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DOI
10.1016/j.bone.2018.10.002

Publication date
2019

Document Version
Final published version

Published in
Bone

License
Article 25fa Dutch Copyright Act

Bone 120 (2019) 61–69

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Full Length Article

The 24-hour serum profiles of bone markers in healthy older men and women

Evie van der Spoel[a,*], Nicole Oei[b,c], Ricardo Cachucho[a,d], Ferdinand Roelfsema[e], Jimmy F.P. Berbée[e], Gerard J. Blauw[a], Hanno Pijl[e], Natasha M. Appelman-Dijkstra[e], Diana van Heemst[a]

[a] Section Gerontology and Geriatrics, Department of Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands
[b] Department of Developmental Psychology (ADAPT-lab), Institute of Psychology, University of Amsterdam, Amsterdam, the Netherlands
[c] Amsterdam Brain and Cognition, University of Amsterdam, Amsterdam, the Netherlands
[d] Section Molecular Epidemiology, Department of Biomedical Data Sciences, Leiden University Medical Center, Leiden, the Netherlands
[e] Section Endocrinology, Department of Internal Medicine, Leiden University Medical Center, Leiden, the Netherlands

ARTICLE INFO

Keywords:
Bone markers
Circadian rhythm
24-hour profile
Cosinor analysis
Sex differences

ABSTRACT

The process of bone turnover displays variations over 24 h, with C-terminal cross-linked telopeptide of type 1 collagen (CTX) and osteocalcin exhibiting a nadir in the afternoon and a peak in the night. In contrast, N-terminal propeptide of type 1 procollagen (P1NP) did not display an apparent 24-hour rhythm. Other emerging novel biomarkers of bone, sclerostin and Dickkopf-related protein 1 (DKK1), are markers of osteocyte activity with limited data available regarding their 24-hour profiles. In this study, we aimed to extend available data on 24-hour profiles of CTX, osteocalcin, and P1NP and to assess the 24-hour profiles of sclerostin and DKK1 in healthy older men and women and to compare these between men and women. We measured these five bone markers in EDTA plasma collected every 4 h during 24 h in 37 healthy older men and women (range 52–76 years). Differences between time points were determined using repeated measures ANOVA and cosinor analyses were performed to determine circadian rhythmicity. The circadian rhythm of CTX was confirmed by the cosinor model, with women showing larger amplitude compared to men. Osteocalcin showed higher levels during nighttime compared to daytime in both men and women. For P1NP levels we observed a small but significant increase in the night in men. Sclerostin and DKK1 did not show a circadian rhythm, but sclerostin levels differed between time points. Because of the large intraindividual variation, DKK1 as measured in this study cannot be considered a reliable marker for diagnostic or research purposes. In conclusion, when measuring CTX, osteocalcin, P1NP, or sclerostin either in clinical practice or in a research setting, one should consider the 24-hour profiles of these bone markers.

1. Introduction

The process of bone turnover displays circadian variations, which are most pronounced for bone resorption as measured with C-terminal cross-linked telopeptide of type 1 collagen (CTX). The circadian rhythm of this key biomarker of bone resorption has been described extensively in literature. CTX is released into the blood circulation when bone collagen is broken down by cleavage of the cross-linked type I collagen by cathepsin K, which is expressed by osteoclasts [1,2]. Serum CTX demonstrates a circadian rhythm with its nadir in the late afternoon and its peak in the second half of the night in healthy individuals [3–6]. The circadian rhythms of the two key biomarkers of bone formation, osteocalcin and N-terminal propeptide of type 1 procollagen (P1NP), have been investigated in several studies. Osteocalcin is produced by osteoblasts at sites of new bone formation. Most of the newly synthesized osteocalcin is incorporated into the bone matrix, while a small fraction is released into the circulation [7]. Its circadian rhythm has first been described in 1985 and similarly as CTX, serum osteocalcin has its nadir in the afternoon and its peak at night [8]. P1NP is enzymatically cleaved off from type 1 procollagen when bone is formed by osteoblasts [9]. Evidence regarding the 24-hour rhythm of P1NP is yet inconclusive; two studies showed that P1NP does not exhibit a discernible 24-hour rhythm while another study did identify a 24-hour rhythm in P1NP with somewhat higher levels in the night [6,10,11].

* Corresponding author at: Section Gerontology and Geriatrics, Department of Internal Medicine, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, the Netherlands. Tel.: +31 71 5266640.
E-mail address: e.van_der_spoel@lumc.nl (E. van der Spoel).

https://doi.org/10.1016/j.bone.2018.10.002
Received 1 June 2018; Received in revised form 28 September 2018; Accepted 3 October 2018
Available online 04 October 2018
8756-3282/

Other emerging novel biomarkers of bone are markers of osteocyte activity, such as sclerostin and Dickkopf-related protein 1 (DKK1). Osteocytes are endocrine mechanosensory cells that, besides regulating phosphate and calcium homeostasis, control bone remodelling by regulating both osteoblasts and osteoclasts via cell-to-cell communication and secreted factors [12]. Sclerostin and DKK1 are negative regulators of bone formation that inhibit osteoblast activity via blocking the Wnt signaling pathway by antagonizing the Wnt/lipoprotein receptor-related protein 5 [13,14]. Since bone formation and bone resorption demonstrate circadian rhythmicity and osteocytes regulate these processes, osteocyte activity will potentially also display a rhythm. Osteocytes regulate bone turnover via secreted regulatory proteins and therefore we hypothesized that these markers demonstrate a circadian rhythm similar to that of other bone markers. Only limited data are however available regarding the circadian rhythm of these emerging markers. No discernible 24-hour rhythm was identified for sclerostin in men [6] while DKK1 levels have not yet been measured during 24 h.

