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Oscillation of Progesterone Receptor Membrane Component 1 during the human menstrual cycle

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SUMMARY

Progesterone receptor membrane component 1 (*PGRMC1*) is a gene located on the q-arm of the X-chromosome. Its product is a versatile protein that is believed to have several different functions depending on its location in the cell. One important function is to bind the hormone progesterone, which has a main role in the regulation of the menstrual cycle. Mansouri and colleagues demonstrated downregulation of *PGRMC1* in two patients with a balanced X;autosomal translocation and premature ovarian failure (POF). This finding led them to identify a missense mutation in the same gene in an additional patient with POF. This disorder affects approximately 1% of women and is characterized by loss of normal ovarian function before the age of 40. These findings make *PGRMC1* a good disease-causing candidate in POF.

The aim of this study was to investigate the expression patterns of *PGRMC1* (both regarding transcription and translation) during the human menstrual cycle and to compare these normal expression levels to levels found in POF patients and in one nursing woman. In addition, I aimed to analyze whether the level of cytochrome P450 (CYP) followed the protein oscillation curve, since it has been found previously that PGRMC1 is a regulator for some of these enzymes.

RNA and protein were extracted from white blood cell samples. The protein levels were analyzed by Western blotting, while the RNA samples were reverse transcribed before their levels were analyzed by quantitative real-time PCR (Q-PCR). I observed a clear oscillation of PGRMC1 protein during the menstrual cycle. In contrast, RNA only seemed to vary a little during the cycle. I found a statistically significant downregulation of the PGRMC1 protein and a tendency of lowered RNA levels in the patients compared to the healthy women. Additionally, the nursing woman showed a similar expression level of protein as the healthy women, but a lowered level of RNA expression. No CYPs could be detected, so no conclusions could be made concerning those. These results support previous findings and strengthen the hypothesis that PGRMC1 is involved in the mechanism underlying POF.

ABBREVATIONS

BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetraacetic Acid
EtBr	Ethidium Bromide
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-Releasing Hormone
HCG	Human Chorionic Gonadotropin
LH	Luteinizing hormone
MES	2-(*N*-morpholino)ethanesulfonic acid
MIM	Mendelian Inheritance in Man
PBS	Phosphate Buffered Saline
PGRMC1	Progesterone Receptor Membrane Component 1
POF	Premature Ovarian Failure
PVDF	Polyvinylidene Fluoride
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
stdev	Standard deviation
TBE	Tris/Borate/EDTA
Q-PCR	Quantitative Real-Time Polymerase Chain Reaction

INTRODUCTION

Chromosomal rearrangements like translocations might be associated with specific diseases and disease-causing genes may be identified by molecular characterisation of the rearrangement (Mansouri, 2006). Mansouri and colleagues investigated a mother and her daughter both of whom had an X;autosomal translocation [t(X;11)(q24;q13)] and were diagnosed with the disease premature ovarian failure (POF) (Mansouri, 2006; Mansouri, M., Schuster, J., Badhai, J., Stattin, E.-L., Lösel, R., Wehling, M., Carlsson, B., Hovatta, O., Karlström, P., Golovleva, I., Toniolo, D., Bione, S., Peluso, J. and Dahl, N., unpublished 2008). Some of the genes next to the breakpoint regions in chromosomes in these women showed altered expression levels. One was the *progesterone receptor membrane component 1* (*PGRMC1*) gene, located about 170 kb from the breakpoint on the X-chromosome, which was significantly down regulated (Mansouri, 2006; Mansouri et al., unpublished 2008). Additionally, a third female with POF was found to carry a missense mutation in *PGRMC1* (Mansouri et al., unpublished 2008). These findings point to a role of *PGRMC1* in the cause of POF.

The menstrual cycle

In most mammals the female has a periodic pattern of ovulation that makes her ovulate only at one specific time during the year, called "heat"(Gilbert, 2006). Women have a variant of this periodic ovulation, with a cyclic ovulation pattern on average once every 29.5 days, but no yearly "heat" (Gilbert, 2006). This cycle is called the menstrual cycle and its regulation is complex and managed by several different hormones, mainly the gonadotropin-releasing hormone (GnRH), the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) and the sex hormones estrogen and progesterone (Gilbert, 2006; Mansouri, 2006; Mader, 2007; Campbell et al., 2000; Campbell et al., 2008; Schoenwolf et al., 2008). These hormones are synthesized in the hypothalamus (GnRH), the pituitary gland (FSH and LH) and the ovaries (estrogen and progesterone) and their expression levels vary throughout the menstrual cycle (figure 1) (Gilbert, 2006; Mansouri, 2006; Mader, 2007; Campbell et al., 2000; Campbell et al., 2008; Schoenwolf et al., 2008). Properties of these hormones are summarized in table 1.

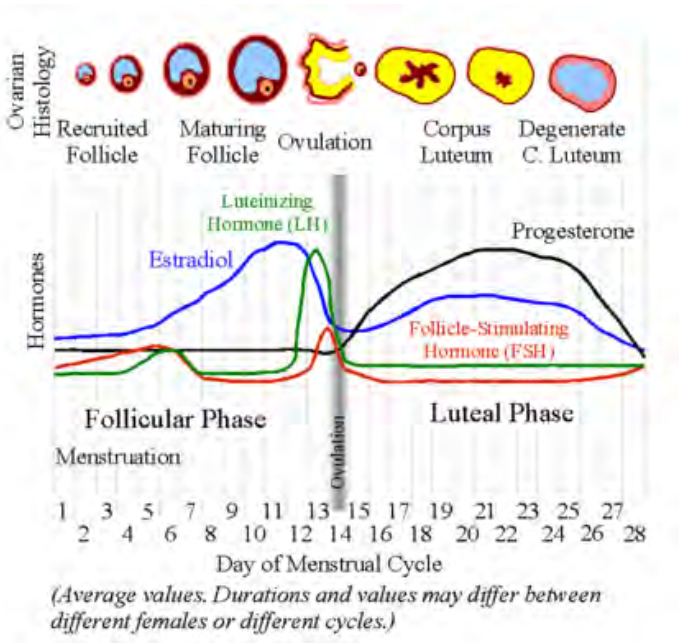

Figure 1. A schematic overview of the events during the menstrual cycle. At the bottom of the figure, the days of the menstrual cycle are given. Above these, the corresponding phases and the occurrence of menstruation (day 1-5) and ovulation (day 14) are indicated. In the middle of the figure the varying hormone levels during the cycle are stated, in correspondence to the proper phase and days. At the top of the figure, the development of the follicle, the ovulation and the degeneration of the corpus luteum that occurs in the ovaries are seen in relation to the hormonal levels and corresponding to the days of the menstrual cycle. (The figure is modified from Wikipedia (http://en.wikipedia.org/wiki/Menstrual_cycle) 2008-05-21)

Table 1. Summary of menstrual cycle regulating hormones.

Hormone	Regulated by	Secreted by	Roles
GnRH	estrogen and progesterone	Hypothalamus	Stimulates secretion of FSH and LH
FSH	GnRH	Pituitary gland	Stimulates secretion of estrogen from growing ovarian follicle
LH	GnRH	Pituitary gland	Stimulates ovulation Stimulates secretion of progesterone from developing corpus luteum
Estrogen	FSH	Ovaries	Stops secretion of FSH
Progesterone	LH	Ovaries	Stops secretion of LH

The menstrual cycle is divided into two distinct phases; the follicular phase (beginning at menstruation and ending at ovulation) and the luteal phase (figure 1). In real life the length of the menstrual cycle varies considerably between individual women (from 20 up to 40 days) and also the lengths of the phases differ (Campbell et al., 2008), but when the

cycle is discussed, usually a standardized 28-day cycle in which the phases have specific lengths is assumed (figure 1).

The menstrual cycle begins when the endothelial tissue from the uterus, called the endometrium, is detached and shedded together with about 35 mL of blood and the unfertilized egg, which occurs about once a month (Campbell et al., 2000; Campbell et al., 2008; Gilbert, 2006; Schoenwolf et al., 2008). This menstruation period covers on average day 1-5 of the cycle (Campbell et al., 2000; Mader, 2007; Schoenwolf et al., 2008). At this stage, the level of progesterone and estrogen is low (Mader, 2007), which function as a signal to the hypothalamus. The hypothalamus responds by releasing GnRH, which stimulates the release of the gonadotropins FSH and LH, which in turn stimulates the ovaries to secrete estrogen (Campbell et al., 2000; Campbell et al., 2008; Gilbert, 2006; Mader, 2007; Schoenwolf et al., 2008). This first half of the cycle is called the follicular phase given that the FSH promotes the development of a follicle in the ovary, and it is this follicle that releases estrogen (Campbell et al., 2000; Campbell et al., 2008; Mader, 2007). In the beginning the follicle is small and secretes small amounts of estrogen, but as it grows the release of estrogen increases (Campbell et al., 2000). At the end of this stage (about day 14 of a standardized cycle) ovulation is initiated, additionally stimulated by the gonadotropins, mainly LH (Gilbert, 2006; Mader, 2007; Mansouri, 2006). The rise of estrogen level is a feedback signal to the anterior pituitary to stop secretion of FSH, thus ending the follicular phase after ovulation (Campbell et al., 2008; Mader, 2007).

