# Impact of the Menstrual Cycle on Circulating Cell-free DNA

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**Abstract.** *Background: To determine serum levels of circulating cell-free DNA (cfDNA) throughout the stages of endometrial proliferation and apoptosis during the menstrual cycle. Materials and Methods: cfDNA was measured in 176 blood samples from 17 healthy volunteers taken at three different time points (menstruation, follicular and secretory phase). Additionally, blood samples from 20 newly diagnosed breast cancer patients were analysed. Quantitave real-time PCR was performed in order to quantify cfDNA fragments of 106 bp. Results: In healthy individuals, levels of cfDNA did not differ significantly during the menstrual cycle. In breast cancer patients, the median cfDNA level was significantly higher compared to healthy individuals, irrespective of the cycle phase ($p<0.001$). Conclusion: The female cycle does not influence cfDNA serum level measurements. Considering the diagnostic or prognostic value of cfDNA in cancer patients, different time points of blood sampling in premenopausal women seem to be negligible.*

Cell-free DNA (cfDNA) circulates freely in the plasma of normal, healthy individuals (1). Increased levels of circulating cfDNA have been detected in the plasma or serum of patients with various medical conditions like cancer, infectious disease, stroke, trauma, and myocardial infarction (2-5). In cancer patients, cfDNA concentrations are higher compared to those present in healthy individuals or in patients with non-malignant disease (6, 7). Therefore, increased cfDNA levels have potential clinical utility for screening, diagnosis, prognosis and monitoring treatment response in cancer patients (8-12).

Current evidence suggests that circulating DNA in the blood stream originates from necrotic and apoptotic cell death (1, 13, 14). In fertile women, the endometrium and mammary gland go through sequential waves of proliferation and apoptosis during the menstrual cycle. The regulation of this apoptosis is driven by hormonal and non-hormonal factors (15). The aim of our study was to determine changes in total cfDNA in healthy blood donors within the menstrual cycle using real-time PCR. To consider the impact of these changes, cfDNA levels in breast cancer patients were also determined and compared to those of the healthy blood donors.

## Materials and Methods

*Patients, sample collection and DNA isolation.* Female blood donors at the Institute of Experimental Haematology and Transfusion Medicine, University of Bonn had to meet the following inclusion criteria: generally healthy and free of medical condition, not receiving permanent medication, not taking oral contraceptives, no current desire to become pregnant, no history of a surgical intervention to treat irregular uterine bleeding. Blood was sampled at the beginning of the menstrual bleeding, at the anticipated middle of the cycle and at the second half of the cycle.

Breast cancer samples were collected at the Department of Obstetrics and Gynecology, University of Bonn. Blood from patients with suspected breast cancer was sampled before core needle biopsies were performed. If histology confirmed the diagnosis, patients were enrolled in the trial irrespective of age or stage of disease.

The study protocol was approved by the institutional review board. Written informed consent was provided by all patients.

*Measurement of cell-free DNA levels by quantitative real-time PCR (QPCR).* Blood samples were collected in serum S-Monovette Gel tubes (Sarstedt, Nürnbrecht, Germany) containing a clot activation additive and a barrier gel. Clotting of serum samples was allowed for at least 60 minutes before centrifugation (1,800 × g for 10 minutes) and supernatants were stored at –80˚C.

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*Key Words:* Circulating cell-free DNA, cfDNA, serum level, menstrual cycle, breast cancer.

