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Running title **Variations in Factor XIII level during menstrual cycle**

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Abstract

Introduction Factor XIII (FXIII) has an important role in the control of bleeding through fibrin cross-linking. While many physiological factors affect plasma FXIII level, the effect of the menstrual cycle is not fully understood.

Aim: To examine changes in plasma FXIII activity during the normal menstrual cycle and to assess correlation between FXIII activity during the menstrual phase and menstrual blood loss.

Methods: In this longitudinal study, a total of 32 healthy normal women of reproductive age were recruited. Menstrual blood loss was measured using the pictorial blood-assessment chart (PBAC). A bleeding score questionnaire was also completed. Blood samples were taken during the menstrual, proliferative, periovulatory, secretory , and premenstrual phase for the assessment of FXIII level using a quantitative ammonia release assay.

Results: The mean±SD FXIII level was lowest during menstrual and periovulatory phases (114 ± 23 and 114 ± 21 IU/dL respectively). The mean FXIII level during the secretory and premenstrual phases were higher than the menstrual phase (p=0.036). The secretory mean FXIII level was also significantly higher compared to the periovulatory phase (p=0.02). There was no significant correlation between FXIII level during the menstrual phase and age (p=0.53) or PBAC score (p=0.53). There were no significant differences in FXIII level during the menstrual phase between women with PBAC score of at least 100 (n=14; mean 116 IU/dL) and women with PBAC scores less than 100 (n=18; mean 113 IU/dL). There was no correlation between FXIII level and bleeding scores.

Conclusion: FXIII activity was lower during menstrual and periovulatory phase of the cycle. However, the small difference between mean values (8 IU/dL) would be unlikely to have a significant impact on diagnosis of FXIII deficiency and clinical management.

Keywords: Coagulation Factor XIII, Menstrual cycle, Menorrhagia, Ovulation.

Introduction

A wide variation has been reported in the levels of coagulation factors in normal individuals and patients affected with bleeding disorders. Different physiological and pathological factors have been shown to result in such variation, including: age, sex, blood group, smoking status, stress and physical exercise[1,2]. In women, sex hormones also affect the level of coagulation factors. This has been demonstrated by the presence of cyclical changes in some coagulation factors during the menstrual cycle and by the effects of hormonal contraception and hormone replacement therapy [3].

The effect of the changes of female sex hormones on coagulation factor levels during the menstrual cycle may have important clinical implications for the diagnosis and management of women with bleeding disorders [3,4]. However, for most coagulation factors, these changes have been assessed in a small number of studies with conflicting results, the majority having only a small number of women [5].

In a cross-sectional study of 123 women, a strong cyclical variation was shown for von Willebrand factor (VWF) antigen and activity levels as well as fibrinogen level during the menstrual cycle [4]. These cyclical variations were dampened for VWF and completely disappeared for fibrinogen with the use of combined hormonal contraceptives [4]. In the same study, factor XI showed no variation during the menstrual cycle, but factor XIII (FXIII) was not assessed . On the other hand, no difference in VWF was seen during menstrual, follicular and luteal phases in a cross-sectional study with no association between the level of estradiol, or progesterone and VWF, or factor VIII [6].

FXIII may have an important role during the menstrual phase of the cycle, in the process of healing the endometrium and controlling the menstrual blood loss through fibrin cross-linking, thus improving the mechanical strength, rigidity and elasticity of the clot to prevent it

from being degraded through fibrinolysis [7]. However, the effect of the menstrual cycle on FXIII levels is not fully understood and it is not clear whether endometrial healing leads to an increase in FXIII consumption, thus altering the plasma level. Variations in FXIII level during the menstrual cycle, if present, would need to be considered when performing screening tests for FXIII deficiency.

The aim of this study was to examine possible changes in plasma FXIII activity during the normal menstrual cycle and to assess any association between level of FXIII and menstrual bleeding.

Materials and Methods

Study design:

This was a longitudinal study of 32 women of reproductive age. Demographic and clinical data was collected (including; age, ethnicity, previous number of pregnancies and miscarriages, body mass index (BMI), and smoking status) through an interview with the women.

