned between serum separator tubes and tubes containing EDTA, an anti-coagulant. Blood in serum separator tubes was allowed to clot and then centrifuged, after which the sera were carefully removed and stored in dark brown cryovials stored at −70°C until further analysis. The EDTA-containing tubes were centrifuged at 3,300g for 10 minutes at room temperature, after which the plasma was removed, aliquoted and stored as above. The buffy coats from the EDTA-containing tube were also collected and flash frozen at −70°C. At baseline [approximately 11 am], random urine samples were collected in brown containers, kept on ice until they were aliquoted, and stored frozen [−70 C].

Determination of serum melatonin and cortisol

As intrinsic markers of SCN activity, serum melatonin and cortisol levels were determined using Melatonin ELISA kit (IBL International, Toronto, Ontario, Canada) and Cortisol ELISA kit (ALPCO Immunoassays, Salem, NH), respectively, per the manufacturer's instructions. Samples were run in duplicate for each analyte and time point. Results were extrapolated using a four-parameter logistics fit calibration curve constructed with five standard concentrations for cortisol (0.5–30 μg/dL) and four standard concentrations for melatonin (7–250 pg/mL). Total hormone levels for the entire 21 hour study period were determined by area under the curve (AUC) analysis using the trapezoid method. In this method, hormone levels for 3 hour intervals between blood draws were averaged and multiplied by the length of time that passed (i.e., 3 hours), and the values were summated to obtain the entire serum hormone concentration over the 21 hour study period for each subject.

Expression of clock-controlled and heme metabolism genes

RNA was isolated from peripheral blood mononuclear cells (PBMCs) from buffy coat samples using miRNeasy Mini Kit (Qiagen, Valencia, CA) in accord with manufacturer's instructions. First-strand complementary DNA was synthesized using iScript™ cDNA synthesis kit (Bio-Rad, Hercules, CA). The reverse transcription reaction was incubated at 42° for 30 minutes and stopped by heating to 85° for 5 minutes. 50 ng of final product were used as template for PCR. qRT-PCR was performed using Taqman® Probe-Based Detection × with an ABI Prism 7500 Fast Real-Time PCR System using Taqman® gene expression assays and Taqman® Gene Expression master mix (Applied Biosystems, Foster City, CA). Templates were amplified by 40 cycles of denaturation at 95°C for 15 seconds, annealing of primers and probe together with extension at 60°C for one minute in triplicate reactions. Fluorescence data were acquired during the combined anneal/extension step. Suitable negative control reactions were run to confirm absence of DNA contamination. Samples were run in triplicate and fold change values were calculated using comparative Ct analysis and normalized to an invariant control (TBP [TATA-box binding protein]).

Analyses of urinary and plasma porphyrins

Urinary porphyrins were initially purified using a solid phase extraction process and subsequently analyzed by reversed-phase ultra-high performance liquid chromatography/ fluorescence detection (UPLC/FLD), as previously described (35). Briefly, 1.0 mL urine samples were purified by solid phase extraction using 1mL (30 mg) Oasis HLB cartridges prior to injection. [Baseline urine samples were available for this analysis from only 6 of the 10 total subjects enrolled; samples from the other 4 subjects had been exhausted.]

Porphyrins were then separated and quantified by reversed-phase UPLC/FLD system (Shimadzu Nexera/Prominence Hybrid UPLC system, Kyoto, Japan) equipped with a Thermo Hypersil-Gold C_{18} (5 μm, 4.6 mm × 250 mm; Thermo Scientific, Waltham, MA) column. The mobile phase consisted of 50 mM aqueous ammonium formate (pH 3.0, A) and acetonitrile (B), with a flow rate of 1.5 mL/min and a linear gradient profile as follows: 0 min, A 80%, B 20% to 30 min, A 20%, B 80%. The final 20/80 mixture was run for an additional 5 minutes (total run time/sample = 35 min). Porphyrins were detected and quantified by fluorescence with a red-sensitive detector (Shimadzu Prominence RF-20A XS, Tokyo, Japan) at excitation wavelength of 398 nm and emission wavelength of 600 nm. Four porphyrins showed good linearity at a concentration range of 25–250 pmol/mL, namely, 8-carboxyl porphyrin (uroporphyrin), 7-carboxyl porphyrin, 6-carboxyl porphyrin, and 5-carboxyl porphyrin, while two porphyrins showed good linearity at a concentration range of 50–500 pmol/mL, namely, 4-carboxyl porphyrin-I (coproporphyrin-I) and 4-carboxyl porphyrin-III (coproporphyrin-III). Porphyrin concentrations were normalized to urinary creatinine as determined by adding heavy isotope-labeled creatinine (+3 Da) for selected reaction monitoring quantification using high-performance liquid chromatography/mass spectrometry (LC-MS; ion transitions: creatinine 114 m/z → 44 m/z, isotope labeled creatinine 117 m/z → 47 m/z). For plasma samples, 500 μL portions were initially diluted with the same volume of water and followed by SPE with the same protocol as above.

