

## Non-reproductive Effects of Anovulation

### Bone Metabolism in the Luteal Phase of Premenopausal Women Differs between Ovulatory and Anovulatory Cycles

#### Nicht reproduktive Effekte der Anovulation

Unterschiede zwischen ovulatorischen und anovulatorischen Zyklen im Knochenstoffwechsel von prämenopausalen Frauen

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#### Key words

- hormones
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#### Schlüsselwörter

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#### Abstract



**Introduction:** Several authors have linked subclinical ovulatory disturbances in normal length menstrual cycles to premenopausal fracture risk and bone changes. This study systematically examined the influence of ovulation and anovulation on the bone metabolism of premenopausal women.

**Participants and Methods:** In 176 cycles in healthy premenopausal women, FSH, 17 $\beta$ -estradiol (E2) and progesterone (P4) as well as bone alkaline phosphatase (BAP), pyridinoline (PYD) and C-terminal crosslinks (CTX) were measured during the follicular and during the luteal phase. The probability and timing of ovulation was self-assessed by a monitoring device. In addition, bone density of the lumbar spine was measured by quantitative computed tomography (QCT) at baseline and at the end of the study. Analysis was restricted to blood samples taken more than three days before the following menstruation.

**Results:** 118 cycles out of the 176 collected cycles were complete with blood samples taken within the correct time interval. Of these, 56.8% were ovulatory by two criteria (ovulation symbol shown on the monitor display and LP progesterone > 6 ng/ml), 33.1% were possibly ovulatory by one criterion (ovulation symbol shown on the monitor display or LP progesterone > 6 ng/ml), and 10.2% were anovulatory by both criteria). Ovulation in the previous cycle and in the same cycle did not significantly influence the mean absolute concentrations of the bone markers. However, bone formation (BAP) was higher in the luteal phase of ovulatory cycles than in anovulatory cycles (n.s.) and the relative changes within one cycle were significantly different for bone resorption (CTX) during ovulatory vs. anovulatory cycles ( $p < 0.01$ ). In 68 pairs of cycles following each other directly, both ovulation in the previous cycle and ovulation in the present cycle influenced CTX, but not the differences of other bone markers.

#### Zusammenfassung



**Einleitung:** Sowohl Knochenstoffwechselveränderungen wie auch ein erhöhtes Risiko für spätere Frakturen sind von verschiedenen Autoren mit subklinischen ovulatorischen Störungen (in menstruellen Zyklen normaler Länge) in Verbindung gebracht worden. Diese Pilotstudie untersuchte systematisch den Einfluss der Ovulation und Anovulation auf den Knochenstoffwechsel prämenopausaler Frauen.

**Studienteilnehmerinnen und Methodik:** Bei gesunden prämenopausalen Frauen wurden in 176 Zyklen FSH, 17 $\beta$ -Estradiol (E2) und Progesteron (P4) sowie knochenalkalische Phosphatase (BAP), Pyridinolin (PYD) und C-terminale Crosslinks (CTX) jeweils während Follikelphase und Lutealphase gemessen. Die Ovulations-Wahrscheinlichkeit und der Zeitpunkt der Ovulation wurden mittels Zyklusmonitor bestimmt. Die Knochendichte der Lendenwirbelsäule wurde mit quantitativer Computertomografie (QCT) zu Beginn und am Ende der Studie gemessen. Analysiert wurden die Zyklen, in denen die Lutealphasen-Blutproben mehr als 3 Tage vor der nächsten Menstruation entnommen worden waren.

**Ergebnisse:** In 118 von 176 gesammelten Zyklen waren die Blutproben im korrekten Zeitintervall. 56,8% wurden auf der Basis von 2 Kriterien (Ovulationssymbol auf der Monitoranzeige und Progesteron > 6 ng/ml) als ovulatorisch gewertet, 33,1% als möglicherweise ovulatorisch (nur Ovulationssymbol auf der Monitoranzeige oder LP Progesteron > 6 ng/ml) und 10,2% als anovulatorisch (nach beiden Kriterien) klassifiziert. Die relativen Unterschiede innerhalb eines Zyklus (LP – FP) waren für den Knochenabbau-Marker CTX in ovulatorischen vs. anovulatorischen Zyklen signifikant vermindert ( $p < 0,01$ ). In 68 Zykluspaaren, die direkt aufeinander folgten, beeinflussten sowohl die Ovulation im vorangegangenen als auch die Ovulation im aktuellen Zyklus signifi-

**Conclusion:** Ovulatory cycles reduce bone resorption in their luteal phase and that of the following cycle. The interaction between ovulation and bone metabolism is complex. Since anovulation may occur in low estrogen states such as pre-anorexic dietary restraint, as well as with high estrogenic circumstances e.g. from functional perimenopausal ovarian cysts, the association with bone changes has been variable in the literature. Accumulating physiological and clinical evidence however point towards a role for ovulation in enhancing bone formation and limiting bone resorption.