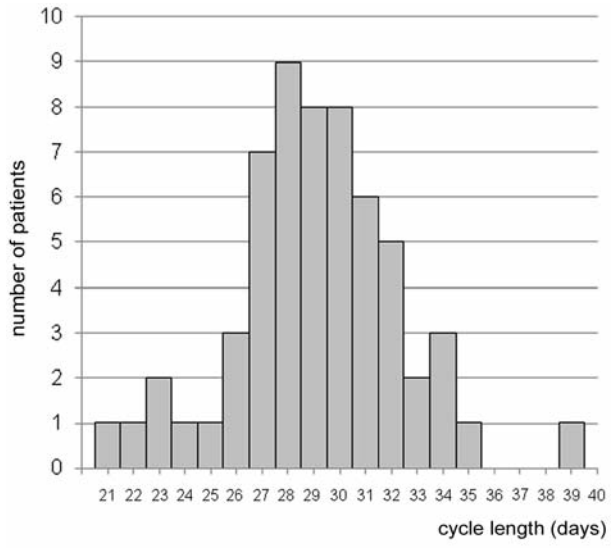


Figure 1. *Different cycle lengths in 59 monitored cycles of 17 women.*

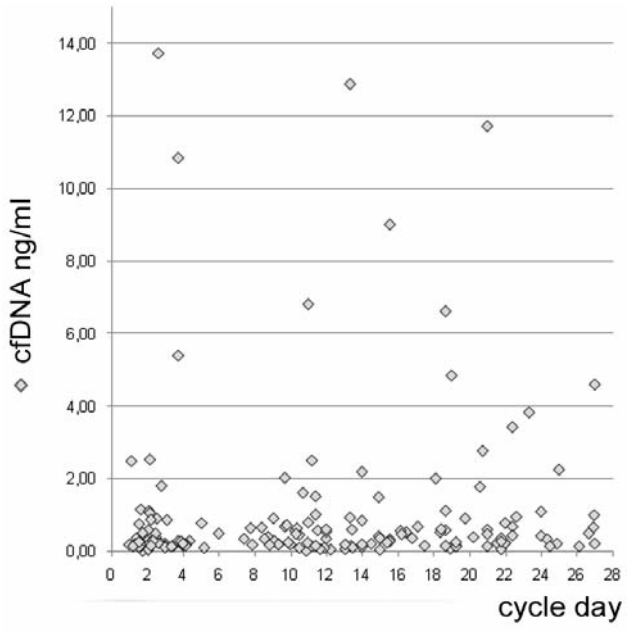


Figure 2. *cfDNA quantification by real-time PCR in serum of healthy young women after adjustment to a 28-day cycle. For clarity, one outlier value of 70.14 ng/ml is not shown.*

*DNA isolation and cf DNA quantification*. Cf DNA was isolated and quantified as described previously (16). In brief, cfDNA was isolated from 0.2 ml serum using the ChargeSwitch gDNA Kit according to the manufacturer's instructions. To quantify the amount of circulating DNA, a primer set amplifying a 106 bp fragment of the beta-actin gene (*ACTB*-106) was designed. The primer sequences were forward 5'-TCG-TGC-GTG-ACA-TTA-AGG-AG-3' and reverse 5'-GGC-AGC-TCG-TAG-CTC-TTC-TC-3'. QPCR was performed in triplicate on an ABI Prism® 7900HT. Each 10 μl reaction consisted of 1 × SYBR® GreenER™ quantitative PCR SuperMix, 0.2 μmol/l forward and reverse primers, and 1 μl DNA sample. QPCR was performed at 90˚C for 10 minutes, followed by 40 cycles at 95˚C for 15 seconds and 60˚C for 60 seconds. Melting curve analysis was performed to confirm PCR product specificity. Each run included serial dilutions of an external standard and water blanks.

*Statistical analyses*.

*Cycle adjustment*. The day of blood sampling was calculated and adjusted to a 28-day cycle, assuming that the luteal phase duration is constant (14 days). The day of blood sampling was adjusted using the formula: Women with a cycle length of more than 28 days:

$$\text{adjusted day of cycle} = \frac{14 \times \text{day of the cycle at the time of blood sampling}}{\text{length of the follicular phase (=cycle length} - 14 \text{ days)}}$$

For example, in a patient with an average period length of 32 days, the ovulation is estimated to occur around the 19th day, calculated from the last menstrual period (follicular phase length=18 days, and luteal phase length=14 days). If the date of blood sampling falls on the 16th day in this cycle, her day in the present cycle with respect to a 28-day cycle would be as follows: 14 × 16/18=12.44 days
Women with a cycle length less than 28 days:

$$\text{adjusted day of cycle} = \frac{\text{day of the cycle at the time of blood sampling} \times 28}{\text{cycle length}}$$

*Changes of cfDNA throughout the menstrual cycle*. After adjustment of the day of the cycle at the time of blood sampling, three different time points were compared: samples from the first day until the fourth day of menstrual bleeding were used as baseline samples. Samples from day 9 to 14 represented the follicular phase and samples from day 20 to 28 represented the secretory phase.