Analyses of plasma and urine ALA and PBG

To quantify ALA and PBG in plasma, we used heavy isotope internal standards processed as described above, separated by ultra-high performance liquid chromatography (UPLC), and quantified by tandem mass spectrometry (MS), as previously described (36–38). Briefly, 1.0 mL portions of plasma samples were purified using 1 cc (30 mg) Oasis MCX cartridges prior to injection into the liquid chromatography system. The LC-MS system consisted of a Waters UPLC (Milford, MA) equipped with a Phenomenex Kinetex C_{18} (1.7 μm, 2.1 × 150 mm) column and a Thermo-Fisher TSQ Quantum Ultra mass spectrometer, triple quadrupole MS with electrospray ionization (Kyoto, Japan). The spray voltage and capillary temperature were optimized at 3500 V and 272°C, respectively.

The optimal collision energy for ALA and PBG was 25% and 15%, respectively. Heavy isotope-labeled ALA (+2 Da) and PBG (+2 Da) were added as internal standards for relative quantification of endogenous ALA and PBG, which were eluted over a linear gradient mobile phase consisting of 0.1% (v/v) FA in water and 0.1% FA in acetonitrile. The quantification of ALA and PBG was achieved using single reaction monitoring of parent ions to product ions (ion transitions: ALA 132 m/z → 86 m/z, isotope labeled ALA 134 m/z → 88 m/z; PBG 210 m/z → 122 m/z, isotope labeled PBG 212 m/z → 124 m/z). Total integrated plasma ALA or PBG concentrations during the entire 21 hour study period were determined by AUC analysis using the trapezoid method as explained above.

Statistical Analyses

A two-way ANOVA with a Student Newman-Keuls (SNK) post-hoc test was used to identify differences in hormone concentrations or mRNA levels and times of day among subject groups. A one-way ANOVA with a SNK post-hoc test was used to evaluate differences in porphyrin concentrations among subject groups. A one-way ANOVA with a SNK post-hoc test or student's t-test was used to determine differences in total hormone concentrations in AUC analysis among subject groups. Periodograms of hormone levels or ALA/PBG concentrations plotted against time were created using SigmaPlot 12.3 (Systat Software, Inc., San Jose, CA). Circadian rhythmicity for hormone levels or ALA/PBG concentrations were evaluated using the single cosinor analysis as previously described (39, 40), using Cosinor Periodogram 2.3, a statistical software program originally developed by

R. Refinetti [Univ South Carolina, Columbia, SC (www.circadian.org/softwar.html)] (41). Cosinor analysis is a statistical method of determining a least squares line of best fit for a cosine function as described by Nelson et al (42). Descriptive variables provided by cosinor analysis include period length, rhythm-adjusted mean (MESOR), amplitude, and cosine maximum (acrophase). All data are presented as means ± SE and a value of $P<0.05$ was considered statistically significant.

RESULTS

Summary description of subjects studied

Three control subjects and six subjects with AIP completed the full blood draw schedule. Selected demographic, clinical, and laboratory are summarized in Table 1. Of note is that all AIP subjects reported having persistent sleeping problems, whereas none of the control subjects reported similar difficulties.

Time course and levels of serum cortisol

Cortisol levels were highest in the early morning period in both control and AIP subjects, with significantly more serum cortisol at 8 am than at all or almost all other time points within the respective study groups [Fig 1A]. Mean values of serum cortisol were not different between control and all AIP subjects at any time point (two-way ANOVA). However, average serum cortisol in AIP subjects with biochemical activity showed a trend for lower serum cortisol levels in the early morning period, which was significantly lower in these subjects than in controls at 5 am [Fig 1B]. Furthermore, total serum cortisol through the entire 21 hour study period, as measured by AUC analysis, was significantly lower in AIP subjects with biochemical activity (124.47±9.8 mcg/dL) than in control subjects (168.78±21.7 mcg/dL). Individual subject serum cortisol levels are shown in Supplemental Figure 1A. These results suggest that AIP subjects with biochemical activity have decreased levels of serum cortisol and blunted early morning increases in this analyte.

To determine if serum cortisol in AIP subjects demonstrates altered circadian oscillation, we performed single cosinor analysis on serum cortisol levels for all subjects, by the methods of Refinetti et al (41). While 2 of the 3 control subjects showed significant circadian oscillation for serum cortisol, only 2 of the 6 AIP subjects showed significant circadian oscillations (data not shown). We next performed single cosinor analysis on mean serum cortisol in control subjects, which showed significant circadian oscillations [Supp Table 1]. In contrast, cosinor analysis on mean levels in AIP subjects did not show significant circadian oscillations. Of importance, AIP subjects excreting normal concentrations of PBG (<4.0 μM), showed significant circadian oscillations of serum cortisol, whereas clinically active AIP subjects ([urinary PBG]>4.0 μM), did not (Supplemental Table 1). In sum, these results indicate that biochemically active AIP subjects have blunted early morning increases and impaired circadian oscillations of serum cortisol.

