



## Short communication

Assessment of diurnal changes and confounding factors that affect circulating cell death biomarker levels: A short communication<sup>☆,☆☆</sup>

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## ABSTRACT

There is increasing use of circulating cell death biomarkers in patients and clinical trials. Knowledge of the potential noise and confounders in assays are vital for biomarker interpretation. The daily and diurnal variability and effect of menstruation and exercise on nucleosomal DNA (nDNA), total cytokeratin 18 (tK18) and apoptotic specific cytokeratin 18 (ck18) were assessed in 3 cohorts of healthy volunteers; 12 pre-menopausal women to establish the effect of menstruation, 12 men to perform exercise and 12 post-menopausal women. All 36 subjects were evaluated to establish daily and diurnal variability. Estimates of variability were derived in a linear mixed effects model and presented as the back transformed coefficient of variation (%CV).

Minimal variation was seen in ck18 (11%CV) and tK18 (11%CV) but higher variability was seen in nDNA (85%CV). K18 results appeared stable throughout the day but a possible peak in nDNA was seen at 15:00. Menstruation had minimal effects but exercise led to immediate short-lived elevations in cell death biomarkers.

There is no evidence of significant daily variability in K18 assays. We recommend subjects should not exercise for 6 h before blood sampling.

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## 1. Introduction

There is increasing evidence of the utility of circulating cell death biomarkers in oncology, immunology, toxicology, sepsis, cardiology and hepatology [1–4]. Recent research has focussed on measurement of circulating nucleosomal DNA (nDNA) and cytokeratin (K) 18 fragments [5–7].

During apoptosis DNA is cleaved into nucleosomes, and necrosis also results in elevated nDNA due to secondary apoptosis of macrophages [8]. Circulating nDNA levels are higher in

auto-immune disease and cancer suggesting increased production, decreased clearance or both [4].

Cytokeratins are components of the epithelial cytoskeleton that are cleaved during apoptosis. The M30 ELISA detects an apoptotic specific K18 neo-epitope (ck18) whilst a companion ELISA (M65) detects total soluble K18 (tK18); including apoptotic fragments and full-length K18 released during necrosis [7].

Knowledge of the assay 'noise' (the technical variation, biological variability and potential confounders) is vital in biomarker interpretation [9,10]. The use of cell death biomarkers without this knowledge could lead to potentially misleading results, severely limiting the utility of these promising assays for clinical decision making. As cell death biomarkers change rapidly following some types of therapy [11,12], it is important to distinguish drug induced change from day to day (daily) and hour to hour (diurnal) variation. Exercise and menstruation could also potentially affect apoptotic biomarkers [13–15]. Therefore this study set out to determine the diurnal and daily variation and the effects of the menstruation or exercise on serum ck18, tK18 and nDNA in healthy subjects, to help assess their suitability for routine clinical use.

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## 2. Materials and methods

Three cohorts of healthy volunteers were enrolled; 12 pre-menopausal women to establish the effect of menstruation, 12 post-menopausal women to act as controls for this analysis and 12 men to establish the effect of moderate exercise. All 36 subjects were evaluated to establish daily and diurnal variability.

### 2.1. Study inclusion and exclusion criteria

Subjects were selected from the AstraZeneca Healthy Volunteer Panel. Obese subjects (BMI > 32 for women; > 35 for men), subjects with cancer, auto-immune disease, hepatitis and human immunodeficiency virus were excluded due to elevated cell death biomarkers in these conditions [3,4,6]. Women taking hormone replacement therapy were also excluded. Male subjects were excluded if considered by the screening physician to be unable to perform a period of moderate physical exercise. Menopausal status was checked in the post menopausal women by assessment of FSH, LH, oestradiol and progesterone at screening.

### 2.2. Conduct of study

The protocol received approval from Yorkshire Independent Research Ethics Committee. Following written informed consent subjects spent two 24 h periods 4 weeks apart at the Clinical Pharmacology Unit (CPU) at AstraZeneca, Alderley Edge where serum was collected every 6 h starting at 9 AM to give broad coverage throughout a 24 h period, whilst minimising blood draws. All subjects attended the CPU twice weekly during the 4 weeks to establish the daily variation, and to assess changes in the pre-menopausal women during the follicular and luteal phases of the menstrual cycle. In addition, during both residential stays, men performed a period of exercise consisting of cycling between 60 and 70 revolutions per minute (rpm) on an ergometer (ERG 911 S) starting at 25 W. Every 3 min the resistance was increased by 25 W. The exercise ended if the subject became distressed, failed to maintain 60 rpm for 20 s or heart rate exceeded 85% of age predicted maximum rate (226-subject age beats per minute). Additional blood samples were taken immediately before and after exercise.

