

# Variations along the 24-hour cycle of circulating osteoprotegerin and soluble RANKL: a rhythmometric analysis

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## Abstract

**Summary** The variability of serum osteoprotegerin (OPG) and soluble RANKL (sRANKL) along the 24-h cycle was assessed in 20 healthy women. No rhythmic variations of serum OPG, sRANKL or sRANKL/OPG ratio were detected as a group phenomenon. Timing of sampling is unlikely to influence the results of measurements of circulating OPG and sRANKL.

**Introduction** Physiological bone turnover shows diurnal variations. The aim of the study was to assess variability of OPG and sRANKL serum levels along the 24-h cycle.

**Methods** Blood was collected from 20 healthy women (median age 31 years, range 25–65 years) at 4-h intervals between 08:00 and 24:00 and at 2-h intervals between 24:00 and 08:00. Serum albumin, cortisol, osteocalcin (OC), C-

terminal telopeptide of type I collagen (CTX), OPG and total sRANKL were measured. Temporal variations were assessed by the COSINOR model.

**Results** Circadian rhythms of cortisol and albumin documented a normal synchronization within the circadian structure. Serum OC and CTX showed rhythmic variations, peaking at night-time. Rhythmic variations of serum OPG, sRANKL and sRANKL/OPG ratio were not detected as a group phenomenon. On an individual basis, rhythmic changes were detected in ten patients for OPG and eight patients for sRANKL, with very small amplitudes and heterogeneous acrophases.

**Conclusions** The absence of consistent rhythmic variations of circulating OPG and sRANKL levels may reflect the absence of rhythmic variations of their expression in the bone microenvironment. Were this the case, the nocturnal rise of bone resorption should be accounted for by different, not RANKL/OPG-mediated factors. Since circulating OPG and sRANKL may derive from sources other than bone, rhythmicity could be masked by non-rhythmic or non-synchronized rhythmic expression in these sources. Timing of sampling is unlikely to influence the results of measurements of circulating OPG and sRANKL.

**Keywords** Bone turnover markers · CTX · Osteocalcin · Osteoprotegerin · RANKL

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## Introduction

The signaling system consisting of RANK ligand (RANKL), its receptor RANK and its decoy receptor osteoprotegerin (OPG) has a key role in the pathophysiology of the bone

microenvironment [1, 2]. Given the availability of specific antibodies, interest has grown in the measurement of circulating OPG and soluble RANKL (sRANKL), which could give insight into the regulatory mechanisms of the system and provide novel markers in a number of diseases [3, 4].

Physiological bone turnover increases at night-time. Putative synchronizing hormones include parathyroid hormone, melatonin, glucocorticoids and food-dependent peptides, such as glucagon-like peptide 2 [5–12]. Expression of clock genes in osteoblasts has recently been demonstrated, and is consistent with the concept of a bone peripheral clock [13, 14].

Rhythmic changes of RANKL and/or OPG expression in the bone microenvironment could contribute to variation of bone resorption and be of relevance for timing of administration of new anti-RANKL agents. With regard to serum levels of sRANKL and OPG, variability along the 24-h cycle should be taken into account when they are investigated as markers of skeletal or vascular diseases. Only a few studies have addressed the issue of possible rhythmicity of circulating OPG [15–17], whereas there are no data on serum sRANKL. The aim of the study was to assess the variability of OPG and sRANKL serum levels along the 24-h cycle in healthy women.