Time course and levels of serum melatonin

For both control and AIP subjects, serum melatonin showed a trend of peaking during the nighttime hours, with AIP subjects having significantly higher plasma melatonin at 5 am than during daytime hours [Fig 2A]. At all time points studied, save one (8 pm), the mean values of serum melatonin in AIP subjects with biochemical activity were lower than for the AIP subjects without activity. However, the differences were relatively small and were not significant at the 5% level [Fig 2B]. Total AUCs for serum melatonin over the 21 hour time period were also not different among subject groups (data not shown). Serum melatonin levels for all subjects are shown in Supplemental Figure 1B.

To determine if serum melatonin in AIP subjects demonstrates altered circadian oscillation, we performed single cosinor analysis on serum melatonin levels of individual subjects. All 3 control subjects showed serum melatonin circadian oscillations, while 5 out of 6 AIP subjects showed significant oscillations (data not shown). Circadian oscillations for average serum melatonin were also confirmed in control and AIP groups, as well as in AIP subgroups [Supp Table 1]. In sum, we did not find evidence for altered serum melatonin between control and AIP subjects, nor sub-groups of AIP subjects.

Time course and levels of plasma ALA & PBG

Subjects with biochemically active AIP showed higher levels of PBG in plasma at all time points studied [Fig 3B]. In contrast, mean serum concentrations of ALA were similar in controls and subjects with AIP, and among the latter, there were no differences between the sub-group with vs without biochemical activity [Fig 3A]. Similarly, there were no differences in AUC over the 21 h study period (data not shown).

Total serum PBG concentration over the course of the experiment, as determined by AUC analysis, was significantly higher in AIP subjects with activity (84.8 ± 15.1 μM) than in AIP subjects without activity (10.4 ± 0.8 μM) or controls (0.757 ± 0.2 μM). There was also a strong trend for total serum PBG concentration over the course of the experiment to be higher for AIP subjects than for control subjects (P=0.064). Although there was no significant circadian oscillations as determined by cosinor analysis in serum PBG concentration for any subject (data not shown), of considerable interest, the levels in subjects with active AIP showed a sine wave type of pattern with a period of ~6 h [Supp Fig 2B]. In addition, serum PBG levels in the AIP subjects without biochemical activity also showed a similar sine wave pattern, albeit with blunted amplitude [Supp Fig 2B].

Urinary and plasma porphyrin concentrations at baseline

Average urinary porphyrin concentrations in spot urine samples taken at baseline are shown in Supp Fig 3. AIP subjects showed a strong trend for an approximately 1–2 orders-of-magnitude increase in urinary uroporphyrin and 4-coproporphyrin-I and -III, although these trends were not significant, perhaps related to our small sample sizes (n=2 for each group). Plasma porphyrin concentrations were undetectable, (< 25 nM or < ~0.14 mcg/dL, the lower limit of detection for the UPLC/FLD assay used).

Expression of clock-controlled genes and selected genes of heme synthesis

We also investigated whether AIP subjects had altered expression of genes involved in circadian function by quantifying mRNA levels of selected clock-controlled genes in PBMCs collected at 11am, 5pm, 11pm, and 5am. PER2 expression was significantly increased at 5am [Fig 4A] and CRY1 expression was increased at 11pm [Fig 4B], although neither gene showed differences between control and AIP subgroups at individual time points. Expression of *NR1D1*, the gene that encodes Rev-erb alpha protein, did not change significantly over time and was not significantly different between subject groups at any time point [Fig 4C].

Because intracellular heme levels have been shown to oscillate in a circadian pattern (43), we investigated whether expression patterns of selected genes of heme synthesis showed circadian variability in control and AIP subjects. As shown in Figure 5, all three such genes studied *(ALAS1, ALAS2*, and *PBGD*) showed a strong trend for increased expression at 11pm over control in AIP subjects with biochemical activity, with two of these genes, *ALAS2* and *PBGD*, significantly increased [P < 0.05].

DISCUSSION

The major findings of this pilot study are that (1) asymptomatic AIP subjects with biochemical activity, defined as increased urinary and plasma levels of PBG, have lower levels of serum cortisol than normal controls or AIP subjects without biochemical activity, and they also exhibit significantly blunted increases in these levels in the morning [5 am, 8 am] [Fig 1B]. (2) AIP subjects with biochemical activity show no increases in serum ALA but do show clear increases and striking sine wave fluctuations in plasma PBG throughout the course of the day [Supp Fig 2]. (3) Overall, however, there is not a major abnormality in circadian oscillations of serum cortisol, melatonin, ALA, or PBG in post-menopausal women with asymptomatic AIP; and (4) there are no abnormalities or differences in circadian expression of selected clock-controlled genes [*PER2, CRY1, NR1D1*] nor of genes of heme synthesis [*ALAS1, ALAS2, PBGD*] in PBMCs of women with AIP, compared with controls.