Several factors, including age, sex, and postmenopausal status, have been investigated as potential modulators of the levels and circadian rhythm of bone turnover markers. Serum levels of CTX in both men and women decreased with age until 40–50 years followed by a gradual increase in men and a sharp increase in women after menopause. CTX levels were lower in premenopausal women than in men, but higher in postmenopausal women [15–17]. In contrast to the serum levels, the circadian rhythm of serum CTX was not influenced by age, sex, or menopausal status [18]. For osteocalcin, levels decreased with age in both men and women, but increased slightly after age 65 for men and were increased after menopause in women. Osteocalcin levels are higher in young men compared to premenopausal women, but lower in older men compared to postmenopausal women [17,19,20]. Similar to CTX, the circadian rhythm of osteocalcin was not influenced by age, sex, or menopausal status [21–24]. P1NP levels decreased after 20 years of age in men, but remained relatively stable afterwards. In women, levels of P1NP decreased until menopause and then started to increase. P1NP levels were lowest in premenopausal women, compared to men, and highest in postmenopausal women [15,16]. No difference in the circadian rhythm of P1NP was found between younger and older healthy men [6]. Whether the circadian rhythm of P1NP is also independent of sex is not known yet. Sclerostin serum levels correlated positively with age in men, premenopausal, and postmenopausal women, with postmenopausal women having higher levels than premenopausal women [25–29]. Sclerostin levels were higher in men than in women [26,28,30]. The circadian rhythm of sclerostin has only been examined in men, so the difference in circadian rhythm between men and women is not known. Both in younger and older men, no circadian rhythm of sclerostin has been observed [6]. For DKK1, levels were higher in female than in male geriatric patients [30]. DKK1 levels were somewhat higher in older individuals than in younger individuals [31]. Since the circadian rhythm of DKK1 has not been investigated yet, no factors influencing the circadian rhythm have been described.

In this paper, we investigated for the first time the 24-hour profiles of CTX, osteocalcin, P1NP, sclerostin, and DKK1 in the same participants, comprising 20 healthy older men and 17 healthy older women. Our objectives were to extend available data on 24-hour profiles of CTX, osteocalcin, and P1NP and to assess the presence or absence of a circadian rhythm for sclerostin and DKK1 in healthy older men and women and to compare the 24-hour profiles of these five bone markers between men and women.

2. Methods

2.1. Study participants

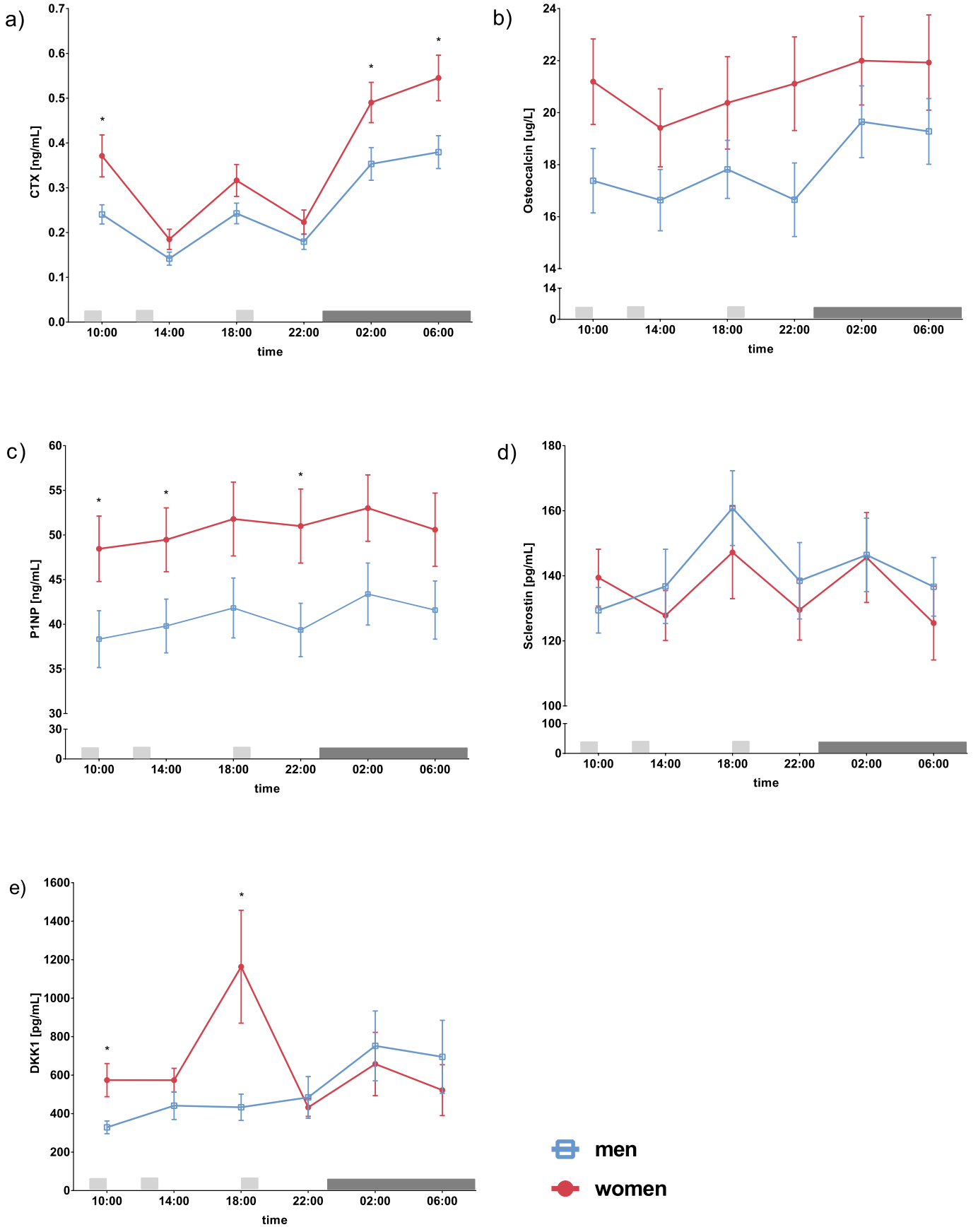
In the Switchbox Leiden Study we collected 24-hour blood samples from 38 healthy older (range 52–76 years) individuals comprising 20 men and 18 women with the aim to assess 24-hour fluctuations in circulating hormones and biomarkers, between June 2012 and July 2013. For the present analysis, one woman was excluded since she had self-reported osteoporosis and was using alendronic acid during the study. Consequently, 37 participants were included in the analyses. Participants of the Switchbox Leiden study were recruited from the family-based Leiden Longevity Study in which 421 long-lived families are included, comprising at least two nonagenarian siblings fulfilling the age criteria (men ≥ 89 years and women ≥ 91 years) without selection on health or demographics together with their offspring and the offspring's partners [32]. Participants of the Switchbox Leiden study had a stable body mass index (BMI) between 20 and 34 kg/m^2 and although not formally asked, based on the age range, the majority of women was most likely postmenopausal. Exclusion criteria were among others, having chronic renal, hepatic or endocrine disease, or using any hormone medication (including oral, nasal, and inhalation corticosteroids). Detailed information on in- and exclusion criteria can be found elsewhere [33]. The Switchbox Leiden Study was approved by the Medical Ethical Committee of the Leiden University Medical Centre and performed according to the Helsinki declaration. All participants gave written informed consent for participation.