## Subjects, materials and methods

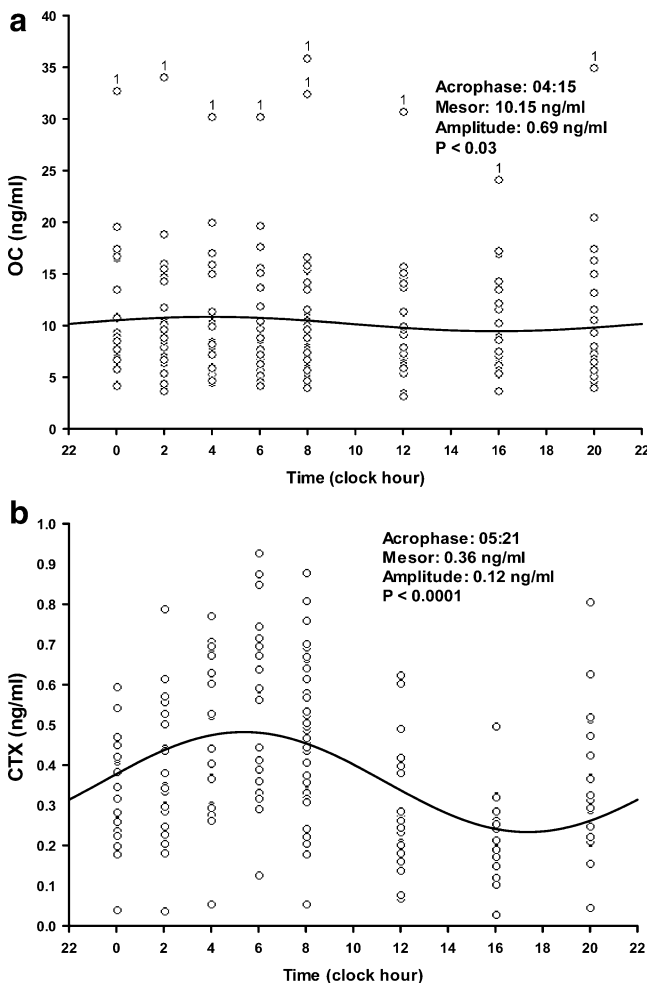
Twenty healthy women volunteered to participate in this study (median age 31 years, range 25–65 years); 17 women were pre-menopausal. The subjects studied were members of the medical staff or their relatives, were non-smokers and were not under the influence of bone-active medications. Pre-menopausal women were eumenorrhoeic. General laboratory screening gave no indication of specific organ dysfunction. Bone mineral density was not assessed, since subjects had no specific risk factors for osteoporosis. The study was performed in accordance with guidelines on good clinical practice and the Declaration of Helsinki, and subsequent relevant amendments, and was approved by the local ethics committee. All subjects gave written informed consent.

Blood samples were collected at 4-h intervals between 08:00 and 24:00, and at 2-h intervals between 24:00 and 08:00. All subjects were given a standard hospital diet with three equicaloric meals at approximately 07:00, 13:00 and 19:00. They were freely ambulant but avoided moderate to heavy exercise throughout the 24-h sampling period; they were not hospitalized during the day and usually returned home between one urine and blood collection and the next one. They were hospitalized at night. They laid down to sleep at 23:00 and awoke at 07:00. Routine biochemical analysis was performed on

fresh samples; aliquots were stored at  $-20^{\circ}\text{C}$  for up to 6 months before being assayed. Serum osteocalcin (OC) was measured by radioimmunoassay using a monoclonal antibody that binds intact OC and fragments 1–43, 20–49 and 20–44 (Osteocalcina Myria, Technogenetics, Milan, Italy). Serum C-terminal telopeptide of type I collagen (CTX) was measured by enzyme-linked immunosorbent assay (ELISA) (Nordic Bioscience Diagnostic, Herlev, Denmark). ELISAs for both OPG and sRANKL were performed directly by Immundiagnostik AG (Bensheim, Germany). OPG was measured by a pair of specific anti-human OPG antibodies (capture monoclonal antibody and detection biotinylated goat polyclonal antibody). For sRANKL, samples were first added an OPG solution and then put into microplate wells coated with a goat polyclonal anti-human OPG antibody. Subsequently, a biotinylated monoclonal anti-human sRANKL antibody was added. All the samples were shipped at the same time and were assayed in duplicate with reagents from the same lot. Measurement was repeated for duplicate coefficients of variation (CVs)  $>20\%$  and values out of the calibration curve. Measuring ranges, minimum detectable concentrations and intra- and inter-assay coefficients of variation were as follows: OC 2.2–64.3 ng/ml, 0.3 ng/ml, 3.5% and 5.6%; CTX 0.156–2.562 ng/ml, 0.01 ng/ml, 2.5% and 9%; OPG 3.13–25 pmol/l, 0.14 pmol/l,  $<10\%$  and  $<10\%$ ; sRANKL 2.2–60 pmol/l,  $<0.5$  pmol/l,  $<5\%$  and  $<10\%$ .

For each individual data series, the coefficient of variation was calculated and the single COSINOR analysis was performed. A predetermined period ( $\tau=24$  h for circadian, 12 h for circasemidian) was chosen to fit a cosine curve to the data series. Correlation coefficient constant and mean square error determine the goodness-of-fit of the cosine curve [18]. The analysis yields three main parameters: midline estimating statistics of the rhythm (MESOR) of the fitted cosine curve, acrophase (occurrence of the estimated peak time, which may not be coincident with the time at which maximum concentration was observed), and amplitude (half the difference between the highest and the lowest points of the fitted cosine curve). Data obtained from single COSINOR analysis were further analyzed by the population-mean COSINOR procedure so that statistically validated parameters for the group could be obtained. Significance was determined by the zero-amplitude (no rhythm) test. Data were analyzed as absolute values for the considered analyte and then as a percent value of each relevant MESOR, so that inter-individual variability was minimized.