## Introduction

Osteoporosis is a serious systemic disease of the skeleton which affects almost 30% of postmenopausal women. Pain, fractures, postural deformities and enormous costs for the health care system are the consequences. Data which link irregular menses or prolonged bleeding days to a higher risk of suffering fractures later in life have been recently published [1,2].

A normal length menstrual cycle may either be ovulatory, or have a short luteal phase in association with late ovulation within the cycles, or be anovulatory. Recent epidemiologic data from the HUNT study in Norway suggest that anovulation in young women without hormonal contraception has a point prevalence of 30% [3], while Li et al. reported ovulatory disturbances between 13% and 82% in a meta-analysis of six studies in 436 women published between 1990 and 2010. This meta-analysis also reported negative changes of spinal bone density (approximately -0.9%/year) with ovulatory disturbances [1].

Bone markers reflect the dynamic processes of bone metabolism. They show early changes in resorption or formation rapidly and thereby complement the much slower assessment of morphologic changes by bone density measurement [4]. Their use in studies to assess therapeutic or adverse effects on bone is widely established. Nevertheless, they – as many other biomarkers – underlie a circadian rhythm [5] and show daily variations of 10 to 15%.

Quantitative computed tomography (QCT) offers the advantage of separate volumetric measurements of cortical and trabecular bone. As hormones act mainly on trabecular bone, this method is ideal for assessing early osteoporotic changes. Moreover, QCT is less prone to artefacts like osteophyte growing or aortic calcification than other techniques [6]. The radiation exposure is in a range of 25–60  $\mu$ Sv. It is higher than with DEXA (1–2  $\mu$ Sv). Nevertheless it is still low compared with the average yearly radiation exposure of 2–4 mSv in Germany.

Until recent years, an accurate diagnosis of ovulation in a menstrual cycle was only possible by means of frequent ultrasound controls or daily serum hormone measurements. Because both methods need frequent visits by the study subjects to the study center, former studies involved only short study periods, of rarely more than two months, or non-continuous observations, taking samples once a year. Using a commercially available cycle monitoring system based on daily self-measurements of urinary estrone 3-glucuronide and LH concentrations, study visits were limited to two per cycle and the timing of the luteal phase visit

was coordinated to take place 6 to 9 days after the LH surge shown as the symbol of likely ovulation on the monitor display. By these means, this prospective observational study was able to examine the influence of ovulation on bone metabolism of premenopausal women, studying hormonal and bone parameters longitudinally for the first time.

**Diskussion:** Die Interaktion zwischen Ovulation und Knochenstoffwechsel ist komplex. Ovulatorische Zyklen reduzieren den Knochenabbau in der Lutealphase des gleichen Zyklus und der des nachfolgenden Zyklus. Dadurch dass Anovulation mit niedrigen Estrogenspiegeln (z.B. prä-anorektische Ernährung) ebenso auftritt wie in Verbindung mit hohen Östrogenspiegeln (z.B. bei perimenopausalen Ovarialzysten), ist ihre Wirkung auf den Knochen in verschiedenen Kollektiven unterschiedlich beurteilt worden.

**Fazit:** Die hier vorliegenden physiologischen und klinischen Daten sprechen dafür, dass die Ovulation beim Knochenaufbau und bei der Verminderung von Knochenabbau eine Rolle spielt.

was coordinated to take place 6 to 9 days after the LH surge shown as the symbol of likely ovulation on the monitor display. By these means, this prospective observational study was able to examine the influence of ovulation on bone metabolism of premenopausal women, studying hormonal and bone parameters longitudinally for the first time.

## Participants and Methods

Participants were cycling women over 40 years without known risk factors for osteoporosis or known vitamin D deficiency, they had all signed informed consent after the study protocol had been approved by the ethics committee of the TUM medical faculty.