The second half of the cycle is called the luteal phase because LH promotes the transformation of the follicle into the corpus luteum, which secretes progesterone (Campbell et al., 2000; Campbell et al., 2008; Mader, 2007; Schoenwolf et al., 2008). The rise of progesterone level (just like the rise of estrogen level earlier) works as a feedback signal to the anterior pituitary to stop secretion of LH. Subsequently the corpus luteum degenerates and stops producing progesterone (Campbell et al., 2000; Campbell et al., 2008; Mader, 2007). At the end of the luteal phase the levels of progesterone and estrogen drop again, resulting in a new menstruation and the start of a new cycle (Campbell et al., 2000; Mader, 2007; Schoenwolf et al., 2008).

If the egg released at ovulation becomes fertilized, the result will be a zygote that develops into an embryo and is implanted into the endometrium (uterine lining) (Campbell et al., 2000; Campbell et al., 2008). The woman has become pregnant. The developing embryo starts producing the hormone HCG (human chorionic gonadotropin). HCG acts in the same way as LH and thereby maintains the corpus luteum (Campbell et al., 2000). The corpus luteum continues producing progesterone and estrogen, which in turn preserves the endometrium and thus prevents menstruation from taking place (Campbell et al., 2000).

Premature ovarian failure (POF)

Premature ovarian failure (POF) (MIM 311360)[1] is a complex and diverse disease (Goswami and Conway, 2007; Kang et al., 2008; Massin et al., 2008). It is defined as menopause (or at least abnormal function of the ovaries) before the age of 40 (Mansouri, 2006; Goswami and Conway, 2007; Nippita and Baber, 2007; Sinha and Kuruba, 2007) but can appear as early as in the teens. Normal menopause usually occurs in women between 45 and 50 years of age (Mader, 2007) as the ovaries start loosing their responsiveness to FSH and LH (Campbell et al., 2008). This results in a decline in estrogen production by the ovaries, which in turn causes ovulation and also menstruation to cease (Campbell et al., 2008).

POF affects 0.9-1.2% of the female population, depending on ethnicity. Among Caucasian women the prevalence is about 1% (Nippita and Baber, 2007). It results from premature loss of ovarian function and thus loss of menstrual cycle, which in turn results in low estrogen and high gonadotropin (FSH and LH) levels (Mansouri, 2006; Sinha and Kuruba, 2007; Nippita and Baber, 2007; van der Stege et al., 2008; Goswami and Conway, 2007). One of the symptoms that these women have to deal with is infertility. Nevertheless, in up to 10% of cases women with POF have succeeded in becoming pregnant (Goswami and Conway, 2007; Nippita and Baber, 2007; Sinha and Kuruba, 2007). Other symptoms of this disease are mostly due to the lack of estrogen and are thus similar to those associated with menopause, e.g. hot flashes and mood swings (Sinha and Kuruba, 2007). However, in POF patients these symptoms are often more severe than usual. Therefore, additional health implications due to abnormal hormone levels have to be considered. For instance, osteoporosis (low bone density) and heart disease may be an effect of lowered estrogen levels during a long period of time (Nippita and Baber, 2007; Sinha and Kuruba, 2007). The diagnosis is made upon measurements of FSH levels, which usually are elevated, together with varying symptoms like irregular or absent menstruation (Goswami and Conway, 2007; Mansouri, 2006; Nippita and Baber, 2007; Sinha and Kuruba, 2007).

It has been postulated that sporadic cases of POF can have a lot of different causes, and the disorder has been associated with both autoimmune and metabolic diseases. In some cases the disease is inherited (Goswami and Conway, 2007; Mansouri, 2006; Mansouri et al., unpublished 2008; Nippita and Baber, 2007; Sinha and Kuruba, 2007). It is believed that familial POF can be caused by several different genetic components (Kang et al., 2008; Nippita and Baber, 2007). Several genes have been associated with the disease, especially genes that are involved in ovarian development and function (Massin et al., 2008; Nippita and Baber, 2007). Additionally, it has been shown that X;autosome translocations often lead to POF supporting the theory of genetic cause, but no causative gene has been identified (Goswami and Conway, 2007; Mansouri, 2006; Mansouri et al., unpublished 2008; Nippita and Baber, 2007; Sinha and Kuruba, 2007). Until today, no curative treatment is known. Instead, hormonal replacement therapy is used to treat the

[1] Mendelian inheritance in man (MIM) is a database that catalogues all known diseases with a genetic component. The online version is called online mendelian inheritance in man (OMIM). In the database, every disease and gene is assigned a six digit number of which the first number classifies the method of inheritance.

symptoms of hormonal deficiency (Goswami and Conway, 2007; Nippita and Baber, 2007; Sinha and Kuruba, 2007).

Progesterone receptor membrane component 1 (PGRMC1)

Progesterone receptor membrane component 1 (PGRMC1) is a membrane component of the progesterone receptor. It is a rather small (~22 kD) single transmembrane protein that binds progesterone (Losel et al., 2007) and thereby mediates progesterone's anti-apoptotic action (Cahill, 2007; Losel et al., 2007; Mansouri et al., unpublished 2008; Peluso et al., 2006, 2008a, 2008b). Previous studies suggest that the progesterone binding site is located in an area composed of the transmembrane domain and part of the C-terminus (Peluso et al., 2008b).

Since it has been found that PGRMC1 has an important function in supporting the viability of ovarian granulosa cells (Cahill, 2007; Losel et al., 2007) it is not far fetched to consider that changes of *PGRMC1* gene expression could have an impact on ovarian function. The *PGRMC1* gene is located on the X-chromosome (locus Xq22-q24) and it is expressed in various cell types (Losel et al., 2007). The regulation of PGRMC1 is poorly understood, even though it is believed that progesterone might be one of the regulating factors (Cahill, 2007; Losel et al., 2007). PGRMC1 is mainly localized in the intracellular membrane of the cells (Cahill, 2007; Losel et al., 2007) but has been found at other locations as well. In addition, PGRMC1 acts as a positive regulator for a number of cytochrome P450 (CYP) enzymes. The involvement of different CYPs in these various hormonal pathways might in turn have a regulating effect on the ovaries and the menstrual cycle.

Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) is a method measuring transcript levels using complementary DNA (cDNA). Specific primers for the genes of interest are used and the product of the amplified cDNA is measured at the end of each PCR cycle (instead of at the end of the whole procedure) by use of fluorophore incorporation, giving a real-time measurement. The relative gene expression can then be calculated in comparison to internal reference genes, most commonly the housekeeping genes *β-actin* and *GAPDH*.

Aim

The purpose of this study was to investigate the oscillation of PGRMC1 levels, both transcription of RNA and translation of protein, during the normal human female menstrual cycle by analysis of a healthy control population. The aim was also to analyze the expression levels of PGRMC1 (both RNA and protein) in POF-patients and compare them to the oscillation curves. Furthermore, this study aimed to investigate whether the expression of CYP-enzymes followed the oscillation of PGRMC1.

The questions asked were:

1. Is there oscillation in RNA or protein? Do the levels of PGRMC1 vary in a defined manner during the menstrual cycle?
2. Can I see from PGRMC1 levels whether it is regulated by specific hormones? Can I determine the function or regulative effect of PGRMC1?
3. Are there low levels of PGRMC1 in POF patients? Is PGRMC1 involved in the disease mechanism?

RESULTS

I extracted RNA and proteins from white blood cells from 13 women with POF, 10 healthy control women and one nursing woman. All samples from the healthy controls were assigned phase in a standardized 28-day menstrual cycle, based on measurements of hormonal levels in their blood.

RNA analysis

Analysis of RNA expression from the *PGRMC1* gene during the standardized menstrual cycle showed a tendency of variation (figure 2). The lowest level of gene expression occurred during the preovulation phase (day 13-14). After that the level of RNA increased until the late follicular phase (day 8-12), followed by a drop in RNA level (figure 2).

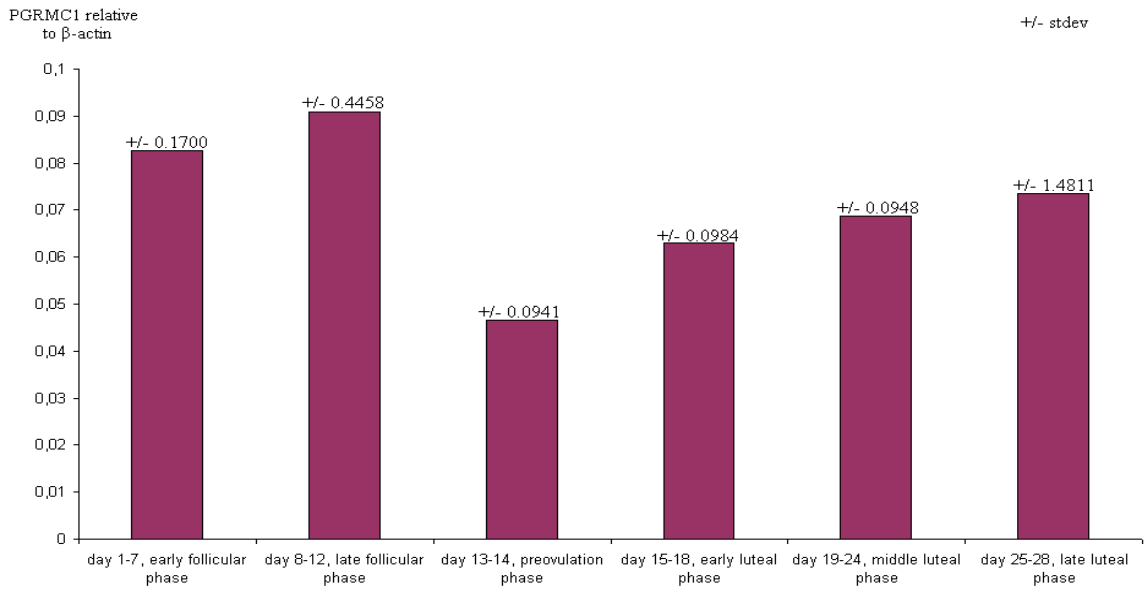

Figure 2. Oscillation curve of progesterone receptor membrane component 1 RNA during a standardized 28-day menstrual cycle. PGRMC1 RNA levels were measured by Q-PCR analysis and normalized to β-actin levels in a set of healthy women during the different phases of a standardized menstrual cycle. The x-axis show the different phases of the cycle and the y-axis show the relative mean (ratio) of PGRMC1 compared to β-actin.