Correlations among variables were assessed by Spearman R analysis.  $P<0.05$  was considered as significant. All statistics tests were performed with SPSS for Windows software, version 12.0.2, and STATISTICA for Windows, version 6.1.



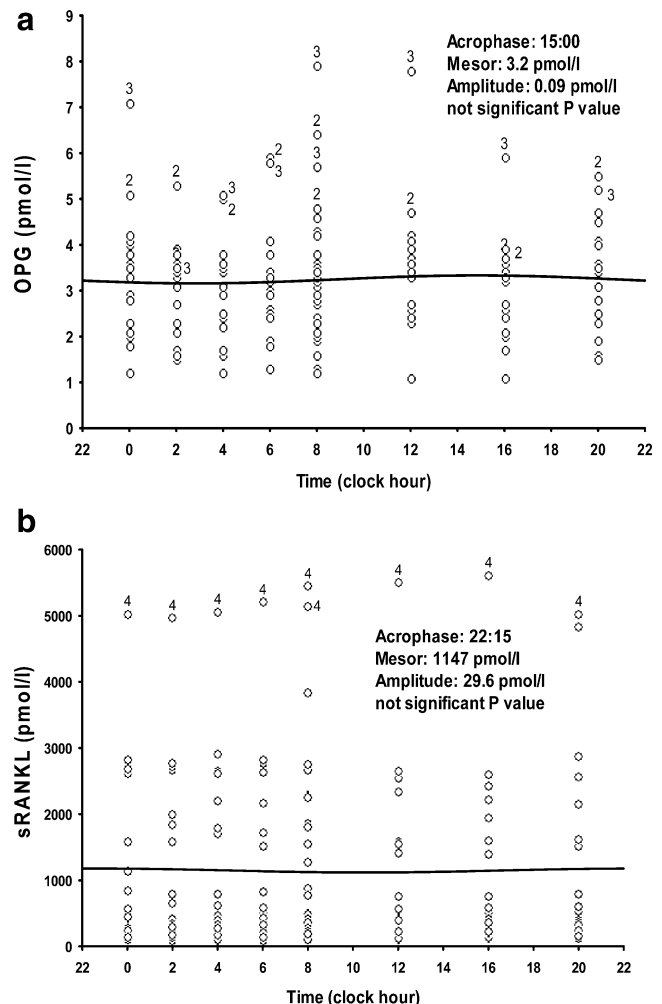
**Fig. 1** Variations in serum OC (**a**) and CTX (**b**) along a 24-h period. Circles represent individual values at specific time points; curves are cosine curves extrapolated by a population-mean COSINOR procedure. The highest values of serum OC all belonged to the same subject (labeled 1)

## Results

Circadian rhythms of serum cortisol (MESOR 116.2 nmol/l, amplitude 60.9 nmol/l, acrophase 09:30,  $P < 0.0001$ ) and albumin (MESOR 4,103 mg/dl, amplitude 214 mg/dl, acrophase 15:30,  $P = 0.04$ ) were well apparent. Serum OC and CTX showed significant rhythmic variations, with acrophases at night-time (Fig. 1).

Serum OPG and sRANKL showed wide inter-individual variability (serum concentrations at 08:00, for OPG median 3.3 pmol/l, range 1.2–7.9 pmol/l; for sRANKL 478 pmol/l, 95–5,444 pmol/l) and intra-individual variations (individual coefficient of variations, for OPG 14%, 6–30%; for sRANKL 16%, 5–51%). No correlation between serum OPG and sRANKL was found with age. Rhythmic variations of serum OPG, sRANKL and sRANKL/OPG

ratio could not be detected with statistical significance as a group phenomenon (Fig. 2). On an individual basis, rhythmic changes, with very small amplitudes (OPG 10.8%, 5.5–22.2%; sRANKL 11.8%, 5.6–22.2%) and heterogeneous acrophases, were detected in ten patients for OPG and in eight patients for sRANKL. No difference in age or menopausal status was found between subjects with and those without rhythmic changes. Similar results were obtained when data were adjusted for inter-individual variability and computed as percent changes from MESOR, and when the three post-menopausal women were excluded from analysis. No correlation was found between serum OPG, sRANKL levels and sRANKL/OPG ratio, on the one hand, and serum CTX or OC, on the other.