Differences in serum cfDNA levels between healthy individuals and breast cancer patients were analyzed using the Mann-Whitney-test. Serum levels of steroids were correlated with cfDNA levels using the Spearman test. Correlations between clinical parameters and cfDNA levels were performed using the Mann-Whitney and Kruskal-Wallis tests, as appropriate. Bonferroni adjustments for multiple tests were performed with a statistical significance at the 5% significance level, as appropriate. Statistical tests were performed using SPSS 12 (SPSS, Chicago, IL, USA).

## Results

A total of 176 blood samples were collected from 17 healthy blood donors. Three blood samples were collected during each of the 59 menstrual cycles, one during menstruation, one in the follicular phase and one in the secretory phase. Blood samples from 4 consecutive cycles were available from 11 participants (132 samples), 3 consecutive cycles from 4 participants (35 samples, one set was incomplete), and the blood samples from two cycles and from one cycle were available for the last two participants (9 samples).

Table I. *Serum levels of cfDNA and steroid hormone levels in healthy blood donors at different time points of the menstrual cycle (after cycle adjustment) and in breast cancer patients.*

| | cfDNA (ng/ml) | | | |
|---|---|---|---|---|
| | n | Mean±standard deviation | Range | Median |
| Menstruation (day 1 to 4) | 57 | 2.16±9.46 | 0.01-70.14 | 0.22 |
| Follicular phase (day 9 to 14) | 39 | 1.06±2.27 | 0.01-12.87 | 0.47 |
| Secretory phase (day 20 to 28) | 32 | 1.30±2.23 | 0.05-11.71 | 0.48 |
| Breast cancer patients | 20 | 6.63±6.76 | 1.16-29.31 | 5.41 |

| | | Estradiol (pg/ml) | | Progesterone (ng/ml) | | Testosterone (ng/ml) |
|---|---|---|---|---|---|---|
| Menstruation (day 1 to 4) | n=57 | 36.77±17.58 | n=56 | 0.52±0.97 | n=57 | 0.31±0.20 |
| Follicular phase (day 9 to 14) | n=39 | 115.06±80.07 | n=39 | 0.61±1.50 | n=38 | 0.43±0.16 |
| Secretory phase (day 20 to 28) | n=32 | 98.34±47.97 | n=33 | 6.24±4.69 | n=33 | 1.26±2.63 |

The mean age of the participants was 25 years (range 19 to 34 years; median 25 years) and the mean cycle length was 29 days (range 21 to 39 days; median 29 days) (Figure 1).

No significant changes in cfDNA levels were observed throughout the menstrual cycle. When compared to cfDNA levels during menstruation, the levels of cfDNA in the follicular phase was higher in 30 samples and lower in 28 samples. The levels of cfDNA in the secretory phase were increased in 30 samples and decreased in 28 samples, compared to the cfDNA levels in the follicular phase. Values of cfDNA levels after cycle adjustment are shown in Figure 2. Mean values of cfDNA and steroid hormone levels are shown in Table I. There were no significant differences between menstruation, follicular phase and secretory phase. Serum levels of cfDNA were not correlated with estradiol, progesterone or testosterone serum levels (Spearman rho correlation coefficient 0.17, 0.16 and 0.17, respectively).