2.2. Study protocol

Participants were admitted to the research centre during 24 h. A catheter was placed in a vein of the forearm of the non-dominant hand of the participant. Blood sampling started around 09:00 h and blood was collected every 10 min. For the current study we used EDTA plasma samples collected with four hour intervals (around 10:00 h, 14:00 h, 18:00 h, 22:00 h, 02:00 h, 06:00 h). The participants received standardized feeding at three fixed time intervals during the day (between 09:00 h–10:00 h, 12:00 h–13:00 h and 18:00 h–19:00 h), each consisting of 600 kcal Nutridrink (Nutricia Advanced Medical Nutrition Zoetermeer, The Netherlands). Participants were not allowed to sleep during the day, and except for lavatory use, no physical activity was allowed during the study period. Lights were switched off for approximately 9 h (circa between 23:00 h to 08:00 h). Full details on the 24-hour blood sampling procedure have been described previously [34]. Anthropometric measurements, comprising BMI, height, fat mass, lean body mass, and waist circumference, were performed in the research centre using a scale, measuring tape, and a Bioelectrical Impedance Analysis meter at a fixed frequency of 50 kHz (Bodystat® 1500 Ltd., Isle of Man, British Isles). Data on usual bedtime and getting up time during the past month were obtained using the Pittsburgh Sleep Quality Index questionnaire [35].

2.3. Biochemical analysis

β-isomerized C-terminal cross-linked telopeptide of type 1 collagen (CTX), N-terminal propeptide of type 1 procollagen (P1NP), osteocalcin, sclerostin, and Dickkopf-related protein 1 (DKK1) were all measured in EDTA plasma samples collected every 4 h. Especially for DKK1 it is recommended to perform measurements in plasma instead of serum, as DKK1 is present in blood platelets. During the clotting process, platelets release their content, including DKK1, resulting in significantly higher DKK1 levels in serum compared to plasma [36]. Measurements of CTX, P1NP, and osteocalcin were performed with Cobas® kits and the fully automated E170 module of Modular Analytics from Roche Diagnostics (Almere, The Netherlands) at the Department of Clinical Chemistry and Laboratory Medicine of the Leiden University Medical Centre in The Netherlands, which is accredited according to the National Coordination Committee for Quality Assurance for Health Care Laboratories in The Netherlands. CTX had an inter-assay coefficient of variation (CV) of 2.59% at 0.34 ng/mL and 2.16% at 0.782 ng/mL, osteocalcin of 0.91% at 20.1 μg/L and 1.33% at 93.0 μg/L, and P1NP had an inter-assay CV of 1.61% at 37.58 ng/mL and 1.83% at 210.77 ng/mL. Sclerostin was measured with a 96-well multi-array



(caption on next page)

Fig. 1. Bone markers over 24 h.
The mean (SE) of a) C-terminal cross-linked telopeptide of type 1 collagen (CTX), b) osteocalcin, c) N-terminal propeptide of type 1 procollagen (P1NP), d) sclerostin, and e) Dickkopf-related protein 1 (DKK1) are presented every 4 h starting at 10:00 during 24 h for men (blue, light) and women (pink, dark). Stars (*) represent significant differences between men and women. Light bars represent meal times and dark bars represent the period when the lights were switched off. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