**Fig. 2** Variations of serum OPG (**a**) and total sRANKL (**b**) along a 24-h period. Circles represent individual values at specific time points; curves are cosine curves extrapolated by a population-mean COSINOR procedure. The highest values of serum OPG and sRANKL, respectively, belonged to three different subjects (labeled 2, 3 and 4)

## Discussion

Pre-analytical and analytical factors may contribute to the striking differences of OPG values reported in different studies [4, 19–21]. Possible variations of circulating OPG and sRANKL along the 24-h cycle have not been adequately investigated. In this work, rhythmometric data of serum cortisol and albumin served to assess synchronization within the circadian structure as it is held in chronobiological studies. Serum OC and CTX peaked at night, as previously reported [5–9]. Notably, OC circulates as both the intact molecule and a number of fragments, generated both by serum proteases in blood circulation and, to a lesser extent, by osteoclastic cysteine proteases during bone resorption [22]. Since different assays show different specificities for the various fragments [23], and our assay detects different fragments besides the intact molecule, we cannot exclude the fact that measured OC might somehow reflect bone resorption in addition to formation. While rhythmicity of serum OC has been linked to cortisol secretion [6], diurnal variation in bone resorption is mainly due to food intake through the release of food-dependent peptides, such as glucagon-like peptide 2, and is diminished during fasting [10–12].

As far as serum OPG and sRANKL are concerned, we did not find a significant rhythm, as a group phenomenon, for these two analytes. On an individual basis, rhythmic changes were detected in a subgroup of subjects, with small amplitudes and heterogeneous acrophases; such inconsistent changes are unlikely to be relevant physiologically or as a source of significant pre-analytical variability. Notably, both OPG and sRANKL showed wide inter-individual variability, and substantial intra-individual variations were observed in a small proportion of subjects. Available information on variation of circulating OPG along the 24-h cycle is scanty and contradictory. Joseph et al. [15] did find a significant rhythmicity for OPG in a group of four men and four women. In a group of six women and three men, Tarquini et al. [16] found a circasemidian, rather than circadian, variation in circulating OPG, with a quite small amplitude (<5%); we failed to confirm such a circasemidian rhythm in our population. Most recently, Ohta et al. [17] have studied 15 young female volunteers from whom blood was collected three times, at 6 h intervals. The authors did not find an appreciable diurnal variation. Differences in study design (gender, age, sampling sequence, assays and data processing) could explain discrepancies.

Our study is limited to a group of mainly pre-menopausal women, in different phases of the ovarian cycle; rhythmic changes may still occur in luteal vs follicular phase, in elderly women, or in men. Moreover, we did not specifically address the effects of food intake (the main determinant of diurnal variation of bone resorption) on the analytes measured.

Finally, we assessed total sRANKL, and we have no data on the free form of the molecule. RANKL is produced as a 45 kDa membrane-associated protein, as a 31 kDa soluble protein by proteolytic cleavage, or as a 39.5 kDa soluble protein following expression of the hRANKL3 isoform [24]. In murine models, the membrane form is more active than the soluble ones [24–26]. sRANKL is present in blood circulation both in the free form and as a complex with OPG, but the sensitivity of available assays is still insufficient to detect the free form of sRANKL in a significant proportion of healthy individuals [3, 4]. However, we cannot exclude rhythmic changes of free sRANKL levels, as a result of rhythmic variations of potential (presently unknown) sRANKL-binding molecules other than OPG.

A possible explanation for our results could be viewed in long plasma half-lives of the molecules. No data are available on sRANKL, and the only data on OPG plasma half-life come from a single animal study. In rats intravenously injected with human monomeric or homodimeric OPG, the elimination curves of the two forms in serum were well fitted to the two-compartment model, with  $\beta_{1/2}$  values of less than 30 min for both forms [27]. If these values roughly apply to humans, the absence of variation in OPG along the 24-h cycle is not accounted for by its half-life.

The absence of rhythmic variations could be due to substantially stable release of these molecules from the bone microenvironment into the bloodstream. Were this the case, one could suggest that the nocturnal rise in osteoclast activity is mediated by pathways independent of RANKL/OPG balance. Alternatively, since circulating OPG and sRANKL derive from several sources [1–4], rhythmic changes of gene expression and release of these products in the extracellular space could, indeed, occur in specialized cells such as those resident in bone. The circulating pool, however, could mask variations of tissue-specific isoforms, with either non-rhythmic or non-synchronized isoforms coming from other tissues. Indeed, OPG is expressed in both endothelial and vascular smooth muscle cells, and a number of Authors have recently suggested that serum OPG might be a marker of atherosclerotic disease, rather than bone metabolism [3, 4, 28].

Whatever be the explanation for our results, they are of relevance for studies dealing with circulating OPG and sRANKL, as timing of sampling is unlikely to influence the results of measurements.

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