There were some variations in RNA levels between individuals among the patients (figure 3). The levels in the patients appeared to be downregulated compared to the levels in the controls, but due to the high standard deviations this could not be statistically confirmed (Appendix table A5).The RNA level in the nursing woman (AK) appeared to be in the same range as in the patients (figure 3).

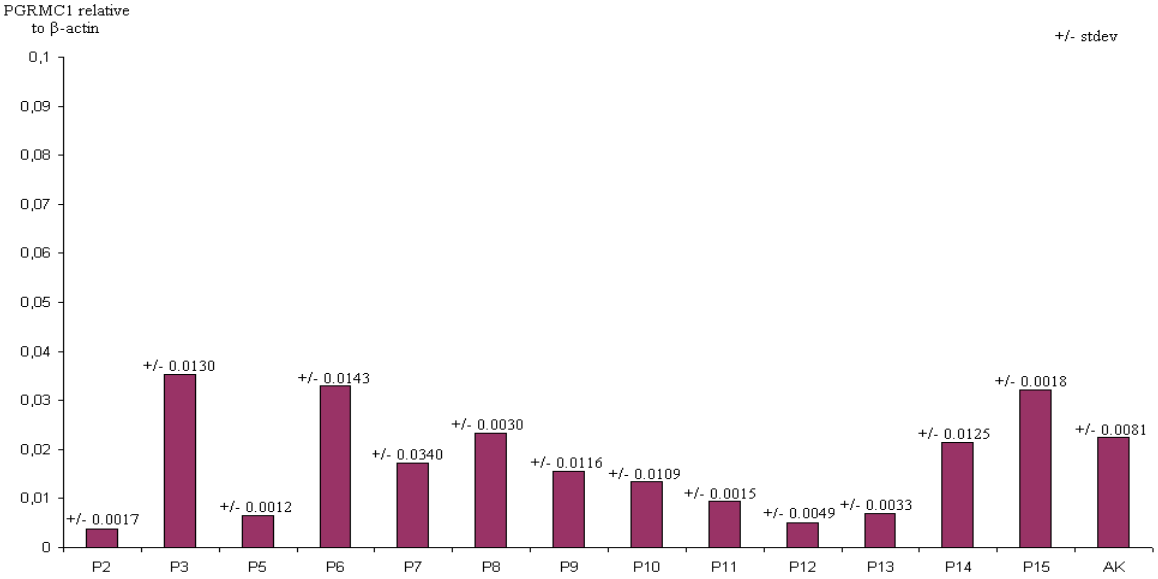

Figure 3. Progesterone receptor membrane component 1 RNA levels in premature ovarian failure patients and one nursing woman. PGRMC1 RNA levels were measured by Q-PCR analysis and normalized to β-actin levels in 13 POF-patients and one nursing woman. The x-axis shows the different individuals (P=patient and AK=nursing woman). The y-axis states the expression level of PGRMC1 relative to β-actin.

Protein analysis

Protein levels were determined with antibodies specific for PGRMC1, two different CYPs (7B and 21A) and the reference β-actin. Unfortunately, I was not able to detect either of the two CYPs in any of my western blots. This indicated that the CYP-enzymes are either not expressed or expressed at levels below the sensitivity of the method in the cell type analyzed.

The PGRMC1 protein expression levels showed a clear pattern of oscillation during the menstrual cycle (figure 4). The highest levels of PGRMC1 proteins were found at the late follicular phase while the lowest levels appeared in the early luteal phase and the late luteal phase. The observed levels of PGRMC1 protein were significantly different ($p = 0.0251$) between the late follicular phase (day 8-12) and the late luteal phase (day 25-28) (Appendix table A4).

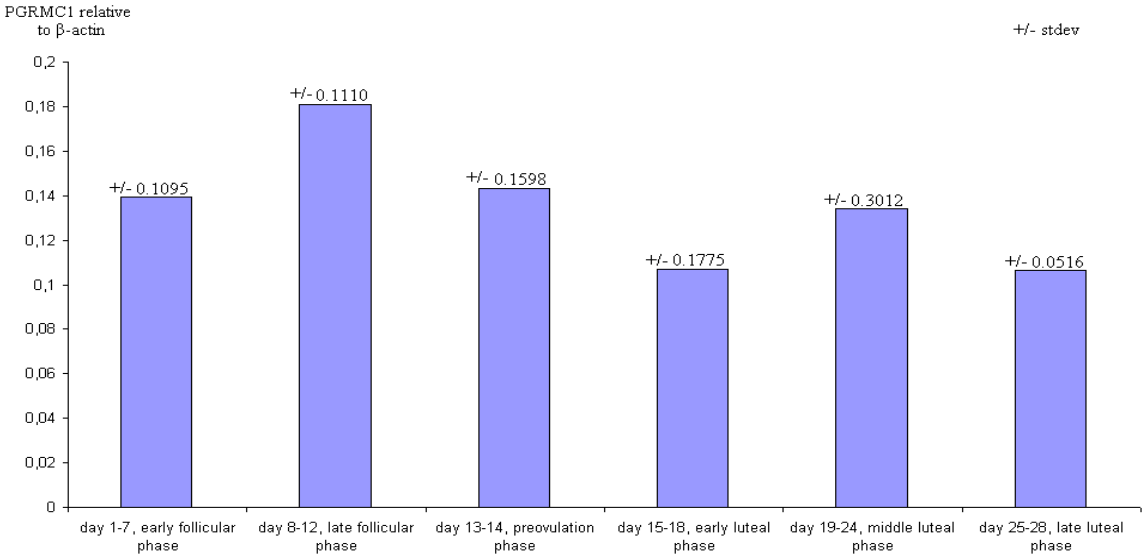

Figure 4. Oscillation curve of progesterone receptor membrane component 1 protein during a standardized 28-day menstrual cycle. PGRMC1 protein levels were measured by western blot and normalized to the internal reference β-actin in a set of healthy women during different time points in a standardized menstrual cycle. The x-axis show the different phases of the cycle and the y-axis show the relative mean (ratio) of PGRMC1 compared to β-actin.

Protein levels in the patients varied somewhat (figure 5). In four cases (P2, P3, P5 and P10) no PGRMC1 protein could be detected at all. However, in the nursing woman (AK) an elevated protein level was observed in comparison to the levels in the patients. Interestingly, the protein level in the nursing woman was similar to that seen in the healthy control women.

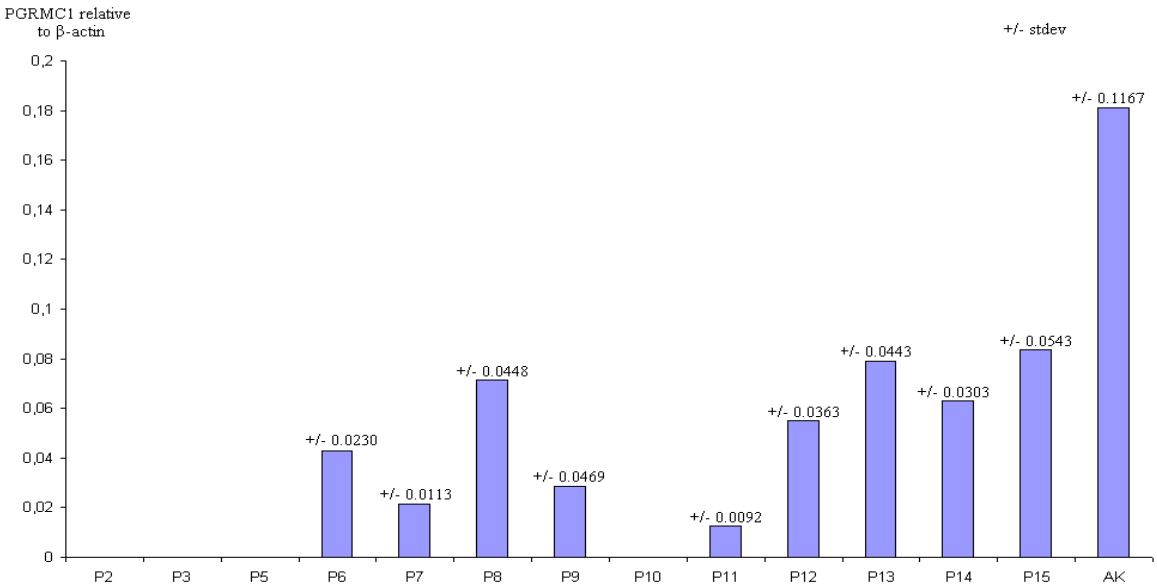

Figure 5. Progesterone receptor membrane component 1 protein levels in premature ovarian failure patients and one nursing woman. PGRMC1 protein levels were measured by western blot and normalized to β-actin levels in a set of POF patients and one nursing woman. The x-axis shows the different individuals (P=patient and AK=nursing woman). The y-axis indicates PGRMC1 protein levels relative to β-actin.