Serum samples for FSH, 17 $\beta$ -estradiol and progesterone were taken between 9 and 12 in the morning both in the follicular phase and in the luteal phase of every cycle. Due to the information provided by the ovulation monitor, it was possible to determine

1. whether an LH-surge had occurred in the current cycle, and
2. whether the day of sampling was within the correct interval of 6–9 days post LH-surge.

For a differentiated description of the changes in bone metabolism during the menstrual cycle, a panel of four bone markers was measured twice in each menstrual cycle. Bone specific alkaline phosphatase (BAP) in serum was chosen due to its stability and long half-life to reflect bone formation, and urinary pyridinolin (PYD), urinary desoxypyridinolin (DPD) and serum-carboxyterminal-telopeptide (CTX) were used to measure bone resorption. Second morning urine samples were collected between 9 and 12 hours a.m. in the morning to avoid circadian fluctuations.

## Cycle monitoring and probability of ovulation

The monitor used to determine the probability of ovulation established a ratio estrone 3-glucuronide and LH concentrations in urine with daily self-measurement usually starting on cycle day 9 and displays the probability of ovulation in the next 1–2 days semiquantitatively as one, two or three of three levels. Due to easy handling, the monitoring system of the cycle was operated without problems by study participants. The accuracy of the cycle monitor in detecting ovulation has been published to be 98% as verified by vaginal ultrasound in women aged 18 to 39 years with regular cycles up to 42 days [7]. Since for this study, women over age 40 and with possibly irregular cycles were to

**Table 1** Means and standard deviations of serum hormones by ovulation occurrence (n = number of cycles measured); possible ovulation was assumed if the cycle monitor had displayed the symbol for likelihood of ovulation or if a progesterone value of greater than 6 ng/ml occurred in the luteal phase. Certain ovulation was defined if both criteria were fulfilled. Anovulation was assumed if neither criterion had been fulfilled.

	Hormone	Certain ovulation (n = 67)	Possible ovulation (n = 39)	Anovulation (n = 12)	p
Follicular phase	FSH (IU/l)	8.38 ± 6.29	12.21 ± 13.54	13.99 ± 12.43	< 0.05
	17β-Estradiol (pg/ml)	166.57 ± 127.07	158.16 ± 141.44	177.58 ± 150.78	0.967
Luteal phase	Progesterone (ng/ml)	0.35 ± 0.12	0.44 ± 0.23	0.50 ± 0.29	< 0.01
	FSH (IU/l)	4.02 ± 2.37	9.43 ± 18.42	12.44 ± 13.96	< 0.01
	17β-Estradiol (pg/ml)	143.45 ± 49.01	118.94 ± 54.51	92.94 ± 46.30	< 0.01
	Progesterone (ng/ml)	12.60 ± 4.89	7.82 ± 5.67	3.04 ± 2.42	< 0.01

be examined, a progesterone concentration of greater than 6 ng/ml was defined as a second criterion for certain ovulation.

As luteal phase progesterone (P4) production is only fully established 4 days after ovulation (or 6 days after the LH-peak) and the pre-menstrual decline of both progesterone and estrogen begins 3 days prior to menstruation, serum samples from the luteal phase were additionally corrected retrospectively for the correct timing interval.

### Bone density measurement

The trabecular compartment bone density of the lumbar vertebrae 1 to 3 of all patients was measured by quantitative computed tomography (QCT) at baseline as well as after two years. In accordance with institutional regulations, the radiological studies were approved by the BfS (Bundesamt für Strahlenschutz).

### Statistical analysis

Data were documented on paper, collected in Excel tables and analysed using SPSS software. Differences were considered to be statistically significant when the likelihood of chance was rejected with more than 95%, expressed as a p-value of less than 0.05.

The bone metabolism parameters were analyzed in two ways: apart from comparing the average group values, the intra-individual changes of bone markers between follicular and luteal phase were examined, assuming that other factors influencing levels of bone markers (like immobilization, vitamin D, nutrition etc.) would not change in the individual over the period of one cycle. By this approach, the additional interaction of ovulation vs. anovulation on bone markers on top of other long-term factors was observed.