Women were recruited from hospital staff through leaflet and poster invitations at Royal Free Hospital NHS Foundation Trust in London, UK as well as the Medical Thrombosis and Hemophilia treatment Center in Duisburg, Germany, from January to October 2012. The study was approved by the hospital ethical committees and informed consent was obtained from participating women. Women were included in the study if they had a regular menstrual cycle of 26-35 days duration, and were not using hormonal contraception, including *Mirena*® IUS (levonorgestrel-releasing intrauterine system), and were not using non-steroidal anti-inflammatory drugs. Exclusion criteria also included personal or family history of bleeding tendency or thrombo-embolism.

Menstrual blood loss was measured using the pictorial blood-assessment chart (PBAC) [8]. The women were given oral and written instructions on the use of PBAC and were asked to complete the chart during the period of blood sample collection. Women were required to document the number of sanitary pads and/or tampons used each day (24 hour) based on the degree of saturation of the pads/tampons, they were also asked to record the number and size of blood clots if present. The completed PBAC sheets were collected at the end of the menstrual period and the total numerical score were calculated by a single gynaecologist (LS). The total score was calculated by adding up the pad/tampons counts obtained from each

day of the period. The scores assigned for tampons were '1' for each lightly stained tampon, '5' if moderately stained and '10' if it was completely saturated with blood. The towels were given ascending scores of '1', '5' and '20'. Small and large clots scored '1' and '5', respectively PBAC score ≥ 100 were considered a heavy menstrual bleeding [8].

A bleeding questionnaire was also completed and the bleeding score (SH, RAK) was calculated based on specific symptoms; these included epistaxis, cutaneous symptoms, bleeding from minor wounds, muscle haematomas, haemarthrosis, oral cavity bleeding, gastrointestinal bleeding, bleeding after tooth extraction, bleeding after surgery, postpartum haemorrhage, and menorrhagia. The severity of each symptom was subsequently summarised, using a bleeding questionare system ranging from '-1' representing absence of bleeding despite haemostatic challenge, '0' representing complete absence of bleeding symptoms, '1' given when woman reported presence of bleeding, '2' if the bleeding symptoms required evaluation by a physician but no active intervention was needed, '3' if bleeding required some kind of intervention by physician, and '4' if blood transfusion or surgery was required to control the bleeding. A bleeding score value below '3' is considered normal [9].

Laboratory methods:

Blood samples were taken five times during one menstrual cycle as follows: menstrual phase (day 1-5), proliferative phase (day 6-11), periovulatory phase (day 12-17), secretory phase (days 18-23), and premenstrual phase (day 24-29).

After obtaining written informed consent, blood samples were obtained between 11:00 a.m. and 2:00 p.m. Venous blood samples were collected by clean venepuncture, with minimal stasis, into citrate (0.105mol L^{-1}) Vacutainers^{TM} (BD Diagnostics, Oxford, UK), with a ratio

of one part anticoagulant to 9 parts whole blood. Within one hour of collection platelet poor plasma (PPP) was prepared by double centrifugation at ambient temperature (2000g for 10 minutes) and aliquots of PPP were frozen to -80ºC. On the day of assay samples were thawed to 37ºC and mixed.

Factor XIII activity was determined using a chromogenic ammonia release assay (Berichrom® FXIII assay; Siemens Healthcare Diagnostics, Marburg, Germany) performed on a CS-5100 coagulation analyser (Sysmex UK Ltd, Milton Keynes, UK). In this assay, FXIII is converted to its active form, FXIIIa, by the action of thrombin in the presence of calcium ions. Activated FXIIIa cross-links a specific peptide substrate to glycine ethyl ester, thereby releasing ammonia, which is determined in a glutamate dehydrogenase catalysed NADH-dependent reaction. The consumption of NADH is measured spectrophotometrically by the decreased absorbance at 340 nm [10]. FXIII potencies were calculated relative to Standard Human Plasma (SHP, Siemens Healthcare Diagnostics). The manufacturer's stated normal reference range was 70-140 IU/dL. The coefficient of variation at a FXIII level of 87 IU/dL was 2%. All samples were assayed in a single analytical run, thereby removing the effect of inter-assay variation.