Statistics

Data obtained from patient samples were compared to data from the healthy women using the two-tailed t-test with two-sample equal variance. A p-value of <0.05 was considered significant.

The analysis of RNA levels showed no significant difference between any of the cycle phases (Appendix table A4). On the other hand, I observed significant difference between the late follicular phase (day 8-12) and the late luteal phase (day 25-28) when investigating the PGRMC1 protein levels: p=0.0251.

I found strong significance in difference between patients and healthy women in tests regarding the protein expression – the significance was even stronger when I excluded the nursing woman (table 2). Regarding the RNA, there were no significant difference between patients and healthy women, independent of the nursing woman's presence in the data set (table 2).

Table 2. Statistical data analysis.

Analysis	Data set 1	Number of observations in data set 1	Data set 2	Number of observations in data set 2	Protein p-value	RNA p-value
1	All healthy women	76	All patients + AK	14	0.0048	0.3169
2	All healthy women	76	All patients	13	0.0033	0.3336

Results of four different t-tests, two with reference to the proteins and two regarding the RNA. T-tests were performed comparing a data set pooling all the healthy women (n= 76) with a data set of the patient samples with or without the nursing woman (AK) (14 and 13 observations, respectively). Significant p-values are indicated in red.

There were significant differences between healthy women and patients in each separate phase during the menstrual cycle (both with and without the nursing woman) in all t-tests as well, when comparing protein levels (Appendix table A5). The RNA level comparisons between phases and patients showed varying results. However, most tests (4 out of 6) indicated a significant difference between the data set of healthy women and the ones with POF (with or without the nursing woman) (Appendix table A5). In all protein cases the p-value was reduced when the nursing woman was excluded from the patients, but the opposite was the case in all RNA comparisons, where the p-value was increased (Appendix table A5). I also tested for differences according to age of the POF-patients and according to their FSH-values, but none of these tests showed any significance. (Appendix table A3 and A5).

Comparisons

To get a comprehensive view over the different oscillation curves (and perhaps see an association between hormonal oscillations and PGRMC1 oscillations), estrogen and progesterone levels calculated as percent of the highest value for each woman were plotted together with the curves showing the oscillation of PGRMC1 RNA and protein (figure 6). There was no obvious correlation between the levels of RNA and any of these hormones. The protein increase preceded the estrogen increase at first, but during the second half of the cycle the protein correlated with the progesterone peak instead.

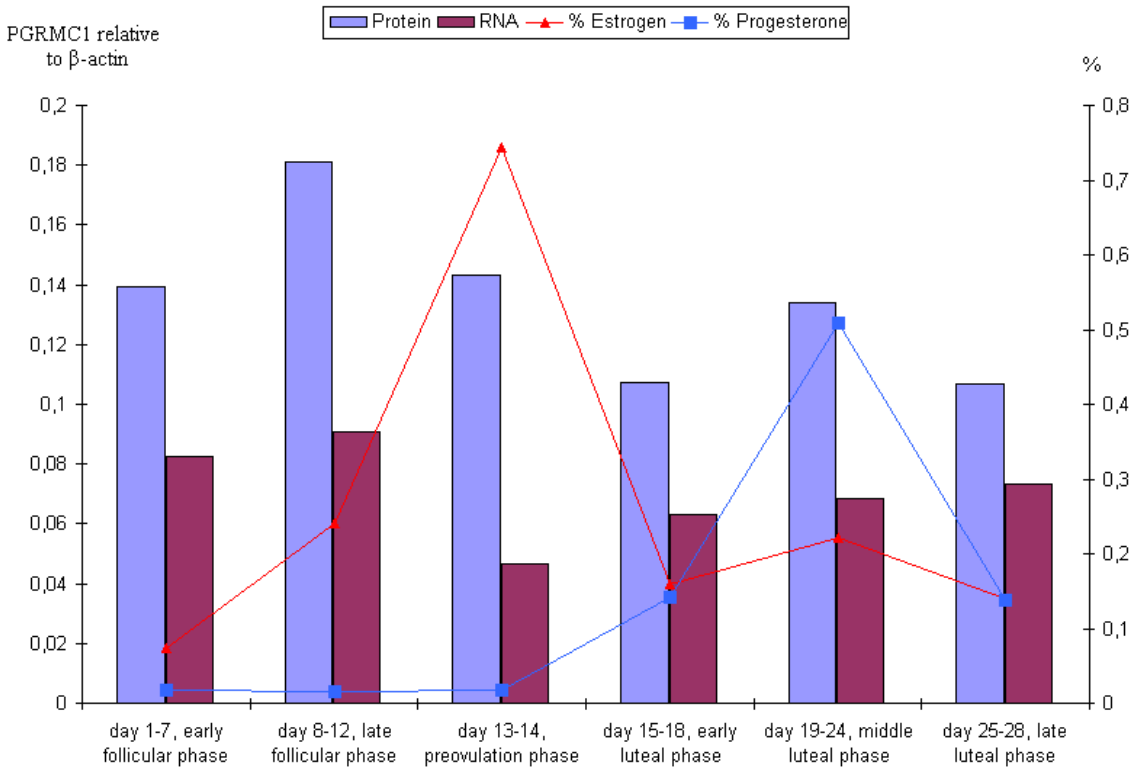

Figure 6. Comparative plot of PGRMC1 RNA and protein oscillation curves together with oscillation curves for estrogen and progesterone. PGRMC1 RNA was measured by Q-PCR and PGRMC1 protein was measured by western blot in a set of healthy women. In both methods, results were normalized to β-actin levels. The x-axis show the different phases of the menstrual cycle. The left y-axis show the relative ratio of PGRMC1 compared to β-actin and the right y-axis show a percent scale for the hormonal measurements. The hormones were calculated as percent of the highest value for each distinct woman, and then combined according to the phases.

The FSH values for the patient samples were calculated as percent of the highest value in any of the patients to be able to plot those values against the PGRMC1 RNA and protein levels in the patients. This plot (figure 7) showed no correlation between FSH values and either of the PGRMC1 RNA or protein levels in these patients.

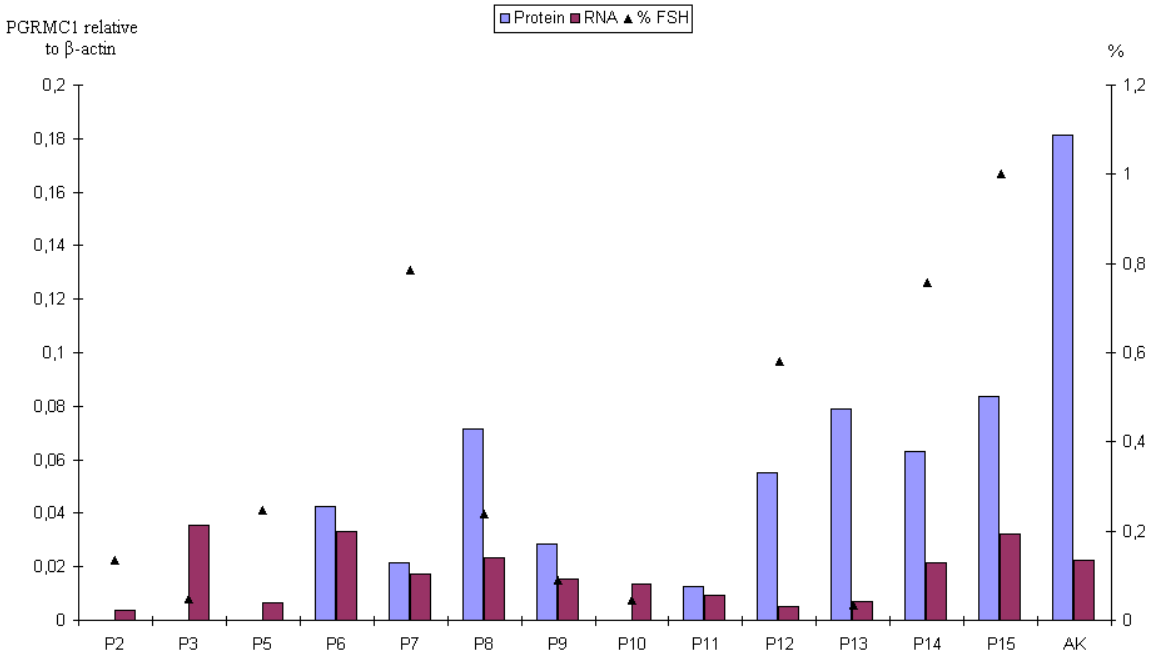

Figure 7. Comparative plot of PGRMC1 protein and RNA in POF patient samples, together with relative FSH measurements. PGRMC1 RNA was measured by Q-PCR and PGRMC1 protein was measured by western blot in 13 POF-patients and one nursing woman. In both methods, results were normalized to β-actin levels. The x-axis shows the different individuals (P=patient and AK=nursing woman). The left y-axis show the relative ratio of PGRMC1 compared to β-actin and the right y-axis show a percent scale for the hormonal measurements. The hormone was calculated as percent of the highest value among patient measurements.