A mixed model analysis examined the influence of a rising progesterone threshold to discriminate anovulatory from ovulatory cycles. A progesterone value of 6 ng/ml had been chosen as cut-off between ovulatory and anovulatory cycles, since 5.8 ng/ml was the lowest progesterone value found in the literature to be indicative of ovulation. Yet many, mainly reproductive medicine, papers use far higher values of progesterone as a certain proof of ovulation. Due to this uncertainty and since the threshold concentration of progesterone for an effect in bone is completely unknown, this approach was chosen. The Mann-Whitney-U-test, which can be applied on unknown distributions was chosen for the explorative data-analysis of bone markers, testing the hypothesis that the individual difference between first (follicular) and second (“luteal”) phase marker values was different for ovulatory vs. anovulatory cycles.

### Results



Nine healthy participants (mean age 45.9 ± 3.48, median 44.2 yrs, range 40 to 50 years) contributed 176 cycles over the course of 22 ± 15.6 months, approximately 19.5 cycles per participant (range 5 to 35). All women had regular cycles between 3 and 6 weeks duration.

Since progesterone production in the corpus luteum begins after ovulation, needs 2–3 days to reach its peak, and declines again in the three days before menstruation, the period during which progesterone values can distinguish between ovulatory and anovulatory cycles is confined to a 10–12 day period during the second half of the cycle. Therefore, 58 cycles, in which menstruation followed three or less days after blood sampling had to be excluded from the final analysis, in order to strictly apply criteria for correct timing of the blood samples (< 3 days before the following menstruation, in order to adequately distinguish ovulatory from anovulatory cycles by low progesterone). Of the remaining 118 cycles, 67 (568%) were ovulatory by two criteria: likelihood of ovulation shown on the monitor display plus a progesterone value of > 6 ng/ml, 39 (33%) were possibly ovulatory (either likelihood of ovulation monitor display or a progesterone value of > 6 ng/ml) and 12 (10.2%) of the cycles were anovulatory by both criteria. Each participant contributed an average of 13.1 cycles to this analysis.

### Hormones and bone markers across the menstrual cycle

Mean estradiol values during the follicular phase were 166.6 pg/ml (median 129.00) in ovulatory cycles, 158.2 pg/ml (median 110.30) in cycles with possible ovulation and 177.6 pg/ml (median 160.4) in anovulatory cycles. During the luteal phase, mean estradiol was higher with 143.5 pg/ml (median 134.2) after certain ovulation than after possible ovulation (mean 118.9 pg/ml; median 113.8; p < 0.05) and lowest in anovulatory cycles with 92.9 pg/ml (median 82.0; p < 0.01 vs. ovulatory cycles).

While estradiol values were very similar in the follicular phases of all three categories, FSH was lower in ovulatory cycles (8.38 ± 6.29 IU/l) than in anovulatory cycles (13.99 ± 12.43 IU/l; < 0.05) during the follicular phase and even more so during the luteal phase (4.0 ± 2.37 vs. 12.44 ± 13.96 IU/l). Despite higher follicle stimulating hormone, estradiol was lower in the luteal phase of the anovulatory cycles (92.9 ± 46.3 pg/ml vs. 143 pg/ml ± 49.0 pg/ml; p < 0.01). As expected, progesterone was highest in ovulatory cycles (12.6 ± 4.89 ng/ml), compared with possibly ovulatory cycles (7.82 ± 5.67 ng/ml) and anovulatory cycles (3.04 ± 2.42 ng/ml; p < 0.01; see [Table 1](#)).

The group mean values of bone markers did not differ significantly between follicular phase and luteal phase ([Table 2](#)). But individual changes of bone markers between follicular and luteal

**Table 2** Bone markers by ovulation occurrence in the same cycle. Mean and SD values of the bone formation marker bone alkaline phosphatase (BAP), and the bone resorption markers urinary pyridinoline (PYD) and desoxypyridinoline (DPD), as well as serum c-terminal telopeptides (CTX) are shown by ovulation state in the same cycle. Possible ovulation was assumed if the cycle monitor had displayed the symbol for likelihood of ovulation or if a progesterone value of greater than 6 ng/ml occurred in the luteal phase. Certain ovulation was defined if both criteria were fulfilled. Anovulation was assumed if neither criterion had been fulfilled.