Statistical method:

Continuous data were presented as mean ± standard deviation (SD). Paired sample t-test was used to measure the mean difference and 95% confidence interval (CI) of FXIII activity in different phases of the menstrual cycle. Comparison of FXIII changes in relation to PBAC score and body mass index (BMI) was performed using an unpaired t-test ($p \leq 0.05$ was considered statistically significant). The statistical package for social sciences, version 20 (SPSS, Chicago, USA) was used.

Results

The demographics of 32 women included in this study are illustrated in Table 1. There were 24 women recruited from Royal Free Hospital in UK and eight women from Medical Thrombosis and Hemophilia treatment Center in Germany. All women included in the study were non-smokers. Only eight women had a previous history of pregnancy with a total of 20 pregnancies; four of them resulted in miscarriage. No woman had more than one miscarriage.

In total, 153 blood samples were collected from the women. Figure 1 shows the distribution of FXIII activity during each phase of the menstrual cycle. The mean±SD FXIII level was at its lowest at the menstrual (114 ± 23 IU/dL) and the periovulatory (114 ± 21 IU/dL) phases of the cycle (Table 2). The FXIII level was significantly higher during the secretory (mean difference 8.0 IU/dL, 95% CI 15.5-0.57, p=0.036) and premenstrual (mean difference 8.61 IU/dL, 95% CI 16.6-0.59, p=0.036) compared withthe menstrual phase. When compared to the periovulatory phase, the mean FXIII level was also higher in the secretory (mean difference 7.78 IU/dL, 95% CI 1.3-14.2), proliferative (mean difference 5.3 IU/dL, 95% CI 1.3-12) and premenstrual (mean difference 9.8 IU/dL, 95% CI -0.01-19.7) phases of the cycle. No study participants showed FXIII levels below the normal range (70–140 IU/dL).

The median PBAC score was 92, ranging from 12 to 920. There was no significant correlation between FXIII activity during the menstrual phase and age (r=0.03; p=0.85) or PBAC score (r=0.11; p=0.53), using Spearman's correlation coefficient. Among the 14 women with PBAC score of at least 100, the mean FXIII activity during the menstrual phase of the cycle was similar to women with PBAC score less than 100 (116 ± 19 vs. 113 ± 26 IU/dL, p=0.72). Bleeding score was '-1' in seven (22%) women, '0' in 22 (68%) women, '1' in one (4%), and '3' in two (6%) women. Among the three women with bleeding symptoms,

FXIII activities during menstrual phase of the cycle were 180 IU/dL , 164 IU/dLfor two women with bleeding score “3”, and 118 IU/dLfor one woman with bleeding score ‘1’.

To study the effect of obesity on the cyclic change of FXIII activity, 24 women were divided into those with BMI of 25 kg/m^2 or less (n=13) and more than 25 kg/m^2 (n=11). No significant difference was observed in FXIII activity at different phases of the menstrual cycle between the two groups, including the menstrual phase of the cycle. The mean FXIII activity was the lowest during the menstrual phase of the cycle in women with BMI of 25 kg/m^2 or less and more than 25 kg/m^2 (111 ± 25 vs. 119 ± 27 IU/dL, p= 0.46).

Discussion

In this study, the mean FXIII activity was significantly higher toward the last two weeks of the cycle compared to the menstrual phase. FXIII level was at its lowest during the menstrual and ovulatory phase. A significant decrease in FXIII level during the periovulatory phase of the cycle has been previously reported in a small study including 10 women. However, FXIII level was not assessed during menstrual phase in the study [11]. The hormonal levels (estrogen and progesterone) are at their baseline during these phases of the menstrual cycle with a peak of estrogen in the follicular phase and peak estrogen and progesterone during the luteal phase. These hormonal fluctuations may explain FXIII variations during the cycle. However, there could also be increased consumption of this factor due to its role in healing, angiogenesis and fibrin stabilisation during the menstrual and ovulatory phase of the cycle. Activated FXIII induces a severe reduction in the thrombospondin-1 (TSP-1) mRNA and reduces TSP-1 levels in the conditioned medium. TSP-1 acts as an angiogenesis inhibitor by preventing endothelial cell proliferation and migration, as well as inducing apoptosis [12]. Further mediators for FXIII pro-angiogenic effects involve upregulation of certain transcription factors, and tyrosine phosphorylation and activation of vascular endothelial