DISCUSSION

PGRMC1 oscillates during the menstrual cycle

The lack of significant variations in PGRMC1 RNA levels showed that there was no oscillation of RNA expression during the menstrual cycle. On the other hand, the PGRMC1 protein expression oscillated during the cycle. Peak levels were seen first during the late follicular phase (day 8-12) and secondly, at a lower level, during the middle luteal phase (day 19-24). Least protein was found during the early and late luteal phases (day 15-18 and 25-28) and these protein levels were just about the same. This shows that there is a difference in PGRMC1 protein (but not RNA) expression between different time points during the menstrual cycle. Thus, PGRMC1 expression appears to be regulated primarily post-transcriptionally.

No clear association could be seen between the PGRMC1 curves (either RNA or protein) and oscillations of the cyclic hormones progesterone and estrogen. The hypothesis that PGRMC1 expression (in particular its transcription) might be regulated by progesterone (Cahill, 2007; Losel et al., 2007) thus is not supported by my data. It has been suggested previously that PGRMC1 binds different ligands depending on its location (Cahill, 2007; Losel et al., 2007). My results suggest that PGRMC1 may be involved in the regulation of estrogen levels, at least during the first half of the menstrual cycle.

If PGRMC1 truly is involved in estrogen regulation (and does bind progesterone, as previous studies have shown), maybe its own regulation is a feedback loop from the levels of estrogen and progesterone in the cell. My results suggest that a low level of estrogen boosts PGRMC1 expression, as do rising levels of progesterone to some extent. These feedback loops between the sex hormones and PGRMC1 would be of high importance for the cell, so it can maintain its capability to bind the hormones and regulate their levels accordingly. Also, this is reasonable in the case of the nursing woman, who showed high to “normal” PGRMC1 protein levels despite absent menstrual cycle, since her estrogen and progesterone expression were maintained by the preserved corpus luteum.

Variability of RNA and protein levels

The observed variations were caused by several combined factors. First, natural occurring variations between individuals had to be considered, since both their gene expression levels and the length of their actual menstrual cycles differed. Second, intra-individual variations due to methodology were of importance. Third, the assignment of cycle days to samples according to hormonal measurements was not exact. Thus, some of the samples maye have been assigned to the incorrect phase, adding to the observed variation.

One additional factor to consider was the freezing of the buffy coat before extraction procedures, which might have compromised the RNA. Q-PCR is a reliable method, well-established in the lab, which normally does not show much variation between replicas.

Yet, my analyses did show some variations within samples, indicating that sampling seems to have influenced the quality of the RNA.

PGRMC1 in POF patients

The patients and the nursing woman had the same levels of PGRMC1 RNA expression. No significant difference was found when comparing these levels to the RNA levels in the healthy women. Significant differences were found, however when comparing the patients' levels to the RNA level in each distinct phase of the healthy women cycle.

Protein levels of the patients and the healthy women were significantly different, both when the nursing woman was included and excluded from the patient data set. The significance increased when excluding the nursing woman, supporting the observation that the protein level in the nursing woman corresponded more closely to the healthy women's expression levels than to the POF patients' protein levels. These patient protein levels also showed significant differences to the protein level in each phase of the healthy women cycle.

These findings support those of Mansouri and colleagues (Mansouri, 2006; Mansouri et al., unpublished 2008) indicating a downregulation of PGRMC1 expression in POF-patients compared to healthy individuals, and suggest that only translation, not transcription is downregulated looking at the cycle in whole. This supports the hypothesis that PGRMC1 might somehow be involved in the expression, regulation or cause of POF and the malfunction of the ovaries.

Future investigation

In the future it would be of interest to support the results of these RNA and protein analyses by adding more healthy women to the study, and thus make the curves more robust.

Regarding the CYPs, it is worth looking into what other different CYPs could be of interest (those involved in steroid synthesis, metabolism and regulation) and maybe test some of them for expression in leukocytes.

I would also like to see more patient samples added to this study; preferably a few samples from each patient taken at different time points during an “imaginary” menstrual cycle. Then it could be concluded whether or not the PGRMC1 levels are downregulated at all times and the comparison to the oscillation curves could be made more parallel. I would also think that this would give more reliable results and I believe it could be of interest to test if there still is an oscillation of PGRMC1, even though the expression level is down regulated in the patients (especially if PGRMC1 does have an affect in POF, as suggested by this study and that of Mansouri and colleagues (Mansouri, 2006; Mansouri et al., unpublished 2008)).

It would also be interesting to include more nursing (or pregnant) women samples by the same principle as just stated regarding the POF-patients. These women are in fact healthy, but also they have lost their menstrual cycle (for the moment). It would be of interest to check whether PGRMC1 actually oscillates as “normal” in these women. This would clarify whether the downregulation of PGRMC1 confirmed in this study is related to the disease, and not to the loss of menstrual cycle alone.

MATERIALS AND METHODS

Blood samples

Blood samples were obtained from 10 healthy women <30 years old, not taking any contraceptives, 13 women diagnosed with POF and one woman who was breast feeding (234 days after delivery) and thus had no menstruation. The healthy women donated blood twice a week over a period of 4 weeks (roughly one menstrual cycle). Progesterone and estrogen levels were measured and the menstrual cycle of the women adjusted to an idealized menstrual cycle (Dr. Inger Sundström Poromaa, Department of women's and children's health, University Hospital, Uppsala) (Appendix table A1). From the POF-patients and the nursing woman one sample each was obtained (since they had no menstrual cycle), and the patients' levels of follicle-stimulating hormone (FSH) were measured (MD Per-Olof Karlström, Department of women's and children's health, University Hospital, Uppsala) (Appendix table A3).

Buffy coat is the fraction of a blood sample that contains the white blood cells, after that the blood has been treated with an anticoagulant (like Ethylene Diamine Tetraacetic Acid, EDTA) and centrifuged to separate its components. Buffy coat pellet was prepared from each of the obtained EDTA blood samples and stored at -20°C until analysis.

Each one of the buffy coat samples was processed the same way (figure 8). Q-PCR and western blots were repeated twice for each sample from the healthy women. For each patient sample, Q-PCR was performed three times and western blots four times.

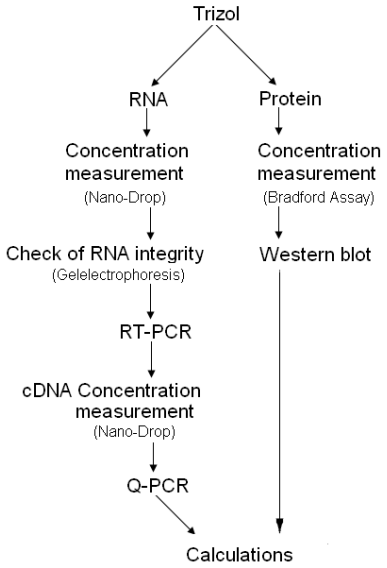

Figure 8. Flow scheme of analyses.

RNA and protein extraction

Total RNA and proteins were isolated from the buffy coat samples using the TRIZOL® reagent (Invitrogen) according to manufacturer's protocol.

Modifications were that the homogenization was done according to the notes for cells grown in suspension and that the patient samples (but not the ones of the healthy women) were washed once in 1x PBS (1 mL) and centrifuged (5 min, 2,000 x g) before addition of TRIZOL®. Instead of 75% and 100% ethanol, 70% and 95.5% ethanol were used. All centrifugations were made at 4°C. RNA was dissolved in RNase-free water. Proteins were air-dried 5 min at 50°C. No sedimentations of insoluble material in protein solutions were done.

Concentration measurements

RNA and cDNA

RNA and cDNA concentrations were estimated from 260 nm absorbance (Nano-Drop, Saveen Werner, computer software ND-1000 V3.3.0). The results were printed using DYMO Label Writer 330.

Proteins

The protein concentration was measured according to the Bio-Rad Protein Assay (Bio-Rad), which is based on the Bradford assay. Eight standards of increasing concentration (1, 2, 5, 8, 10, 15, 20 and 25 μg/mL) were prepared from a stock protein standard solution (Bio-Rad Protein Assay Standard I, Lyophilized Bovine Plasma Gamma Globulin) of 1.48 mg/mL (Bio-Rad). These were used to create the standard curve. Standards, blanks and samples were mixed with the dye (Bio-Rad Protein Assay dye reagent concentrate) according to the Bio-Rad protocol, loaded (in duplicates) in a microtiter plate with flat bottom and scanned at 595 nm in an ELIZA microplate reader (Emax precision microplate reader, Molecular Devices). The results were analyzed with the computer program SOFTmax for Macintosh Version 2.02.

Check of RNA integrity

To check the integrity of the isolated RNA, about 500 ng RNA was separated on a 1% agarose (SeaKem® LE Agarose, Lonza) gel containing EtBr in tris/borate/EDTA (TBE, for recipe see appendix table A2) buffer for 50 min at 100 V. The RNA was then visualized by UV-light and the gel was photographed.

cDNA synthesis

Using the reverse-transcription PCR (RT-PCR) method, cDNA was synthesized from total RNA using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas). I followed the accompanying protocol for synthesis of first strand cDNA suitable for

PCR Amplification, using total RNA (~1 μg) and a random hexamer primer included in the kit.

Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) was carried out using cDNA as template and specific primers for *PGRMC1* (for primer sequences, see table 3).

Table 3. Primer sequences for the three genes used in the Q-PCR reaction.

Gene	Fw/Rev	Primer sequence
PGRMC1	Fw	5'- GGTGTTCGATGTGACCAAAG -3'
	Rev	5'- GATGCATCTCTTCCAGCAAA -3'
β-actin	Fw	5'- CTGGAACGGTGAAGGTGACA -3'
	Rev	5'- CGGCCACATTGTGAACTTTG -3'
GAPDH	Fw	5'- CAACAGCGACACCCACTCCTC -3'
	Rev	5'- CATACCAGGAAATGAGCTTGAACAAA -3'

To each gene a forward (Fw) and a reverse (Rev) primer sequence were used.