Pre-menopausal women donated samples daily during menstruation. Menstruation is the time where maximal apoptosis is seen in the breast and uterus, so any effect on cell death biomarkers should be most obvious. Menstruation also acted as a cue for subjects to attend for sampling at a time when their reproductive cycles were approximately synchronised. This was confirmed by assessment of FSH, LH, oestradiol and progesterone at all sampling time-points.

### 2.3. Assays

Blood was collected in tubes containing a silica clot activator and centrifuged at 2000 g for 10 min to obtain serum. The sample collection, processing and storage were as previously described [1]. The M30 and M65 ELISAs are commercially available assays operated according to the manufacturer's instructions (Peviva, Sweden). In brief 25 µL of sample is analysed in a sandwich ELISA based around a mouse monoclonal antibody to K18 and a horseradish peroxidase (HRP) conjugated secondary antibody (either the M30 antibody against K18asp396 neo-epitope (cK18) or a second monoclonal antibody to K18 (tK18)). A 7 step calibration curve was generated using a recombinant protein fragment and results expressed as U/L. Changes in levels were expressed as the % change compared to baseline as in the previous literature [1,2].

nDNA was assessed using the Cell Death Detection ELISaplus (Roche, Switzerland) according to manufacturer's instructions. In brief 20 µL of sample is analysed in a sandwich ELISA based around an anti-histone antibody conjugated to biotin and an anti-DNA antibody conjugated to HRP, captured on a streptavidin coated plate. Results are reported as optical density measured at 405 nm using Revelation Software on a Dynex MRXII plate reader. Changes in levels were expressed as absolute change in optical density as in the previous literature [1,5]. Full blood count, urea and electrolytes and liver function tests were assessed at all time-points using routine methods by the Safety Assessment Department at AstraZeneca, Macclesfield, UK.

### 2.4. Statistical considerations

The objectives were to derive estimates of variability; therefore no formal power calculations were done. However, cohorts of 12 subjects were considered sufficient for judgements on biomarker variability [16]. Similar numbers were used to judge the noise of these biomarkers in patients with cancer [9]. Variables were log-normally distributed and so were logarithmically transformed. In order to derive estimates of the variability between subjects and from hour to hour and day to day within a subject a linear mixed effects model with random effects for subject and fixed effects for time and day was constructed. The impact of menstruation and exercise was assessed by secondary models with that parameter as a fixed effect. The estimated impact of potential confounding factors on the biomarkers is presented as the possible percentage change in the assay (the back transformed coefficient of variation derived in the model with 95% confidence intervals).

## 3. Results

The demographics of the subjects are shown in Table 1. Two subjects had no evaluable results due to new diagnoses of hypertension (male) and breast cancer (post-menopausal female) during the study.

### 3.1. Levels of circulating cell death biomarkers in subjects.

Levels of cK18 (median 106 U/L (range 74–1000 U/L; inter-quartile range (IQR) 92–139 U/L)) and tK18 (median 281 U/L (range 68–1247 U/L; IQR 229–346 U/L)) were similar to those reported in the literature (Supplemental Tables S1, S2). As nDNA is a quasi-quantitative assay [10], it is difficult to compare results across laboratories; however, results were similar to our previous publications with a median of 0.2 (range 0–1.9 (IQR 0.2–0.6)) [1,2].

### 3.2. Day to day variability

The primary objective was to estimate variability components within and between subjects and days. The main source of variability in cK18 and tK18 levels was accounted for by differences

**Table 1**  
Demographics of subjects (age and body mass index (BMI) expressed as median with range in brackets).

|                 | Age          | BMI              | Race                |
|-----------------|--------------|------------------|---------------------|
| Male            | 49.5 (34–60) | 26.3 (21.4–29.7) | 12 Caucasian (100%) |
| Female          |              |                  |                     |
| Post-menopausal | 60.5 (45–69) | 26.5 (21.2–29.3) | 12 Caucasian (100%) |
| Pre-menopausal  | 44.5 (25–50) | 26.3 (20.8–29.4) | 12 Caucasian (100%) |

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