The clinical characteristics of the 20 breast cancer patients are shown in Table II. Differences in the cfDNA levels in postmenopausal women and in premenopausal women were not statistically significant (mean 5.42 ng/ml *vs*. 3.73 ng/ml; $p$=0.211). Breast cancer patients had significantly higher cfDNA levels compared to healthy blood donors at each of the different time points during the menstrual cycle (Figure 3). The difference remained significant after Bonferroni adjustment for multiple tests.

cfDNA was investigated for its potential use as a tumor marker. Using the optimal cutoff-level of 1.18ng/ml in the breast cancer group, sensitivity for 7 premenopausal breast cancer patients was 100% and specificity was 84.3%. A represantative ROC curve is shown in Figure 4. The value of the AUC-ROC was 0.904 (95% confidence interval 0.845 to 0.964).

Table II. *Characteristics of the breast cancer patient group (n=20).*

| | |
|---|---|
| Age (median) (years) | 55 |
| Range | 40-75 |
| Menopause | |
| Premenopausal | 7 |
| Postmenopausal | 13 |
| Stage/tumor size | |
| pT1 | 9 |
| pT2 | 8 |
| pT3 | 2 |
| pT4 | 1 |
| Grading | |
| G1 | 2 |
| G2 | 7 |
| G3 | 11 |
| Nodal involvement | |
| Node-negative | 15 |
| Node-positive | 5 |
| Primary metastatic disease | 2 |
| Steroid receptor status | |
| Positive | 15 |
| Negative | 5 |
| Her2neu status | |
| negative | 17 |
| positive | 3 |

## Discussion

The results of this study suggest that levels of serum cfDNA remain unchanged throughout different phases of the menstrual cycle. Although the precise mechanism by which DNA is released into the bloodstream remains unclear, circulating nucleic acids present in peripheral blood appear to be originating from apoptosis and necrosis (13). One of the characteristic features of the secretory phase, as opposed

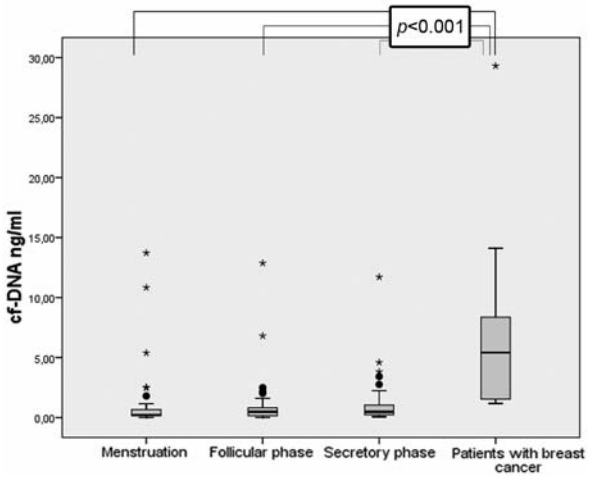


Figure 3. *Box plots showing the concentrations of cfDNA in the serum of the 4 different groups. The upper and lower limits of the boxes indicate the 75th and 25th percentile, the upper and lower horizontal bars indicate the 90th and 10th percentile, the lines inside the boxes indicates the median. For clarity, one outlier value of 70.14 ng/ml is not shown.*