The gene expression level was calculated relative to that of the housekeeping genes *β-actin* and *GAPDH* (for primer sequences, see table 3). The Q-PCR was performed according to the Invitrogen Platinum® SYBR® Green qPCR SuperMix-UDG Protocol, using their kit containing a ready to use SuperMix and ROX as reference (for PCR program set up, see table 4).

Table 4. Q-PCR program setup.

Temp (°C)	Time	
50	2 min	
95	2 min	
95	15 sec	} 45 cycles
60	60 sec	
Melting curve analysis		

All measurements in the Q-PCR were done in triplicate at each run and the machine used was Stratagene® Mx3005P (AH diagnostics). The results were analyzed using the MxPro software.

Western blot analysis

The dissolved proteins from the Trizol extraction were separated according to size by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. A NuPAGE® 4-12% Bis-Tris gradient SDS-gel (1.5 mm x 15 well) (Invitrogen™) was used together with a PageRuler™ Prestained Protein Ladder (Fermentas) and the loading buffer NuPAGE® LDS Sample Buffer (4x) (Invitrogen™). As buffer, 1xRunning Buffer (NuPAGE® 2-(*N*-morpholino)ethanesulfonic acid (MES) SDS Running Buffer (20x) (Invitrogen™)) was used and 0.5 ml NuPAGE® Antioxidant (Invitrogen™) per 200 mL Running Buffer was added in the upper chamber of the module. Each sample (10 μL) was mixed with 5 μL

loading buffer and 1 µL reducing agent (dithiothreitol (DTT), 1M) and heated for 10 min at 70°C before loading to the gel. Electrophoresis was carried out at 150V for 50 min. Once separated, the proteins were transferred from the gel to a polyvinylidene fluoride (PVDF) membrane (Immobilon™-FL Transfer Membrane, pore size 0.45 um, MILLIPORE) in 1xTransfer Buffer (NuPAGE® Transfer Buffer (20x), Invitrogen™) with 10% methanol and 1 mL NuPAGE® Antioxidant per liter Transfer Buffer. The transfer was carried out at 40V for 90 min, after which the membrane (now containing the proteins) was blocked with 5 mL 2.5% bovine serum albumin (BSA) (SIGMA®) in 1x phosphate-buffered saline (PBS) at 4°C over night (for recipe see appendix table A2). To detect the proteins, the membrane was first incubated in 3 mL 1% BSA in 1x PBS with the primary antibodies (table 5) for 1 h at room temperature, and washed three times with 1x PBS (0.1% Tween) (Tween® 20, for molecular biology, SIGMA®). At each washing step, the membrane was kept in the solution (shaking) for 8 min.

Table 5. Primary antibodies used in western blot.

Antibody	Dilution	Company
rabbit anti-PGRMC1	1:5,000	SIGMA
mouse anti-β-actin	1:10,000	abcam
goat anti-CYP7B	1:1,000	Santa Cruz Biotechnology
rabbit anti-CYP21A	1:500	LifeSpan BioSciences

The membrane was then incubated another hour at room temperature, this time with light sensitive secondary antibodies that are specific for the primary ones (table 6). For this incubation, 5 mL 1% BSA in 1x PBS was used. The membrane was washed three times with 1x PBS (0.1% Tween), followed by two washes with 1x PBS. Also this time the membrane was kept in the solution (shaking) for 8 min at each washing step.

Table 6. Secondary antibodies used in western blot.

Antibody	Dilution	Company
anti-rabbit	1:20,000	LiCor
anti-mouse	1:20,000	LiCor
anti-goat	1:20,000	LiCor

Finally, the membrane was scanned (Odyssey, LI-COR BioScience, Westburg) and analyzed with the Odyssey 2.1 software. β-actin was used as an internal reference, so all protein measurements of PGRMC1 and CYPs were normalized to the level of β-actin.

Standardized menstrual cycle

I assumed a 28-day standardized menstrual cycle that was divided into six different phases; early follicular phase (day1-7), late follicular phase (day 8-12), preovulation phase (day 13-14), early luteal phase (day 15-18), middle luteal phase (day 19-24) and late luteal phase (day 25-28). These groupings were made on basis of the varying hormonal levels in the women. Measurements of cyclic hormones (LH, progesterone and estrogen) together with information of the time of previous menstruation were used to assign the distinct samples to the different phases (Dr. Inger Sundström Poromaa,

Department of women's and children's health, University Hospital, Uppsala). This, however, is not an exact approach and results in uncertainty of exact days in the cycle. The established cycle day of one sample can be up to 5 days off from the actual day, meaning that some of the samples might have been placed in "the wrong phase".

Analysis and calculations

Geometric mean and standard deviation were calculated from all values from the different replicas for each sample (Appendix figure A1, A-J and figure A2, A-J).

The resulting values from the healthy women were then sorted into groups according to the phases of the standardized menstrual cycle in which the original sample was taken. All values in each phase were subsequently compiled again using geometric means.

For statistical comparisons between patients and healthy women, student's t-test was used. I used a two-tailed t-test with two-sample equal variance, and considered $p<0.05$ as significant.

ACKNOWLEDGMENTS

I would like to thank all present and former members of the Dahl group.

Thank you Niklas for having me in your group and for the opportunity to take part in this project. Thank you Jens for sharing your project with me and for being my supervisor. You have been inspiring and have had great patients with me. I thank you for the rewarding discussions and all help in general as well. Thank you Anne-Sophie for all the help in the lab and for great discussions.

I would also like to thank everyone at the Department of Genetics and Pathology for help with common equipments.

I especially want to thank the premature ovarian failure patients and the healthy control women for donating samples. Without you this study would not have been possible.

Thank you Dr. Inger Sundström Poromaa (Department of women's and children's health, University Hospital, Uppsala) for collecting samples from healthy women and making the additional measurements.

Thank you MD Per-Olof Karlström (Department of women's and children's health, University Hospital, Uppsala) for collecting samples from the POF-patients and making the hormonal measurements.

Finally, I would like to thank my family and friends for being there for me during this period.

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APPENDIX

Table A1. Compilation of data regarding the control population of healthy women.

Sample	Day of cycle	Sample	Day of cycle	Sample	Day of cycle
1.1	19	5.6	12	9.8	9
1.2	22	5.8	19	10.1	28
1.3	25	6.1	2	10.2	3
1.4	1	6.2	5	10.3	7
1.6	8	6.3	8	10.4	10
1.7	11	6.4	12	10.5	14
1.8	15	6.5	14	10.6	19
2.1	7	6.6	18	10.7	22
2.2	10	6.7	22	10.8	26
2.3	13	6.8	26	11.1	18
2.4	20	8.1	19	11.2	21
2.5	24	8.2	23	11.3	23
2.6	27	8.3	26	11.4	28
2.7	2	8.4	1	11.5	4
2.8	7	8.5	5	11.6	7
3.1	11	8.6	8	11.7	10
3.2	14	8.7	11	11.8	15
3.3	16	8.8	17	12.1	14
3.4	26	9.1	10	12.2	17
3.5	2	9.2	13	12.3	19
3.8	13	9.3	16	12.4	23
5.1	23	9.4	22	12.5	27
5.2	28	9.5	26	12.6	2
5.3	2	9.6	1	12.7	5
5.4	5	9.7	6	12.8	10
5.5	9				

This table presents all the samples used in the study (8 samples per woman in most cases). Each sample is numbered with a primary figure symbolizing the individual and a second figure for the sample number of that woman. The days are the ones in a standardized 28-day menstrual cycle on which the sample was taken and the sample-day parallel was made upon hormone level measurements and information of time of previous menstruation.

Table A2. Recipes of used solutions

Solution	Ingredients	
TBE (10x)	Tris/HCl, pH 8.1	1340 mM
	Boric acid	45 mM
	EDTA	25 mM
PBS (1x)	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4 x 2 H_2O	4.3 mM
	KH_2PO_4	1.4 mM

Table A3. Ages of and FSH-levels in POF patients.

Patients	Age at time of sampling	FSH-level
P2	32	9.9
P3	39	3.6
P5	34	18.2
P6	40	ND
P7	36	58
P8	36	17.7
P9	36	6.6
P10	31	3.4
P11	29	ND
P12	36	43
P13	38	2.5
P14	43	56
P15	34	74
AK	34	ND

P=patient, AK=nursing woman, ND=not determined.
In healthy women this level is approximately 10.

Table A4. Statistical analysis of PGRMC1 levels in different phases of the menstrual cycle.