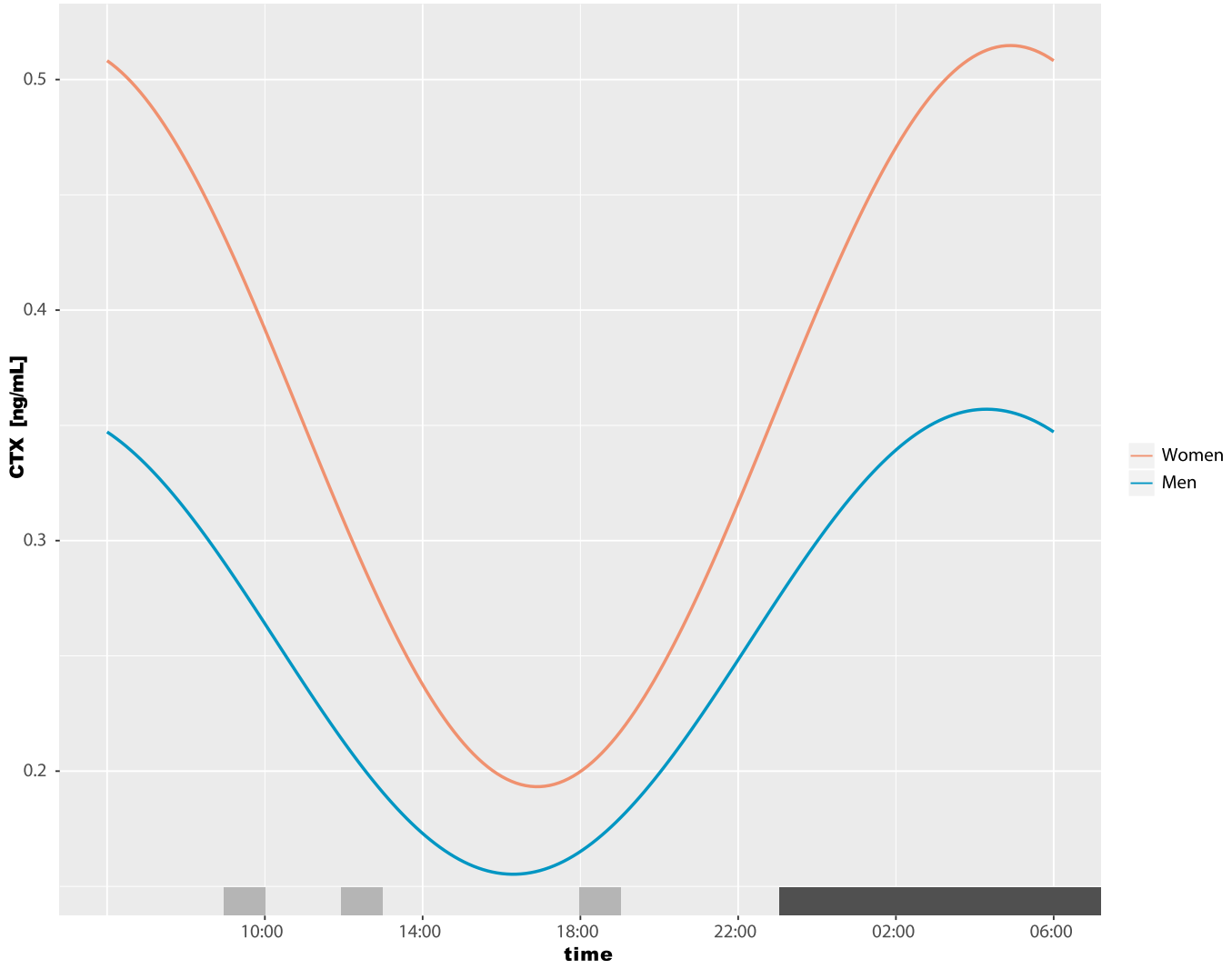


Fig. 2. Cosinor model of CTX for study participants stratified by sex.
A cosinor model fitted to C-terminal cross-linked telopeptide of type 1 collagen (CTX) of 20 men (blue/lower line) and 17 women (red/upper line). Light bars represent meal times and the dark bar represents the period when the lights were switched off. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Human Sclerostin Assay from Meso Scale Diagnostics, with an inter-assay precision of 10%, as previously described [37]. DKK1 was measured by the Quantikine Human DKK-1 Immunoassay from R&D systems. The inter-assay CV of this kit is 7.6% at 243 pg/mL and 4.6% at 1371 pg/mL, and the inter-assay CV was 5.8% in our hands as previously described [38]. 25-Hydroxyvitamin D, phosphate, calcium, and albumin were measured in a fasting morning serum sample, 25-hydroxyvitamin D using the E170 module of Modular Analytics and phosphate, calcium, and albumin using Modular P systems from Roche Diagnostics (Almere, The Netherlands). Calcium concentrations were corrected for albumin concentrations using the following formula: corrected calcium = measured calcium [mmol/L] + 0.02 * (42 − albumin [g/L]).

2.4. Statistical analysis

Characteristics of the study participants were calculated separately for men and women using descriptive statistics. Normally distributed variables were presented as mean with standard deviation and differences between men and women were assessed by independent-samples *t*-tests. Not normally distributed variables were presented as median with interquartile ranges, using the nonparametric independent-samples Mann-Whitney *U* test to assess differences between men and women. Mahalanobis distances were calculated to check multivariate normality [39]. Two probabilities of Mahalanobis distances were below $P < 0.001$ for DKK1, so two participants (one man and one woman) were excluded for the DKK1 analyses. Differences between men and women in 24-hour means of bone markers were calculated using the

Table 1
Characteristics of study participants stratified by sex.

	Men $n = 20$	Women $n = 17$	P value
Age (years)[a]	65.6 (5.3)	64.6 (5.2)	0.57
Offspring of long-lived family, N (%)	10 (50)	9 (52.9)	0.86
BMI (kg/m^2)[b]	25.2 (23.3–27.4)	23.2 (21.9–30.2)	0.32
Height (cm)[b]	178 (175–182)	165 (162–168)	< 0.001
Fat mass (kg)[b]	19.1 (18.0–24.1)	23.5 (20.2–34.8)	0.01
Lean body mass (kg)[b]	60.5 (57.6–66.0)	41.5 (37.9–45.5)	< 0.001
Waist circumference (cm)[b]	97 (92–106)	82 (81–95)	0.003
25-Hydroxyvitamin D [nmol/L]	69.9 (54.8–86.1)	79.1 (61.4–101.1)	0.22
Phosphate [mmol/L][a,b]	0.98 (0.15)	1.12 (0.11)	0.003
Calcium [mmol/L][c]	2.46 (2.36–2.50)	2.46 (2.41–2.50)	0.64
Usual bedtime (h)	23:30 (23:00–23:38)	23:30 (22:45–23:30)	0.48
Usual getting up time (h)	07:45 (07:00–08:15)	08:00 (07:30–08:15)	0.33