Acute attacks of severe pain, usually abdominal, severe constipation, tachycardia, and systemic arterial hypertension are the clinical hallmarks of the acute porphyrias: AIP, hereditary coproporphyria, variegate porphyria and ALA dehydratase porphyria (1–6). AIP is the most severe form of the common heterozygous forms of acute porphyria and is most often associated with recurrent attacks, probably because the normal level of activity of hepatic PBG deaminase is the lowest among the enzymes of heme synthesis distal to ALA synthase (1, 2, 4–6). In women of child-bearing age, such attacks sometimes recur monthly especially during the luteal phases of their menstrual cycles, at which time there is a burst of progesterone, a porphyrogenic female hormone, produced by the *corpus luteum*. Although the pathogenesis of acute porphyric attacks is complex and still understood incompletely, a *sine qua non* is derepression of hepatic ALA synthase-1, leading to sometimes massive overproduction and over-excretion of ALA and PBG [more than 1 millimole/day]. That the major neuro-muscular toxin is ALA or a by-product of ALA [not PBG] is implied by the fact that similar clinical features also occur in lead poisoning and hereditary tyrosinemia type 1, both of which similarly are characterized by marked over-excretion of ALA but not of PBG (1, 2, 4–6, 11).

In addition to severe acute porphyric attacks, many subjects with acute porphyria suffer from disorders of mood and affect, difficulty sleeping, difficulty concentrating, depression and anxiety. Whether these manifestations are directly related to relative hepatic heme deficiency is currently unknown. Similarly, it is unclear whether the current treatment of choice for severe acute attacks, namely, intravenous heme administration, can improve these symptoms. (1, 2, 4–6, 44). Other medical conditions reported by the volunteer subjects (i.e., systemic arterial hypertension, high cholesterol, recurrent urinary tract infections, restless legs syndrome, mild depression) are not known to directly influence the clock, although some clinical and epidemiological studies have established that neurologically-associated disorders, such as marked depression and symptomatic restless legs syndrome, may be associated with clock dysfunction. In this study, subjects with depression that was more than mild, as assessed by the Beck depression inventory, were excluded.

Because of recent evidence linking heme to clock-controlled proteins and circadian rhythms (45), we hypothesized that subjects with biochemically active acute porphyria would prove to have abnormalities in their circadian rhythms. Although our results did not reveal major disturbances, they did show decreases in levels of total serum cortisol in AIP subjects with biochemical activity and, especially, a blunting of the normal circadian increase in these levels that normally occurs in the early morning [Fig 1]. They also showed a trend for decreased nocturnal levels of melatonin. The reasons behind these lower levels and blunted responses remain uncertain. Perhaps, such subjects have subtle defects in the hypothalamic-

pituitary-adrenal axis, as has been described in severely symptomatic, biochemically active subjects with AIP (46). Or, perhaps, these more subtle changes are due to mild relative heme deficiencies, leading to defects in clock-controlled genes and circadian rhythms (24, 43, 45, 47).

Nocturnal levels of serum melatonin have been reported to be decreased in subjects with biochemically active, symptomatic AIP (16). We did not find significantly different nocturnal levels of serum melatonin in subjects with asymptomatic AIP, some of whom showed no biochemical activity at the time of study [Fig. 2]. However, the number of subjects studied here was small, and it may be that AIP subjects with greater biochemical activity and, presumably, greater defects in regulatory heme pools, will show more striking differences from controls.

The synthesis of heme is under tight regulation, chiefly exerted at the level of ALA synthase, the first and normally rate-controlling enzyme of the pathway. Sufficiency or excess of heme leads to repression of ALAS1 mRNA levels (6, 48–50) and to increased proteolytic cleavage of mitochondrial ALAS1 (51). Oscillations in hepatic heme synthesis and in ALAS1 activity and ALA and PBG synthesis have long been known in rodent models of AIP (52–54). The striking oscillations that we observed in plasma levels of PBG [Supp Fig 2B] suggest similar oscillations in humans with AIP.

Strengths of this study include the careful and thorough assessment of the subjects studied and the detailed characterization performed, including serum cortisol and melatonin and plasma ALA and PBG, and mRNA levels in PBMCs. Methods for analyses are state-of the-art.