	Bone marker	Certain ovulation (n = 67)	Possible ovulation (n = 39)	Anovulation (n = 12)	p
Follicular phase	BAP (µg/l)	9.18 ± 1.65	9.21 ± 1.34	8.92 ± 1.98	0.721
	PYD (nmol/mmol creatinine)	39.06 ± 10.80	38.42 ± 10.39	35.21 ± 10.38	0.306
	DPD (nmol/mmol creatinine)	8.28 ± 2.98	7.79 ± 3.07	8.08 ± 1.95	0.564
	CTX (ng/ml)	0.25 ± 0.15	0.22 ± 0.13	0.25 ± 0.16	0.619
Luteal phase	BAP (µg/l)	9.04 ± 1.65	9.31 ± 1.45	8.92 ± 2.47	0.838
	PYD (nmol/mmol creatinine)	38.41 ± 8.32	37.33 ± 8.28	40.75 ± 6.36	0.720
	DPD (nmol/mmol creatinine)	8.40 ± 2.94	7.17 ± 2.36	9.57 ± 2.06	0.934
	CTX (ng/ml)	0.22 ± 0.13	0.21 ± 0.14	0.29 ± 0.17	0.284

**Table 3** Influence of ovulation on marker changes between follicular and luteal phase ( $\Delta$  [delta] = [LP] – [FP] of bone markers). The mean ± SD delta values of the bone formation marker bone alkaline phosphatase (BAP), and the bone resorption markers urinary pyridinoline (PYD) and desoxypyridinoline (DPD), as well as serum c-terminal telopeptides (CTX) are shown by ovulation state in the same cycle. Possible ovulation was assumed if the cycle monitor had displayed the symbol for likelihood of ovulation or if a progesterone value of greater than 6 ng/ml occurred in the luteal phase. Certain ovulation was defined if both criteria were fulfilled. Anovulation was assumed if neither criterion had been fulfilled.

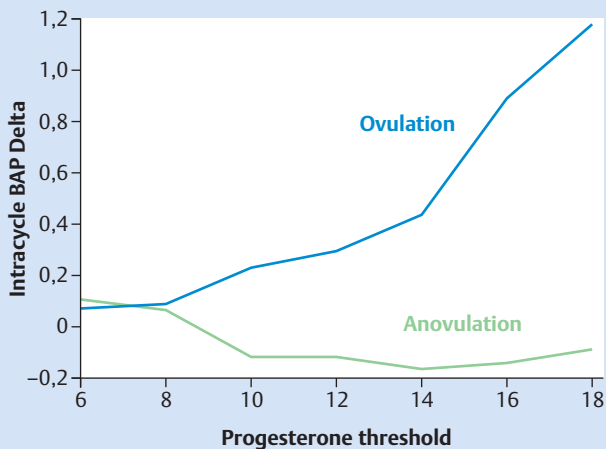
Within-cycle change of bone markers	Certain ovulation (n = 67)	Possible ovulation (n = 39)	Anovulation (n = 12)	p
$\Delta$ BAP (µg/l)	-0.134 ± 1.486	0.103 ± 1.046	0.000 ± 1.128	0.490
$\Delta$ PYD (nmol/mmol Kreatinin)	-0.651 ± 10.512	-1.090 ± 9.800	5.542 ± 10.753	0.185
$\Delta$ DPD (nmol/mmol Kreatinin)	0.116 ± 2.729	-0.621 ± 4.060	1.483 ± 3.007	0.659
$\Delta$ CTX (ng/ml)	-0.036 ± 0.077	-0.013 ± 0.078	0.032 ± 0.070	<0.01

**Table 4** Influence of progesterone cut-off on the distribution of ovulatory and anovulatory cycles and on the within-cycles changes of the bone resorption marker c-terminal telopeptides (CTX). This data-analysis of 84 complete cycles explored the differences in delta CTX (i.e. the difference between follicular phase and luteal phase bone marker values for each individual) with rising progesterone threshold. The left 4 columns show the distribution of anovulatory and ovulatory cycles and respective delta CTX-values for ovulatory and anovulatory cycles if the progesterone concentration in serum was used as the sole criterion for ovulation. The right 4 columns show the distribution and delta values if the ovulation likelihood (a ratio of LH and estrone-3-glucuronide) on the monitor display was added as criterion. Since the threshold for ovulatory progesterone values varies greatly in the medical literature, an explorative data-analysis was done with step-wise elevation of the progesterone threshold from 6 to 18 ng/ml, using the Mann-Whitney-U-Test on bone resorption marker  $\Delta$  CTX. Employing both criteria for ovulation combined (i.e. monitor display for LH-peak and progesterone serum level in the luteal phase), the asymptomatic two-sided significance was  $p = 0.002$ . When using only progesterone level as the sole criterion, no significance was noted ( $p = 0.076$ ).