Unless indicated otherwise, data are presented as median with interquartile ranges.
[a] Data are presented as mean with standard deviation.
[b] Data were not available for one man.
[c] Calcium concentration was corrected for albumin concentration.

nonparametric independent-samples Mann-Whitney U test. We tested whether levels of bone markers were different between time points using repeated measures (RM) ANOVA, which is a model-independent method. RM ANOVA was performed with Time (10:00 vs 14:00 vs 18:00 vs 22:00 vs 02:00 vs 06:00) as within-subjects factor, Sex as between-subjects factor, and bone marker levels as dependent variables. Additionally, to take into account the effect of being offspring of long-lived family or partner, Status (offspring vs partner) was added as between-subjects factor and RM ANOVA analyses were repeated. Post hoc independent-samples t-tests were performed to analyse differences between men and women per time point. When appropriate, paired-samples t-tests were performed to calculate differences between mean values during daytime (time points 10:00, 14:00, 18:00, and 22:00) and mean values during nighttime (time points 02:00 and 06:00). Furthermore, to determine whether a bone marker showed a circadian rhythm, cosinor analyses were performed using the software program R, version 3.4.3 (The R Foundation for Statistical Computing, Vienna, Austria). Cosinor analysis is a model-dependent method which fits a cosinor model to the raw data. First, the rhythm detection test, also called the zero-amplitude test, was performed to test the overall significance of the cosinor model. The cosinor model was fit with sex as covariate using the software package 'cosinor' developed by Michael Sachs and parameter tests were performed using the software package 'cosinor2' developed by Augustin Mutak [40,41]. One of the circadian parameters calculated by the cosinor analysis is the midline estimating statistic of rhythm (MESOR), which is a circadian rhythm-adjusted mean based on the parameters of a cosine function fitted to the raw data. In addition, the amplitude is provided, which is the difference between the maximum and MESOR of the fitted curve. The acrophase represents the phase of the maximal value assumed by the curve [42]. Cosinor analyses were also performed with offspring-partner status as covariate to test whether being offspring of long-lived family or partner influenced the potential differences found between men and women. P values < 0.05 were considered as statistically significant. All statistical analyses, except for the cosinor analyses, were performed with SPSS for Windows, version 23 (SPSS, Chicago, IL, USA). Fig. 1 and Supplementary Fig. 1 were made using GraphPad Prism version 7 (GraphPad, San Diego, CA, USA) and Fig. 2 was made using R, version 3.4.3, and Adobe Illustrator.

3. Results

3.1. Characteristics of study participants

The characteristics of the study participants are presented separately for 20 men and 17 women in Table 1. Men and women were similar in their age, offspring-partner distribution, BMI, and vitamin D and calcium levels. Participants were normal nocturnal sleepers in the month prior to the study day, with similar usual bedtime and getting up time for men and women. As expected, measures of body composition were different between men and women, with men being taller, having less fat mass, more lean body mass, and larger waist circumference. Phosphate levels were higher in women than in men.

3.2. 24-hour profiles of bone markers

Table 2 presents medians of 24-hour means of CTX, osteocalcin, P1NP, sclerostin, and DKK1 with interquartile ranges for men and women separately. Fig. 1 displays at time points 10:00, 14:00, 18:00, 22:00, 02:00, and 06:00 the mean levels of bone markers stratified by sex. Individual 24-hour profiles of all bone markers are plotted in Supplementary Fig. 1. To investigate whether levels of bone markers differed over time, RM ANOVA was performed with Time as within-subjects factor, Sex as between-subjects factor, and bone marker levels as dependent variable. When appropriate, post hoc t-tests were performed to compare 24-hour profiles between men and women. Furthermore, cosinor analyses were performed to determine a circadian rhythm, with Fig. 2 displaying the cosinor model for CTX. Results are presented per bone marker in the following sections.

3.3. CTX

Women had significantly higher 24-hour means of CTX levels compared to men (Table 2). Fig. 1a displays mean levels of CTX for men and women separately at time points 10:00, 14:00, 18:00, 22:00, 02:00, and 06:00. By visual inspection, we observed that CTX exhibited in both

Table 2
24-hour means of bone markers in study participants stratified by sex.

	Men $n = 20$	Women $n = 17$	P value
CTX [ng/mL]	0.23 (0.19–0.30)	0.32 (0.25–0.49)	0.02
Osteocalcin [μg/L]	16.6 (13.7–21.5)	22.0 (16.4–26.7)	0.045
P1NP [ng/mL]	40.1 (29.6–47.5)	52.2 (38.0–61.3)	0.11
Sclerostin [pg/mL]	134 (116–167)	129 (111–163)	0.11
DKK1 [pg/mL]	494 (347–753)	662 (455–855)	0.73

Data are presented as median 24-hour means with interquartile ranges, analyzed by Independent Samples Mann-Whitney U test.
Abbreviations: C-terminal cross-linked telopeptide of type 1 collagen (CTX), N-terminal propeptide of type 1 procollagen (P1NP), Dickkopf-related protein 1 (DKK1).

men and women a decrease at 14:00 followed by an increase at 18:00 and a small decrease at 22:00. After 22:00, CTX increased with highest levels at 06:00. Mean CTX levels differed significantly between time points ($F(2.3,78.2) = 94.4$, $P < 0.001$) and the between-subjects factor Sex was also significant ($F(1,34) = 5.4$, $P = 0.03$). In addition, there was a Time by Sex interaction, ($F(2.3,78.2) = 4.7$, $P = 0.01$), mainly showing higher mean CTX levels in the night in women than in men. Post hoc *t*-tests indicated that women had significantly higher levels of CTX at 10:00 ($t_{df} = 2.7_{35}$, $P = 0.01$), 02:00 ($t_{df} = 2.4_{35}$, $P = 0.02$), and 06:00 ($t_{df} = 2.7_{35}$, $P = 0.01$) compared to men. The trajectory of CTX levels fitted a cosinor model over 24 h ($P < 0.001$), with a mesor estimate (95% confidence interval (CI)) of 0.26 (0.19–0.32) ng/mL in men ($P < 0.001$) and 0.35 (0.32–0.38) ng/mL in women ($P < 0.001$). Fig. 2 displays the cosinor model fitted to CTX in men and women. The amplitude (95% CI) of CTX was estimated as 0.16 (0.12–0.20) ng/mL in women, which was significantly ($P = 0.04$) higher compared to men (0.10 (0.06–0.14) ng/mL). The acrophase estimate (95% CI) was 04:18 (02:46–05:50) in men ($P = 0.02$) and 04:54 (03:51–05:57) in women ($P = 0.03$), which was not significantly different between men and women ($P = 0.49$).