Limitations of this study are that, due to limited funding, we were able to enroll only 10 subjects, and, unfortunately, due to very difficult peripheral venous access, among these, one control subject did not provide blood samples at most of the time points. Then, too, perhaps, it would have been preferred if subjects, prior to the day of study, had been able to spend several days in the sleep center, becoming fully acclimated to it and more comfortable with the staff and surroundings. However, the costs of such a study design would have been prohibitively high, and the willingness of subjects to participate would have been much lower. Another limitation is that we were able to enroll only four subjects with biochemically active AIP, in whom one would expect more striking defects in regulatory heme pools. Three of these four had only slight increases in urinary PBG levels. It remains unknown whether AIP subjects with more marked biochemical activity will show greater abnormalities in serum cortisol or melatonin or plasma PBG levels than were observed here. Also still unknown, but of obvious interest, is whether intravenous heme, the specific treatment of choice for full-blown attacks of acute porphyria (1–7), may also normalize serum cortisol or melatonin and/or ameliorate the difficulties in sleeping [Table 1] or myriad other ‘minor’ symptoms of which AIP subjects often complain.

In summary, in this pilot study, post-menopausal women with asymptomatic AIP with biochemical activity had lower levels of cortisol in the serum, a blunted early morning rise in serum cortisol, and absence of normal circadian oscillations in these levels. In contrast, we did not find evidence of an abnormality of levels of serum melatonin or circadian oscillations in subjects with AIP not in crisis. No abnormalities in circadian rhythms or levels of clock-controlled genes or selected genes of heme synthesis were found in PBMCs, comparing AIP to control subjects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

A	subject with AIP
AIP	acute intermittent porphyria
ALA	5-aminolevulinic acid
ALAS1	5-aminolevulinic acid synthase 1
ALAS2	5-aminolevulinic acid synthase 2
ANOVA	analysis-of-variance
AUC	area under the curve
BDI-II	Beck Depression Inventory II
BMAL1	brain and muscle ARNT-like 1
C	control subject
Cry1	cryptochrome 1
EDTA	ethylene diamine tetra acetic acid
FA	formic acid
HMB	hydroxymethylbilane
HMBS	hydroxymethylbilane synthase
HMOX1	heme oxygenase 1
HPLC	high performance liquid chromatography
IV	intravenous
LC-MS	liquid chromatography-mass spectroscopy
MESOR	midline estimating statistic of rhythm
NCoR-HDAC3	nuclear receptor co-repressor-histone deacetylase 3
NPAS2	neuronal PAS domain-containing protein 2
NR1D1	nuclear receptor subfamily 1, group D, member 1
NSAID	non-steroidal anti-inflammatory drugs
PAS	Per-ARNT-Sim
PBGD	porphobilinogen deaminase
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
Per2	period 2
PBG	porphobilinogen
R	coefficient of determination

SAD	seasonal affective disorder[s]
SCN	suprachiasmatic nucleus
SPE	solid phase extraction
TBP	TATA-box binding protein
UPLC/FLD	ultra-high performance liquid chromatography with fluorescence detection

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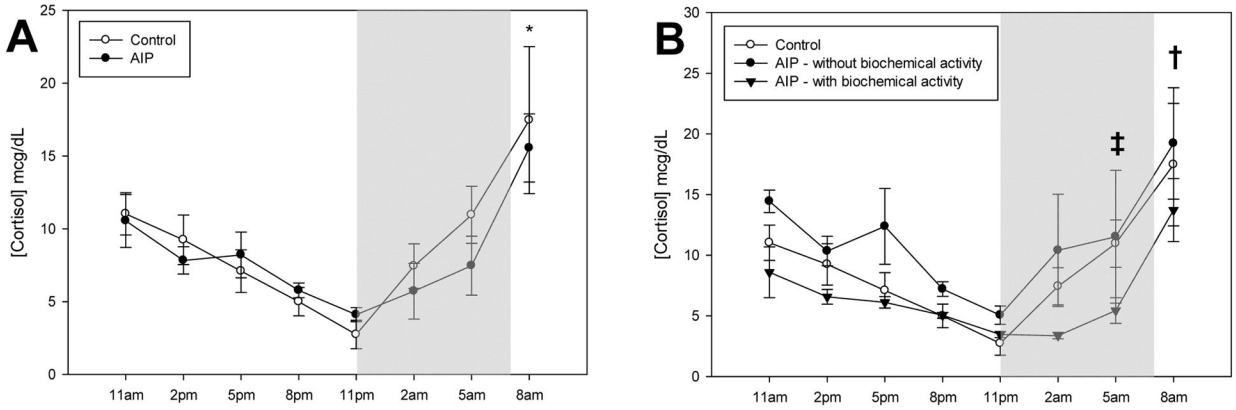

Figure 1. Time course of serum cortisol concentrations in subjects studied

Subjects had blood samples drawn and processed as described in Methods. (A) Concentrations of serum cortisol in control (n=3) and all AIP subjects (n=6). *= Control group significantly different from all other time points within control except for 5am, and AIP group significantly different from all other time points within AIP. (B) Concentrations of serum cortisol in control (n=3) and AIP subjects with (n=4) and without (n=2) biochemical activity for time course studied. †= AIP subjects with biochemical activity significantly different from other time points within group, AIP subjects without biochemical activity significantly different from 8pm and 11pm time points within group, and control group significantly different from all other time points within control except 5am. ‡= AIP subjects with biochemical activity significantly different from control at same time point. Shaded boxes represent times of usual sleep. Data are means ± SE.