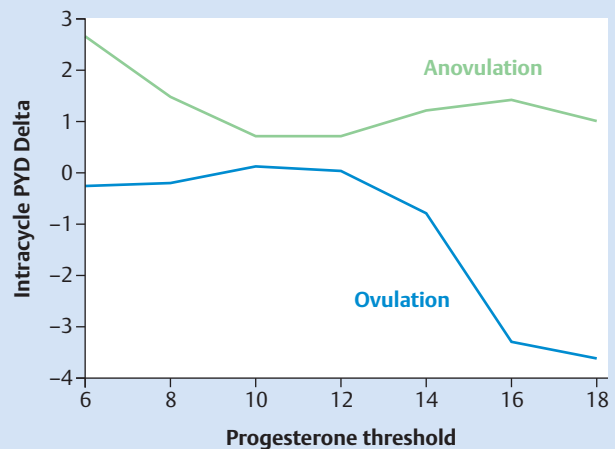
Progesterone threshold	Crit. P2		$\Delta$ CTX		Crit. P2/LH		$\Delta$ CTX	
	n	n	$\Delta$ CTX	$\Delta$ CTX	n	n	$\Delta$ CTX	$\Delta$ CTX
	Anov.	Ovul.	Anov.	Ovul.	Anov.	Ovul.	Anov.	Ovul.
6	19	65	-0.001	-0.029	10	49	0.032	-0.035
8	30	54	-0.007	-0.031	12	40	0.023	-0.039
10	37	47	-0.000	-0.04	15	36	0.021	-0.048
12	44	40	-0.006	-0.04	15	29	0.021	-0.051
14	50	34	-0.01	-0.041	17	25	0.023	-0.047
16	66	18	-0.019	-0.037	22	14	0.011	-0.042
18	73	11	-0.02	-0.036	25	10	0.007	-0.039
Asymptomatic 2-sided significance				$p = 0.076$			$p = 0.002$	

phase were notable, with  $\Delta$  (delta) = [LP] – [FP] ranging from -0.65 in ovulatory cycles to 5.54 in anovulatory cycles for PYD (pyridinoline;  $p = 0.19$ ), and from 0.12 to 1.48 for DPD (desoxy-pyridinoline;  $p = 0.66$ ) as shown in **Table 3**. While the delta for the slowly reacting bone formation marker BAP (bone alkaline phosphatase) and the deltas for the urinary resorption markers crosslinks (PYD/DPD) were not significantly different between ovulatory and anovulatory cycles, the delta for the quickly reacting bone resorption marker c-terminal telopeptides (CTX) in serum was highly significant ( $p < 0.01$ ).

Two variables were uncertain in this context: assuming an effect of ovulation, the question of when a cycle should be considered ovulatory was important. Since 5.8 ng/ml was the lowest progesterone value found in the literature to be indicative of ovulation, 6 ng/ml had been chosen as a conservative cut-off for comparison of hormone values and bone markers (**Table 3**). Yet many, mainly reproductive medicine papers use far higher values of progesterone as a certain proof of ovulation. Also, assuming that progesterone might have any effect on bone, the threshold concentration of progesterone for an effect in bone is completely unknown. In order to explore these two uncertainties, a mixed



**Fig. 1** Intra-cycle changes of bone formation marker bone alkaline phosphatase (BAP) with rising progesterone threshold. The x-axis displays the rising progesterone threshold, the y-axis shows the difference ( $\Delta$  [delta] = [LP] – [FP]) of BAP. Negative values indicate that luteal phase values are lower than follicular phase values. Positive values indicate that luteal phase values are higher than follicular phase values. The blue line depicts the results for ovulatory cycles while the green line shows the deltas for anovulatory cycles. In ovulatory cycles, the bone formation marker BAP was higher during the luteal phase than during the follicular phase in the participating women. This difference seems to grow with higher progesterone thresholds applied for the discrimination between ovulatory and anovulatory cycles.