3.4. Osteocalcin

For osteocalcin, we observed higher 24-hour means of osteocalcin levels in women than in men (Table 2). The 24-hour profiles of osteocalcin are presented in Fig. 1 with women showing a small decrease at 14:00 followed by a linear increase with its highest points at night (02:00 and 06:00). For men, osteocalcin levels were relatively constant during daytime and were increased in the night. Mean osteocalcin levels were significantly different between time points ($F(3.9,135.9) = 8.1$, $P < 0.001$). To test whether this time effect is caused by day-night differences, post hoc analyses were performed comparing osteocalcin levels during daytime (10:00–22:00) with nighttime (02:00–06:00). Men had a mean (95% CI) difference between day and night of 2.3 (1.4–3.3) μg/L, with significantly ($P < 0.001$) lower mean (SD) osteocalcin levels of 17.1 (5.1) μg/L during the day compared to the night (19.5 (5.8) μg/L). Also women had lower osteocalcin levels during the day (20.5 (6.7) μg/L) compared to the night (22.0 (7.0) μg/L) with a mean (95% CI) difference of 1.4 (0.5–2.4) μg/L ($P = 0.01$). However, no significant difference between men and women was observed ($F(1,35) = 2.5$, $P = 0.13$), nor a Time by Sex interaction ($F(3.9,135.9) = 1.2$, $P = 0.29$). The overall fit of the cosinor model was not significant ($P = 0.13$).

3.5. P1NP

24-hour mean P1NP levels were slightly higher in women than in men, although not significantly (Table 2). By visual inspection, we observed that levels of P1NP (Fig. 1c) were relatively constant over 24 h, but exhibited a small linear increase during 24 h with the highest point in the night at 02:00. Mean P1NP levels differed significantly between time points ($F(4.1,139.0) = 7.3$, $P < 0.001$), with a trend to higher levels in women compared to men ($F(1,34) = 3.5$, $P = 0.07$). However, no Time by Sex interaction was observed ($F(4.1,139.0) = 0.5$, $P = 0.75$). Men had significantly ($P = 0.001$) higher mean (SD) P1NP levels in the night (42.5 (14.8) ng/mL) than during the day (39.8 (13.7) ng/mL) with a mean difference (95% CI) of 2.7 (1.3–4.0) ng/mL. Levels of P1NP did not differ significantly ($P = 0.12$) between day (50.4 (15.5) ng/mL) and night (51.8 (15.6) ng/mL) in women. Post hoc *t*-tests indicated that women had significantly higher levels of P1NP levels than men at 10:00 ($t_{df} = 2.1_{35}$, $P = 0.04$), 14:00 ($t_{df} = 2.1_{35}$, $P = 0.045$), and 22:00 ($t_{df} = 2.3_{35}$, $P = 0.03$). There was no significant fit of the cosinor model ($P = 0.59$).

3.6. Sclerostin

Table 2 presents no significant difference between men and women in their 24-hour mean sclerostin levels. Over 24 h, sclerostin levels (Fig. 1d) increased in men with its highest point at 18:00 followed by a decrease. In women, levels fluctuated over the day with peaks at 10:00, 18:00, and 02:00. Levels of sclerostin were not significantly different between men and women at any of the time points sampled. Furthermore, sclerostin levels differed significantly between time points ($F(4.0,139.1) = 2.7$, $P = 0.04$) with no difference between men and women ($F(1,35) = 0.25$, $P = 0.62$) and no Time by Sex interaction ($F(4.0,139.1) = 0.6$, $P = 0.67$). The cosinor model did not significantly fit the sclerostin levels ($P = 0.30$).

3.7. DKK1

24-hour mean DKK1 levels did not differ between men and women (Table 2). DKK1 (Fig. 1e) was relatively constant during daytime with a significant increase in the night for men. In women, mean DKK1 levels were also relatively stable over 24 h, except at 18:00, when there was a sudden increase. However, when individual 24-hour profiles of DKK1 were plotted (see Supplementary Fig. 1) and visually inspected, DKK1 exhibited a large variation between and within subjects. Neither the within-subjects factor Time was significant ($F(3.3,108.0) = 2.2$, $P = 0.09$), nor the between-subjects factor Sex ($F(1,33) = 2.4$, $P = 0.13$). However, there was a significant interaction between time and sex ($F(3.3,108.0) = 3.0$, $P = 0.03$). Post hoc *t*-tests indicated that women had higher DKK1 levels at 10:00 ($t_{df} = 2.8_{33}$, $P = 0.01$) and at 18:00 ($t_{df} = 2.6_{33}$, $P = 0.01$) compared to men. The overall fit of the cosinor model was not significant ($P = 0.51$).

For all bone markers, results did not materially change after adding offspring-partner status as a between-subjects factor in the analyses.