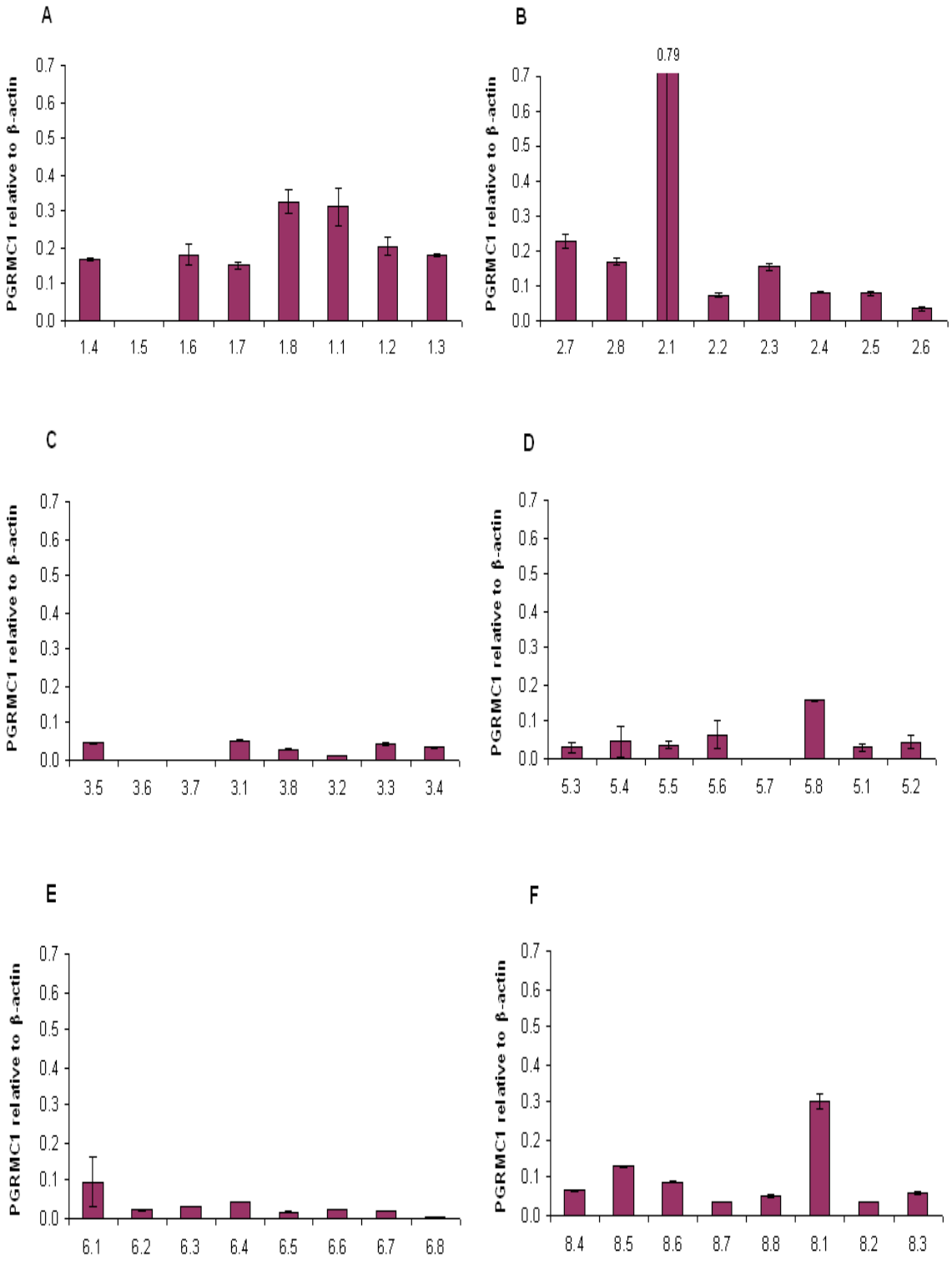
	day 8-12, late follicular phase (n=15)		day 13-14, preovulation phase (n=7)		day 15-18, early luteal phase (n=8)		day 19-24, middle luteal phase (n=16)		day 25-28, late luteal phase (n=11)	
	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA	Protein	RNA
day 1-7, early follicular phase (n=19)	0.2616	0.2731	0.5993	0.6364	0.7675	0.8099	0.5040	0.8213	0.2342	0.2104
day 8-12, late follicular phase (n=15)			0.1911	0.4605	0.6587	0.4566	0.9366	0.3474	0.0251	0.4410
day 13-14, preovulation phase (n=7)					0.6005	0.8850	0.5526	0.6325	0.6490	0.4436
day 15-18, early luteal phase (n=8)							0.7759	0.7493	0.3031	0.4189
day 19-24, middle luteal phase (n=16)									0.3234	0.2597

This table shows all p-values from the t-test calculations of significant differences between the phases in the RNA and protein oscillation cycle. Total number of observations is 76. Significant values are marked in red.

Table A5. Statistical calculations of patients and healthy women using t-test.

Analysis	Data set 1	Number of observations in data set 1	Data set 2	Number of observations in data set 2	Protein p-value	RNA p-value
1	All healthy women	76	All patients + AK	14	0.0048	0.3169
2	All healthy women	76	All patients	13	0.0033	0.3336
3	Healthy day 1-7	19	All patients + AK	14	0.0006	<0.0001
4	Healthy day 1-7	19	All patients	13	0.0003	<0.0001
5	Healthy day 8-12	15	All patients + AK	14	<0.0001	0.1189
6	Healthy day 8-12	15	All patients	13	<0.0001	0.1325
7	Healthy day 13-14	7	All patients + AK	14	0.0092	0.0209
8	Healthy day 13-14	7	All patients	13	0.0021	0.0255
9	Healthy day 15-18	8	All patients + AK	14	0.0126	0.0145
10	Healthy day 15-18	8	All patients	13	0.0082	0.0180
11	Healthy day 19-24	16	All patients + AK	14	0.0466	0.0029
12	Healthy day 19-24	16	All patients	13	0.0414	0.0039
13	Healthy day 25-28	11	All patients + AK	14	0.0010	0.2097
14	Healthy day 25-28	11	All patients	13	<0.0001	0.2273
15	Patients <35 years old	5	Patients >35 years old	8	0.1681	0.3114
16	Patients FSH<7	4	Patients FSH>15	6	0.3388	0.9810

Statistical calculations between different combinations of the data sets were done using the t-test. This table shows all the statistical comparisons done between the control set of healthy women and the patient samples (including or excluding the nursing woman, AK), and patient sub-groupings. Number of observations is the number of samples corresponding to each of the two data sets compared in each calculation. All comparison set ups were done for both protein samples and RNA samples. A p-value of <0.05 is considered significant, and marked with red.


A
PGRMC1 relative to β-actin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.4 1.5 1.6 1.7 1.8 1.1 1.2 1.3
B
PGRMC1 relative to β-actin
0.79
2.7 2.8 2.1 2.2 2.3 2.4 2.5 2.6
C
PGRMC1 relative to β-actin
3.5 3.6 3.7 3.1 3.8 3.2 3.3 3.4
D
PGRMC1 relative to β-actin
5.3 5.4 5.5 5.6 5.7 5.8 5.1 5.2
E
PGRMC1 relative to β-actin
6.1 6.2 6.3 6.4 6.5 6.6 6.7 6.8
F
PGRMC1 relative to β-actin
8.4 8.5 8.6 8.7 8.8 8.1 8.2 8.3