**Fig. 2** Intra-cycle changes of bone resorption marker pyridinoline (PYD) with rising progesterone threshold. The x-axis displays the rising progesterone threshold, the y-axis shows the difference in PYD between luteal and follicular phase ( $\Delta$  [delta] = [LP] – [FP]). Positive values indicate that luteal phase values are higher than follicular phase values. Negative values indicate that luteal phase values are lower than follicular phase values. The blue line shows the respective delta values for ovulatory cycles, the green line for anovulatory cycles. In the individual participants, bone resorption declined in the luteal phase of ovulatory cycles, while remaining higher in anovulatory cycles. The difference seems to grow with higher progesterone thresholds applied for the discrimination between ovulatory and anovulatory cycles.

model analysis of 84 cycles examined the influence of the chosen progesterone threshold (rising in steps of 2 ng/ml from 6 ng/ml to 18 ng/ml) on both the distribution of the ratio of ovulatory to anovulatory cycles and also on the differences in bone markers. **Table 4** shows the results for the bone resorption marker CTX. If a progesterone threshold of 6 ng/ml was used as sole criterion, 65 of the 84 analyzed cycles in this model were ovulatory, while this number declined to 34 with a progesterone cut-off of 14 ng/ml and to 11 using a threshold of 18 ng/ml. Considering further the monitor display for ovulation likelihood (a function of the ratio of pre-ovulatory urinary estrone 3-glucuronide and LH), the number of evaluable cycles was further reduced, and the number of ovulatory cycles was 49 for progesterone > 6 ng/ml and 25 for progesterone > 14 ng/ml. The participant was included as a random effect in this model. Estimation of covariance parameters was 0.000159, meaning that effects of interindividual participant variations were very small.

Since the timing of the follicular phase serum sample preceded ovulation in the same cycle, ovulation was only likely to affect the differences in the intraindividual changes of bone markers by affecting the second phase of the cycle. This contributed to our interest in factors determining specifically the second phase of the cycle, such as the presence or absence of relevant progesterone concentrations.

A graphic demonstration of the influence of the chosen progesterone threshold on the delta of bone markers in ovulatory vs. anovulatory markers is shown in **Figs. 1** (BAP) and **2** (PYD). Bone formation as reflected by (BAP) was elevated only in the luteal phase of ovulatory, but not in anovulatory cycles, while bone resorption markers (PYD, DPD, CTX) were decreased more in ovulatory cycles than in anovulatory cycles (**Fig. 2**).

Rising cut-off progesterone values beyond 12 ng/ml were associated with a steep within-cycle rise in the bone formation marker bone alkaline phosphatase (**Fig. 1**) and a marked within-cycle drop in the bone resorption marker pyridinoline (**Fig. 2**), indicating that – different from anovulatory cycles in which there is almost no delta for the bone markers – ovulatory cycles with values beyond 12 ng/ml for progesterone may have increased bone formation and reduced bone resorption in their luteal phase, compared with their follicular phase. Nevertheless, with the current sample size and variations, the differences for BAP, PYD, and DPD did not reach significance.

Only seven participants had two or more bone density scans during the study. Mean trabecular bone density by QCT was  $157 \pm 33.5$  mg calcium hydroxyl-apatite/cm<sup>3</sup> at the start of the study and  $146.0 \pm 32.95$  mg calcium hydroxyl-apatite/cm<sup>3</sup> at the end of the study. On average, 4.17 mg calcium hydroxyl-apatite/cm<sup>3</sup> or 2.57% of bone density were lost annually by the participants. Non-significant negative correlations with monthly bone density loss were observed for both mean (FP + LP/2) estradiol ( $R^2 = 0.168$ ;  $p = 0.274$ ) and luteal progesterone ( $R^2 = 0.032$ ;  $p = 0.646$ ). No correlation between the rate of ovulations in percent ( $R^2 = 0.008$ ;  $p = 0.815$ ) and bone density change was observed in this very small group. Three participants with an average luteal progesterone value of < 9 ng/ml in 14 cycles had lower bone densities than four participants with average progesterone values of > 9 ng/ml in 50 cycles ( $p = 0.112$ ). No formal interaction analysis was performed due to the small number of participants.

## Discussion



In this study, luteal phase FSH values were normal in the ovulatory cycles, but elevated in cycles classified as “possible ovulation” or “anovulatory”. Estrogen values during the follicular phase were independent of the ovulatory process but on average, study participants’ mean estradiol was near the upper limit of the reference range for premenopausal women. In the luteal phase, they were within the reference range in ovulatory cycles and “possible ovulation cycles” whereas they were low in anovulatory cycles. As progesterone was part of the definition of ovulatory vs. anovulatory, the average values for progesterone were distributed accordingly.