growth factor receptor-2 [13,14]. This proangiogenic function of FXIII might be important in endometrial healing, regeneration and vascular reproliferation following endometrial layer shedding during menstrual cycle as well as ovarian surface at site of follicular rupture during ovulation.

We did not find any correlation between FXIII level during menstrual phase and menstrual bleeding. There was no significant difference in FXIII activity in women with heavy menstrual bleeding (PBAC score equal or more than 100) compared to those with normal menstrual bleeding. However, it is important to note that the PBAC was used in this study to measure menstrual blood loss and this is a simple tool that only allows a semi- objective assessment of menstrual blood loss [15]. The PBAC was chosen rather than a more objective assessment tool, like alkaline hematin, because it is more practical and less expensive. In addition, the number of women in the study is small. Studies with a large number of women are required to determine the correlation between menstrual blood loss and FXIII level.

In our study, bleeding symptoms were reported in only three women with a score of '1' and '3' in one and two women respectively. Bleeding symptoms can be reported by patients in the absence of any congenital or aquired bleeding disorders, with at least one haemorrhagic symptom being reported in up to 25% of normal subjects [16]. There were no abnormal FXIII levels observed in those women with bleeding symptoms. However, the number of women with bleeding symptoms was very small in this study. Even in patients with FXIII deficiency, there is no single bleeding symptom sufficiently specific or sensitive to identify patients with FXIII-B-subnit deficiency or those with heterozygous FXIII deficiency, both of which are characterised by a milder bleeding tendency.

This study showed that there is a trend towards differences in FXIII in various phases of the menstrual cycle. FXIII levels were significantly higher during the luteal and premenstrual phases and the lowest level was seen during the menstrual and ovulatory phases. This could be due to fluctuations in the hormonal levels. However, the difference was small with only 8 IU/dL difference between the highest and the lowest mean FXIII level during the cycle. Based on our study this small change in FXIII level in different phases of the menstrual cycle is unlikely to influence the diagnosis of FXIII deficiency. Therefore, there should be no restrictions on performing FXIII assays based on the phase of the menstrual cycle. Whether this small variation in FXIII levels reflects the normal variations that can occur with repeated measurement of FXIII is a possibility. This can only be determined by further studies assessing this variation in comparison with men and postmenopausal women. The decrease in the level of FXIII seen in the menstrual and ovulatory phases in our study may also reflect the role of FXIII in tissue regeneration, healing and haemostasis during these phases of the cycle. This finding warrants further studies in assessing the role of FXIII in women with normal and abmormal menstrual and ovulatory bleeding.

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Author contributions:

Lava Sharief performed literature review, patient recruitment, data acquisition, entry, analysis, interpretation and the first draft of the manuscript. Ian Mackie and Andrew S Lawrie provided laboratory assistance and supervision, data analysis and interpretation, manuscript critical analysis and approval. Susan Halimeh and Guenther Kappert performed patient recruitment, data acquisition, entry, analysis, interpretation and the first draft of the manuscript. Flora Peyvandi provided study design, critical analysis and manscript approval. Rezan Kadir provided study design, literature review, clinical and academic supervision, critical analysis and edited the approved manscript.