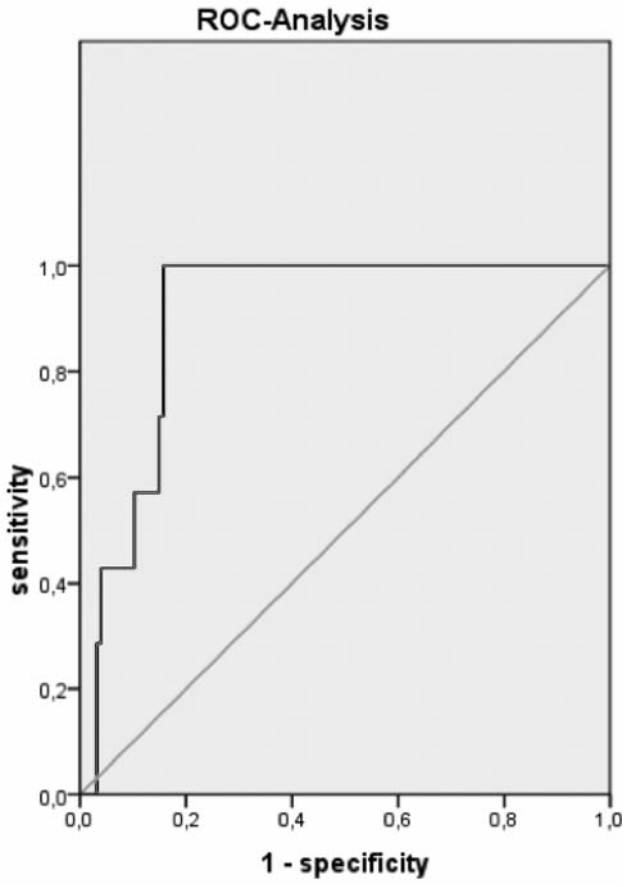


Figure 4. *Receiver operating characteristics (ROC) curve analysis of serum cfDNA for the predicition of malignancy (premenopausal women). Area under the ROC curve=0.904.*

to the proliferative phase, is a progressive increase in the number of apoptotic cells within endometrial glands, with menstruation leading to a desquamation of the layer lining the uterine cavity (17). Therefore, cfDNA levels may undergo changes throughout the menstrual cycle. Factors that could influence the levels of cfDNA have been evaluated in several studies and have shown that the cfDNA levels are not related to gender, age or race (18-21). To our knowledge, cfDNA levels throughout cyclic changes in female patients have not been evaluated so far.

Several reports focus on the potential role of the quantification of cfDNA serum or plasma levels in distinguishing between patients with cancer and healthy individuals. Ellinger et al showed that the levels of cf DNA were increased in patients with various human cancer entities including lung (22), ovarian (1), colon (23), bladder (7), testicular (23), and prostate cancer (1). Similar results have been observed in breast cancer patients (7, 24).

There is a wide range in the reported total concentrations of plasma and serum cfDNA and standardized methods for collecting and processing samples are not yet established (25). In order to establish reference values for cancer patients an additional analysis was performed in a cohort of patients with breast cancer following the same procedures. In agreement with previous findings (7, 23), our results suggest that the cfDNA levels of breast cancer patients were significantly higher compared to healthy donors. However, due to the differences in the patient characteristics, these results should be interpreted with caution. The healthy blood donors were younger compared to the breast cancer group which also included postmenopausal women. A matched pair analysis of the blood samples with regard to age and cycle day in both groups would be more appropriate.

Quantification of cfDNA may serve as a new diagnostic marker especially in cancers where conventional tumor markers cannot provide conclusive results. Furthermore, it may be used as a complementary, noninvasive assay to follow-up patients and high-risk individuals or to evaluate response to chemotherapy or radiotherapy (6, 10, 22).

Serum has approximately 6-fold higher cfDNA level than plasma and for a long time it was assumed that higher levels were due to cell lysis during clotting (14). However, a recent study showed that higher levels of cfDNA in serum are not caused by extraneous contamination (26). Even when blood processing, such as centrifugation, is delayed up to 6 hours after blood withdrawal, cfDNA levels in serum and especially in plasma do not change significantly (27). Although real-time PCR is now the gold standard for analyzing cfDNA, the use of different primer sets as well as the analysis of genomic

(10, 28, 29), retroviral (30, 31) or mitochondrial DNA (32, 33) makes comparison of the different studies difficult. Consequently, standardization and prospective, multicenter studies are necessary before cfDNA analysis can be implemented in routine clinical investigations.

In conclusion, cfDNA may be a promising noninvasive biomarker with diagnostic and prognostic capacities. The menstrual cycle of females is not a factor that influences the levels of serum cfDNA. In female cancer patients therefore, different phases within the menstrual cycle can be disregarded when assessing cfDNA.

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