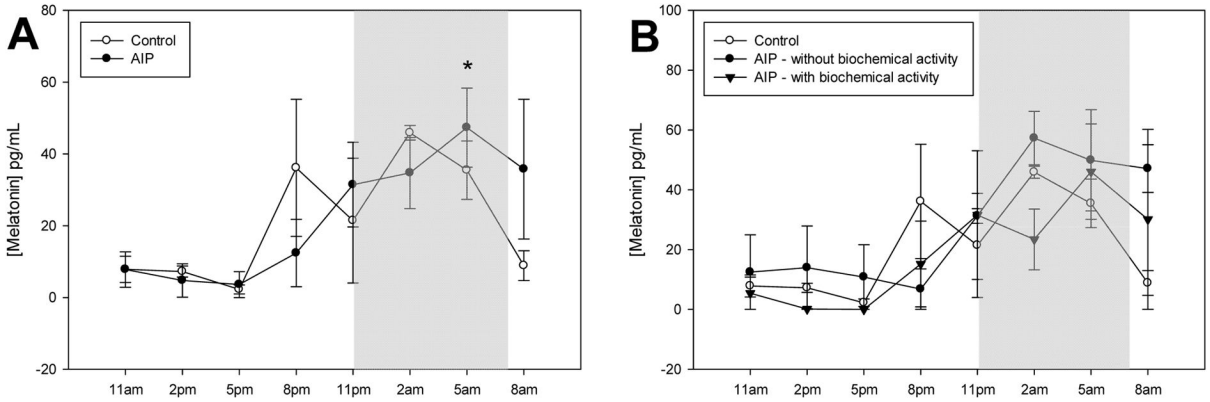

Figure 2. Time course of serum melatonin concentrations in subjects studied

Subjects had blood samples drawn and processed as described in Methods. (A) Concentrations of serum melatonin in control (n=3) and all AIP subjects (n=6). *= mean value for AIP group at 5 am significantly greater than mean values for this at 11am, 2pm, and 5pm time points. (B) Concentrations of serum melatonin in control (n=3) and AIP subjects with (n=4) and without (n=2) biochemical activity. Shaded boxes represent times of usual sleep. Data are means ± SE.

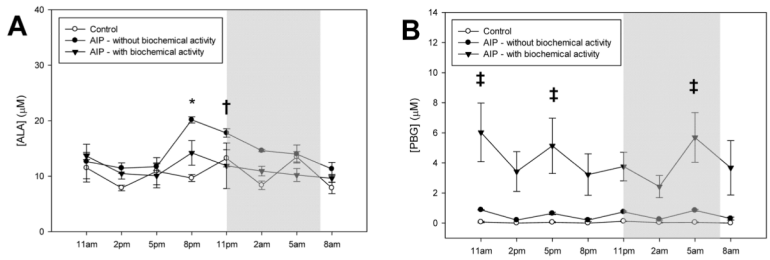

Figure 3. Time course of plasma ALA and PBG concentrations in subjects studied

Subjects had blood samples drawn and processed as described in Methods. Mean concentrations of plasma (A) ALA and (B) PBG in control (n=3) and AIP subjects with (n=4) and without (n=2) biochemical activity. *= All groups significantly different from each other at 8pm time point. †= AIP subjects without biochemical activity significantly different from other subject groups within time point. ‡= AIP subjects with biochemical activity significantly different from other subject groups within time point. Shaded boxes represent times of usual sleep. Data are means ± SE.