4. Discussion

This study is the first which simultaneously measured the 24-hour trajectories of the five bone markers CTX, osteocalcin, P1NP, sclerostin, and DKK1 in the same participants, comprising healthy older men and women. Moreover, DKK1 levels had not been measured over time before and sclerostin levels had not yet been measured over time in healthy older women. In line with previous findings, we observed that CTX had a circadian rhythm with its nadir in the afternoon and its peak in the late night in both men and women. Although the timing of the circadian rhythm was similar between men and women, we are the first to observe a statistically significant larger CTX amplitude in women than in men. Furthermore, in line with previous findings, we observed that the 24-hour profile of osteocalcin exhibited higher levels during nighttime compared to daytime in both men and women. However, no significant circadian rhythm for osteocalcin was identified when a strict cosinor function was fitted. For P1NP levels we observed a small but significant increase in the night in men, but not in women. Sclerostin did not demonstrate a clear circadian rhythm, but levels differed between time points in both men and women. We did not observe a clear rhythm in DKK1 over 24 h in neither men nor women.

The circadian rhythm of CTX with its peak around 05:21 and 05:59 for men and women respectively was similar to that observed in other studies [3–6,18]. We observed a difference between older men and women in the amplitude of CTX over 24 h, with a larger amplitude in women than in men. This was in contrast to other studies, which have shown that the circadian rhythm was independent of sex [11,18]. The circadian rhythm of serum CTX is strongly influenced by fasting and food intake, with an increase in CTX during fasting resulting in a lower variation over 24 h [18,43,44]. CTX levels decreased after intake of food, glucose, fat, and protein. This response is independent of sex or age [43,45]. In response to food intake, glucagon-like peptide-2 (GLP-2) is secreted, which is a gastrointestinal hormone involved in the

maintenance of intestinal epithelial morphology and function [46]. Injection of GLP-2 caused a dose-dependent reduction in CTX levels [45]. In general, CTX levels are higher in postmenopausal women than in men [15,16]. Although not formally asked, based on the age range of women and the higher levels in women than in men, we assume (most) women in our study are postmenopausal. Both men and women received six bottles of Nutridrink consisting of 1800 kcal during the day. In general, women need fewer calories than men, so it could be that food intake caused a stronger reduction during the day in women than in men. Although unlikely, this could explain the nonexistence of a difference in levels between men and women during day time, and also the bigger amplitude in women than in men. However, we cannot exclude the possibility of a chance finding. Studies have shown that ethnicity, low bone mass, bed rest of 5 days, absence of a circadian rhythm of cortisol, absence of a light-dark cycle (blindness), or circadian disruption and sleep restriction did not influence the circadian rhythm of CTX [11,18,47]. Indeed, since we found similar circadian rhythm of CTX compared to literature, we assume that bed rest and potential sleep disturbances, possibly caused by performing blood sampling in the night, did not influence the circadian rhythm of CTX.

For osteocalcin, we found significantly higher levels during nighttime compared to daytime in both men and women. When performing cosinor analysis, no significant circadian rhythm was observed. However, although cosinor analysis is often used to determine circadian rhythms, this model is very strict. If a curve does not fit into a cosine function, one would conclude that no circadian rhythm is observed. However, not all circadian rhythms fit into a cosine function. For example, a curve comprising every 24 h a sharp peak at the end of the night still displays a circadian rhythm but this does not fit into a cosine function. Therefore, we can conclude from our data that osteocalcin, although not detected with cosinor analysis, displays a circadian rhythm with its nadir during daytime and its peak in the night, which is in line with previous studies [5,8,11,21–24]. No differences in 24-hour profile of osteocalcin were found between men and women, which is in line with literature [11,23]. Besides being released into the circulation during bone formation, osteocalcin fragments are also released into the circulation during bone resorption since it is part of the bone matrix. This might explain why osteocalcin has a similar circadian rhythm as CTX and has a stronger circadian rhythm than other bone formation markers [9,48]. Studies have shown that the circadian rhythm of osteocalcin is highly dependent on the circadian variation in serum cortisol [49–52]. The circadian rhythm of cortisol is mostly influenced by the circadian clock, but cortisol levels also depend on other factors such as stress and activity [53]. First, participating in a clinical study is stressful; especially blood withdrawal during 24 h with limited movement could lead to stress and could influence the levels of cortisol. Furthermore, participants of our study performed several tests, including the Trier Social Stress Test and tests for cognitive function, on the day before the blood sampling and were wearing multiple sensors to register physiological parameters during the study. Potentially, sleep disturbances caused by performing the continuous blood sampling could have influenced the cortisol rhythm causing less circadian variation of osteocalcin. Furthermore, participants had unusual food intake during the continuous blood sampling. Although participants received mixed meals containing all essential nutrients, their food intake consisted of liquid meals only. Literature showed that fasting did not significantly change the circadian rhythm of serum osteocalcin [43,44,54]. For osteocalcin levels, one study showed slightly lower osteocalcin levels in the fed state, while another study showed no effect of food intake or GLP-2 injection [45,55]. Therefore, it is unlikely that the unusual food intake in the form of liquid meals has influenced the circadian rhythm of osteocalcin. The circadian rhythm of osteocalcin was not influenced by ethnicity, low bone mass, or by bed rest of 5 days [11,21,22,24]. Therefore, since we found similar circadian rhythm of osteocalcin compared to literature, we assume also that bed rest and limitation in their movement and lack of activity did not influence the circadian rhythm of osteocalcin.

Literature on the circadian rhythm of P1NP is limiting and conflicting [6,10,11]. In our study, P1NP did not exhibit a clear circadian rhythm in neither men nor women, but P1NP levels were higher in the night than during the day in men. Also other studies found that day-night differences were less pronounced for markers of bone formation compared to bone resorption, reviewed by others [9,56]. In general, P1NP levels were higher in women than in men, similar to findings from other studies [15,16]. The participants were mostly bedbound and were limited in their movements during the 24 h in which the blood was withdrawn. Although studies have shown no effect of five days of bed rest on the circadian rhythm of CTX and osteocalcin, it could have influenced the 24-hour profile of P1NP [18,22]. However, the circadian rhythm of carboxyl-terminal propeptide of type 1 procollagen (P1CP), which is closely related to P1NP, was not influenced by five days of bed rest [22]. Furthermore, sleep disruption did not significantly change the circadian rhythm of P1NP [47]. Although the effect of fasting or food intake on the circadian rhythm of P1NP was not investigated, P1NP levels were somewhat lower in the fed state, and P1NP levels decreased mildly during an intravenous glucose tolerance test, but oral glucose ingestion did not have a significant effect on P1NP levels [55,57,58].