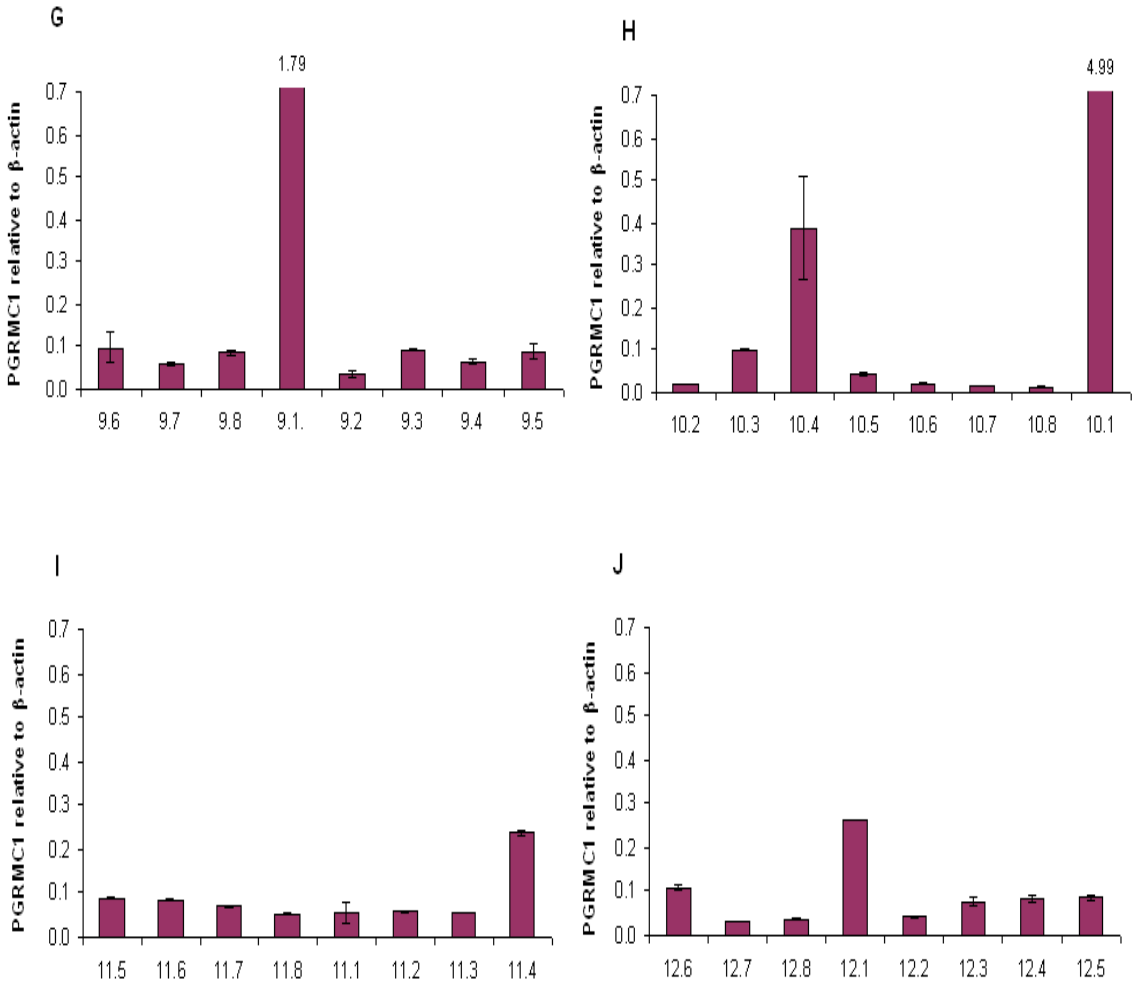
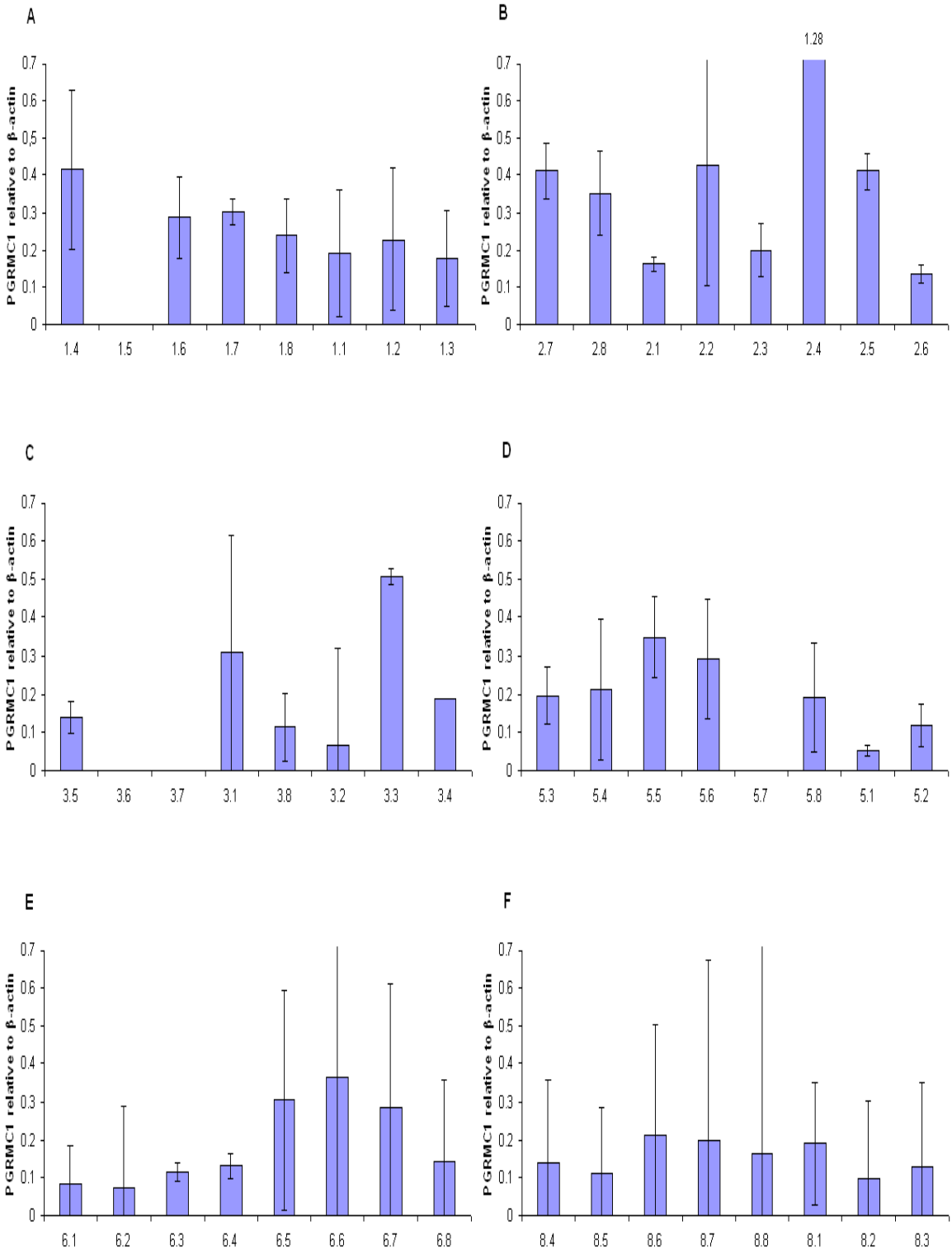


Figure A1, A-J. RNA measurements of all samples in each of the 10 healthy women. The samples have been analyzed two times in triplicate by Q-PCR and the geometric mean has been calculated to get one RNA level corresponding to each sample. The samples have been aligned according to what day of the menstrual cycle they belong to (Appendix table A1), with the left in each figure being the start of the cycle. The x-axis states the number of the woman together with each corresponding sample number. The y-axis display PGRMC1 levels normalized to β-actin. There are three types of variation seen in these graphs; variation within samples (displayed with standard deviations), variations within individuals (during the cycle) and variation between individuals. In cases where the bar is off chart, its value has been written above the bar.


A
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PGRMC1 relative to β-actin
1.28
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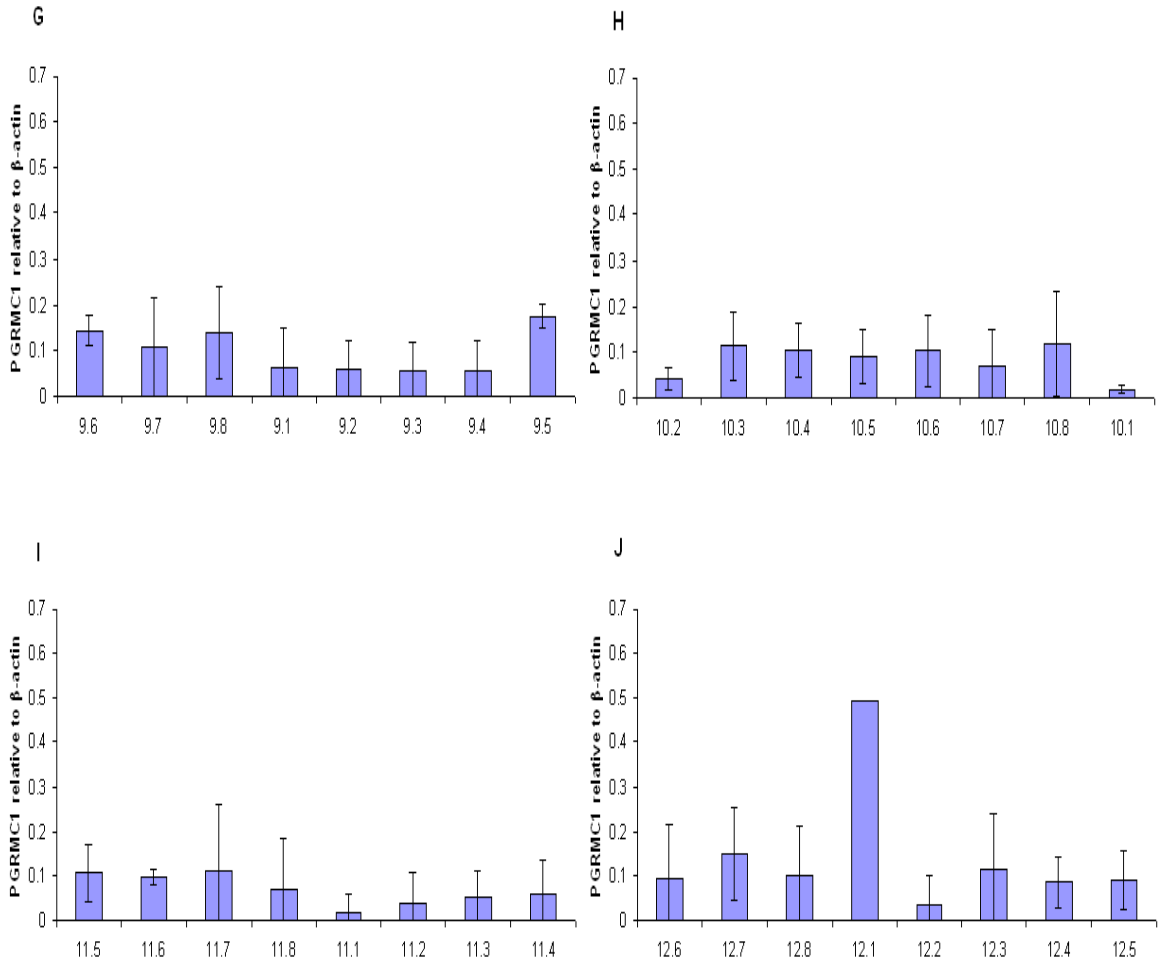

Figure A2, A-J. Protein measurements of all samples in each of the 10 healthy women. The samples have been analyzed two times with Western blotting and the geometric mean has been calculated to get one protein level corresponding to each sample. The samples have been put in order according to what day of the menstrual cycle they relates to (Appendix table A1), with the left in each figure being the start of the cycle. The x-axis states the number of the woman together with each corresponding sample number. The y-axis display PGRMC1 levels normalized to β-actin. There are three forms of variation seen in these figures; variation within samples (displayed with standard deviations), variations within individuals (during the cycle) and variation between individuals. In the case where the bar is off chart, its value has been written above the bar.