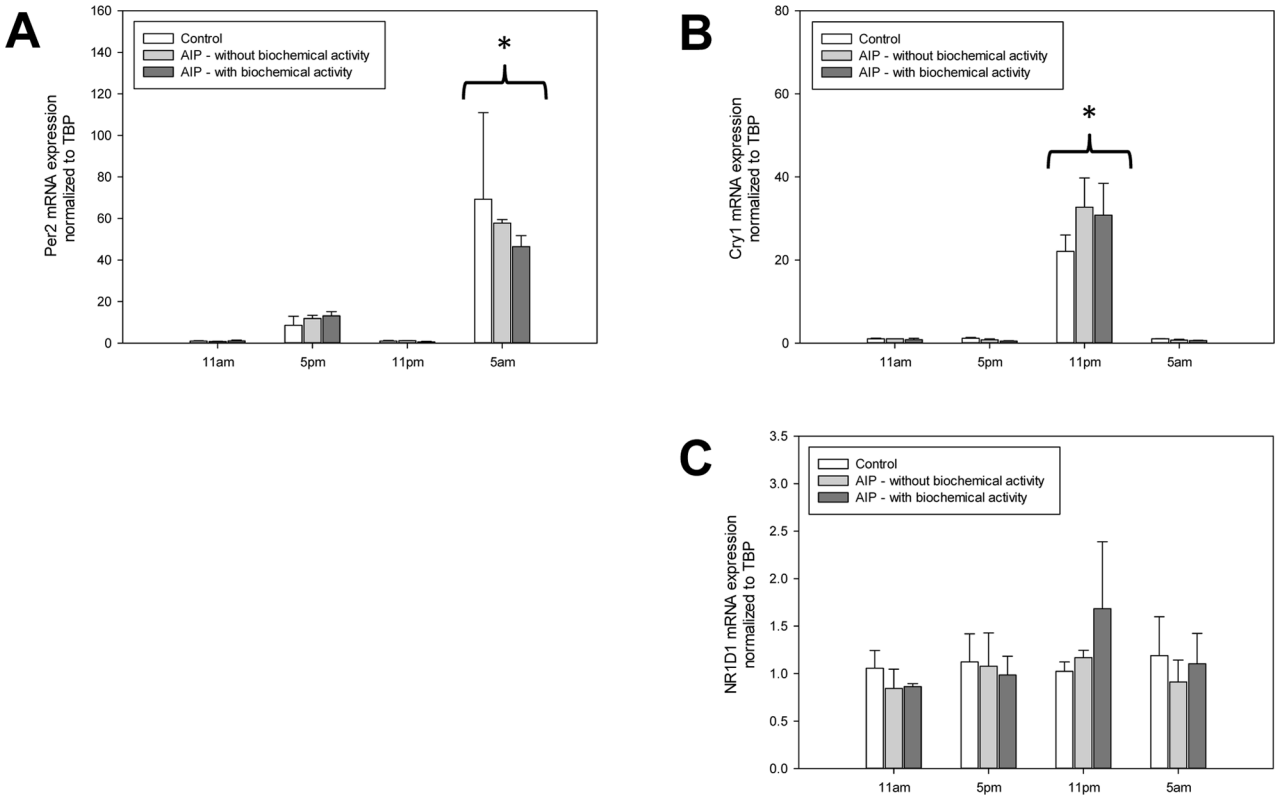

Figure 4. Selected clock-controlled gene mRNA expression in PBMCs in subjects studied

Subjects had blood samples drawn and processed as described in Methods. mRNA expression for (A) PER2, (B) CRY1 and (C) NR1D1 using $\Delta\Delta C_t$ method in PBMCs for control (n=3) and AIP subjects with (n=4) and without (n=2) biochemical activity. *= Significantly different from other time points within respective subject group. Data are means ± SE.