Sclerostin has only been measured once over time by other researchers, and no circadian rhythm of sclerostin was observed in men [6]. We confirmed this in men, but also in women we did not identify a circadian rhythm. However, sclerostin levels differed between time points in both men and women, so there seems to be an effect of time. Sleep disruption did not significantly change the circadian rhythm of sclerostin [47]. However, it is not known whether food intake, limited activity or 24-hour bed rest may have influenced the circadian rhythm of sclerostin.

DKK1 levels have not been measured over time before. Our measurements of DKK1 displayed a large variation between participants, but we also see a large variation of DKK1 within participants. This variation within a participant over time cannot be explained by circadian variation since no circadian rhythm was detected nor a 24-hour profile over time. It is not clear what the cause of this wide variation is. One explanation could be that DKK1 is secreted in a pulsatile fashion, similar to some other regulatory factors such as hormones. For example, when growth hormone (GH) was measured in the same participants in blood sampled every 10 min during 24 h, we could indeed assess its pulsatility. When GH would have been measured every 4 h, however, the individual variation would also be large [59]. DKK1 levels should be assessed more frequently than every 4 h to confirm this hypothesis. Another explanation could be that DKK1 is not bone specific, but also secreted by human preadipocytes and promotes adipogenesis [60]. A study showed that DKK1 is also expressed in platelets [36]. The activity of blood platelets varies over 24 h which could explain the variation in DKK1 levels [61]. However, the variation in platelet activity described in this study is much lower than the variation in DKK1 levels we have measured over 24 h. Moreover, blood platelets are intact in EDTA plasma, in contrast to serum, therefore DKK1 levels present in platelets did not contribute to the DKK1 levels present in plasma. It is unclear whether freeze-thaw cycles of the material could have influenced the results. Based on our previous hands-on experience with the assay, we do not have assumptions that the ELISA assay did not work correctly. This assay has been performed several times in our laboratory and results have been published before [38]. For DKK1, at a frequency of 4 h, we cannot reliably derive information on the absence or presence of the 24-hour rhythm of DKK1.

Sclerostin and DKK1 are different types of bone markers than CTX, osteocalcin, and P1NP. While the latter are by-products of collagen breakdown or incorporated in the bone matrix, sclerostin and DKK1 have regulatory roles. Moreover, since osteocytes are highly sensitive to changes in calcium and phosphate levels and to mechanical loading, levels of their secreted regulatory proteins will be more susceptible to internal and external factors [12]. Indeed, sclerostin and DKK1 levels

changed after mechanical (un)loading [62]. This might explain why sclerostin and DKK1 levels demonstrate large variations over 24 h and did not display similar 24-hour profiles as other bone markers.

Limitations of our study are the unusual (not similar to daily-living) study conditions, including staying in bed with limited movement during the 24 h in the research centre, food intake which only consisted of liquid meals, and sleep which was potentially disturbed by the continuous blood sampling. This could have influenced the results. Furthermore, bone mineral density was not assessed, since bone health was not a primary objective of this study. Although the study conditions are not similar to daily-living conditions, the conditions were standardized for all participants, so individuals were studied in a comparable research setting which is a strength of this study. Nevertheless, standardisation during the study period only does not completely rule out the influence of possible individual factors on the 24-hour profiles of bone markers. This study is the first to measure all these bone markers during 24 h in 37 participants, a relatively large study population for investigating the circadian rhythm of bone markers. Future studies could measure more samples per participants to be able to determine the exact timing of the circadian rhythm of the bone markers. Also a period longer than 24 h could determine the stability of the rhythm over several days.

In conclusion, for DKK1 as measured with a frequency of 4 h in this study, no reliable conclusion on the absence or presence of a 24-hour rhythm can be drawn because of the large intraindividual variation. Furthermore, when measuring CTX, osteocalcin, P1NP, or sclerostin either in clinical practice or in a research setting, one should consider the 24-hour profiles of these bone markers. It has to be taken into account that not only CTX and osteocalcin have a circadian rhythm but P1NP and sclerostin levels, although not exhibiting a circadian rhythm, vary as well during the day. Although levels differed between men and women, timing of the 24-hour rhythm of all bone markers did not differ between men and women. Potentially, sex hormones predominantly influence levels of bone markers, but not interfere with the timing of the 24-hour rhythm. Further research could focus on the association between sex hormones and the circadian rhythm of bone markers.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.10.002.

Acknowledgements

The Switchbox Leiden Study is part of the Leiden Longevity Study, which was founded by Rudi Westendorp and Eline Slagboom. We thank all participants of the Switchbox Leiden Study, the researchers coordinating the blood sampling (Steffy Jansen and Abi Akintola), clinical chemist Judith Gillis, and other researchers (Liesbeth Winter and Ana Zutinic) for their valuable intellectual contributions. The biochemical assays were carried out at the department of Clinical Chemistry and Laboratory Medicine and the Einthoven laboratory from the section of Endocrinology of the Leiden University Medical Center in The Netherlands. Therefore, we would like to thank their personnel for facilitating and performing the measurements (Chris van der Bent, Trea Streefland, Bart Ballieux, and Angela van Tilborg). This research is funded by two European Commission projects, Switchbox (FP7, Health-F2-2010-259772) and THYRAGE (Horizon 2020 research and innovation programme, 666869). Evie van der Spoel is supported by a personal PhD grant from the Leiden University Medical Center.

Declarations of interest

None.

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