Disclosures:

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

Figure Legend:

Figure 1 Distribution of FXIII activity (mean ±SD) during each phase of the menstrual cycle

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Table 1 Demographics of 32 women with normal menstrual cycle included for this study

Variables	Outcome
Country of origin, No. (%)	
Caucasian	16 (50)
Asian	10 (31)
Afro-Caribbean	6 (19)
Women with previous miscarriage No. (%)	
Yes	4 (12,5)
No	28 (87.5)
Previous pregnancies No. (%)	
None	24 (76)
1	2 (6)
2	2 (6)
3	2 (6)
4	2 (6)
BMI (Kg/m^2); Mean ±SD (range)	25 ± 4.5 (19 to 36.5)
Age (years); Mean ±SD (range)	28.8 ± 8 (18 to 42)
Height (cm); Mean ±SD	162 ± 7
Weight (kg); Mean ±SD	66 ±14

SD= standard deviation

Table 2 FXIII level (IU/dL) at different phases of menstrual cycle in 32 healthy women and in comparison to the menstrual phase.

	Week 0 (Menstrual)	**Week 1 (proliferative)**	**Week 2 (periovulatory)**	**Week 3 (Secretory)**	**Week 4 (premenstrual)**
Number of samples	32	32	32	31	22
Mean	114	119	114	120	122
SD	23	26	21	22	27
Lower limit	80	72	77	78	73
Upper limit	180	200	179	171	185
p-value*	-	0.058	0.997	0.036	0.036

* Paired t-test comparing mean FXIII activity of menstural phase to other phases of the cycle.

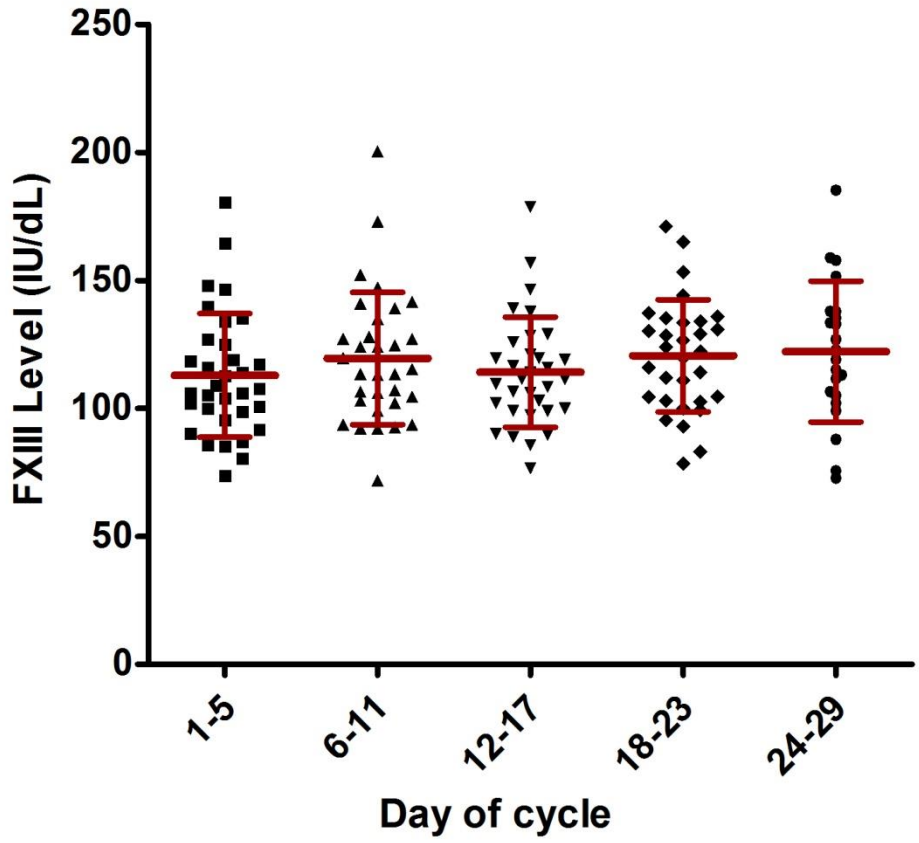

Figure 1 Distribution of FXIII activity during each phase of the menstrual cycle; central horizontal lines show mean FXIII activity and top and bottom lines show standard deviation of the mean.