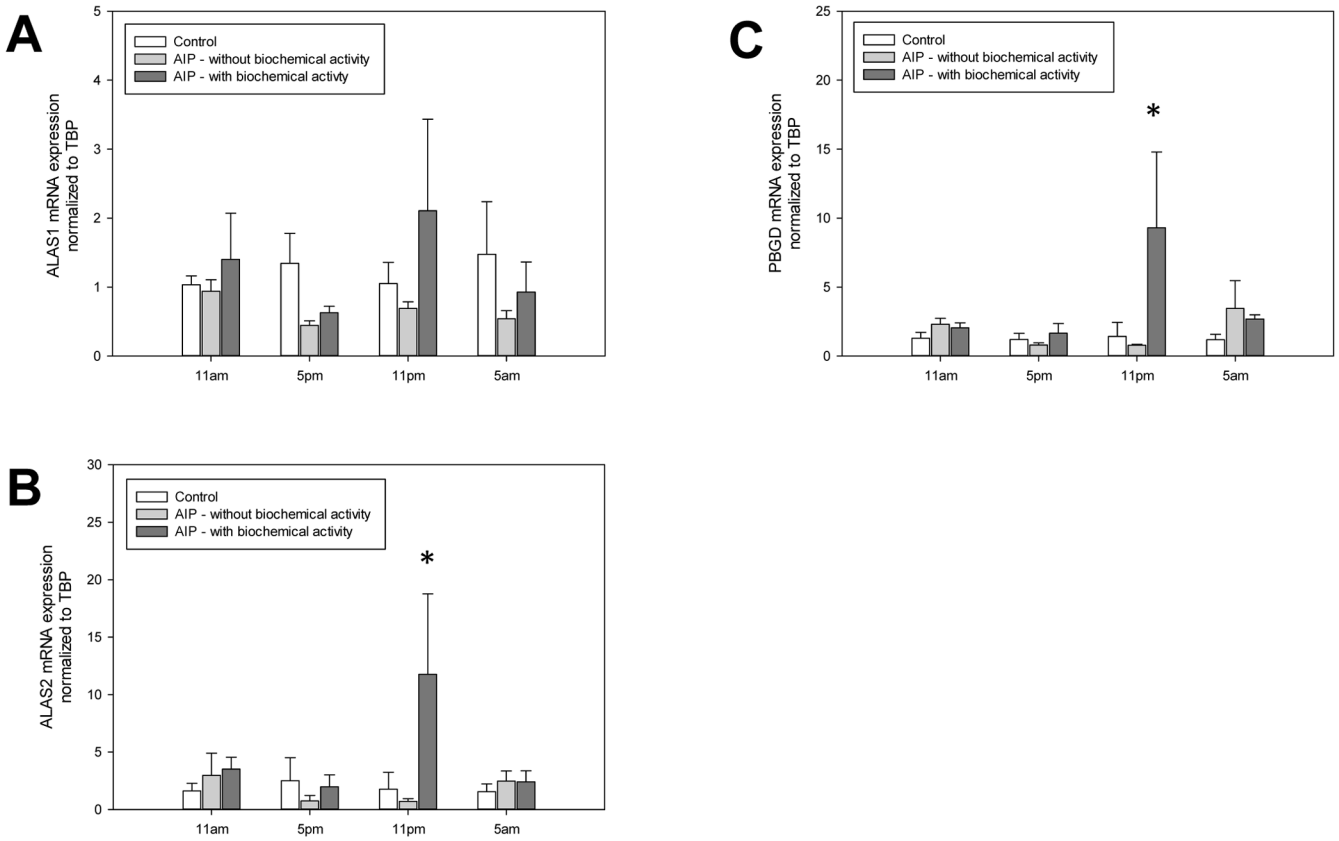

Figure 5. mRNA expression of selected genes involved in heme synthesis in PBMCs in subjects studied

Subjects had blood samples drawn and processed as described in Methods. mRNA expression for (A) ALAS1, (B) ALAS2 and (C) PBGD using $\Delta\Delta C_t$ method in PBMCs for control (n=3) and AIP subjects with (n=4) and without (n=2) biochemical activity. *= AIP subjects with biochemical activity significantly different from other time points within group, and also different from other subject groups within same time point. Data are means ± SE.

Table 1

Selected demographic, clinical, and laboratory features of subjects studied

	Subject	Age (y)	BMI (kg/m^2)	History of Acute Attacks	Base ALA (μM/mM-creatinine)	Line PBG (μM/mM-creatinine)	Urine Total Porphyrins (nM/mM-creatinine)	Beck Depression Score	PBGD Activity in RBCs (nmol porphyrin/L/sec)	Mutational Analysis of PBGD gene	Other Medical Conditions	Sleeping Problems	Avg. h of Sleep/night	Coffee Intake [cups/d]	Cigarette Smoking [ppd]	Alcohol Use
Control Subjects	C1	33	29.2	No	3.2	1.5	107	1	22	Control	None	No	10.5	0	1	None
	C2	45	25.6	No	2.6	1.6	144	1	21	Control	None	No	5	2.5	1/2	None
	C3	60	26.5	No	1.8	1.0	69	0	22	Control	None	No	7	1	None	None
	C4	48	24.1	No	3.0	1.2	91	0	19	Control	None	No	7	0	None	None
AIP Subjects	A1*	68	24.2	No	1.8	1.2	226	5	18	R173W	HTN, High cholesterol, Recurrent UTI, Restless Legs Syndrome	Yes	4.5	3	1	None
	A2*	51	22.8	Yes, 8 y ago	3.9	3.4	175	14	17	Splice-site mutation, VS71g>a in HMBS allele	No	Yes	6	0	None	None
	A3	47	28.3	No	4.2	5.7	331	2	20	R173W	No	Yes	6	2	1	None
	A4	48	37.6	Yes, 1 y ago	5.0	5.2	417	13	16	R173W	Depression, Chronic Pain, GERD	Yes	10	2	1	None
	A5	49	33.7	Yes, 1 y ago	4.6	5.1	279	18	19	Deletion in HMBS allele 730–731delct	Bipolar Disorder Type II	Yes	5	0	None	None
	A6	78	25.4	Yes, 1 y ago	18	24.3	870	10	10	R167Q	No	Yes	4	2	None	None

*Denotes AIP subjects without evidence of biochemical activity [normal urinary excretions of ALA, PBG, porphyrins and normal levels of ALA and PBG in plasma]. Abbreviations: a, adenine; A, subject with AIP; AIP, acute intermittent porphyria; ALA, 5-aminolevulinic acid; Avg, average; BMI, body mass index; C, control subject; ct, cytidine-thymidine; d, day; g, guanosine; GERD, gastro-esophageal reflux disease; HTN, systemic arterial hypertension; IVS, intervening sequence (intron); h, hours; PBG, porphobilinogen; PBGD, PBG deaminase [also known as hydroxymethylbilane synthase (HMBS)]; ppd, pack per day; Q, glutamine; R, arginine; RBCs, red blood cells; UTI, urinary tract infection; W, tryptophan.