These results correspond with the earliest hormonal processes before the beginning of clinical perimenopause. The average age of patients at the beginning of the study was 45 years. McKinlay et al. reported perimenopause to start with cycle irregularities after the 47th year on average and the final menstrual period (FMP) to follow at 51.4 years [8]. Skurnick et al. stated that anovulatory cycles alone are no sign for an impending menopause during the next two years [9].

10.2% of the cycles in this pilot study were without any proof of ovulation during the whole study period. The Daily Hormone Study (DHS) of the Study of Women’s Health Across the Nation (SWAN) included 848 women aged 43–53 years at baseline who collected daily urine for one cycle or up to 50 days annually for 3 years. Anovulatory cycles increased from 8.4 to 24% in the 3 years, while ovulatory-appearing cycles decreased from 80.9 to 64.7% by year three [10].

The average values of bone markers for formation (BAP) as well as for bone resorption (PYD, DPD, CTX) remained within the reference range in the follicular and luteal phase in cycles with ovulation, possible ovulation and anovulation in premenopausal women. The intracyclic changes of the bone resorption marker CTX differed significantly between ovulation and anovulation ( $p < 0.001$ ), with less resorption activity in ovulatory cycles. The bone resorption markers PYD and DPD were lower after ovulation than after anovulation, while average values of the bone structure marker BAP were higher (all  $p = n.s.$ ). For the bone resorption marker CTX a significant difference was also detectable depending on whether the *previous* cycle had been ovulatory or possibly ovulatory vs. anovulatory ( $p < 0.05$ ).

The impact of ovulation on bone metabolism has not been extensively studied. Adami et al. showed significantly elevated bone markers in women with hypothalamic amenorrhoea in a study of 136 premenopausal patients [11]. Nielsen et al. found higher concentrations of the bone formation markers osteocalcin and BAP in the luteal phase in a study of 8 healthy women between 20 and 47 years and postulated a higher activity of osteoblasts during the luteal phase compared with the follicular phase [12]. Shimizu et al. found no changes in the bone markers BAP and N-terminal telopeptide (NTX) during the menstrual cycle in a study with 15 young women [13]. Chiu et al. detected no changes of BAP in serum or DPD in urine in a study on 20 premenopausal women studied for a single cycle. DPD in serum correlated negatively with progesterone, while urinary DPD showed no significant correlations with progesterone. Low progesterone concentrations in the follicular phase seemed to be associated with a higher bone resorption [14]. Gass et al. found that the average concentrations of CTX during the follicular phase were significantly higher than in the luteal phase in an examination of 55 premenopausal women. However, no significant changes for os-

teocalcin and BAP were observed during a single cycle [15]. The results of these international studies are in accordance with the results of our study. The bone resorption marker CTX seems to be influenced by cycle phase, ovulation and progesterone.

The average change in trabecular bone density during this study was 2.57% per year, which is in the range of the least significant change (LSC) for QCT measurements. The number of ovulatory cycles during the study correlated negatively with bone density, participants with mainly ovulatory cycles lost less bone density on average than those with mainly anovulatory cycles, however these differences did not reach significance ( $p = 0.628$ ) due to the small numbers. Patients with high average luteal progesterone concentrations ( $> 9$  ng/ml) had lower trabecular bone density than patients with average luteal progesterone concentration  $< 9$  ng/ml ( $p = 0.112$ ).

The correlation between ovulation and bone density is discussed controversially in the literature. Prior et al. reported reduced bone density in women with anovulatory cycles in a study of 66 premenopausal women aged 21 to 42 years [16]. Ouynag et al. published lower bone density in premenopausal women with oligomenorrhoea in a cross-sectional study of 4771 women between 30 and 49 years of age [17]. Waugh et al. detected a significantly negative effect of ovulation disturbances (anovulatory cycles or luteal phases  $< 10$  days) on annual bone density loss in a prospective cohort study over 2 years with 225 young premenopausal women (average age  $32.4 \pm 4.6$  years) who each contributed an average of 9.8 cycles over the two years [18].

In a cross-sectional analysis of bone density data within the Daily Hormone Study (DHS, a sub-study of the Study of Women’s Health Across the Nation (SWAN)), Grewal et al. examined 643 women with a mean age of almost 47 years and thus very similar to the present study, but monitoring only one menstrual cycle of up to 50 days per participant per year over 3 years [11]. They found no significant associations between BMD levels and log urinary LH and log urinary pregnanediol-glucuronide (PdG), AUC, after adjustment for age, BMI, race, menstrual-cycle length, and menopausal status. Since anovulation was found to be over four-fold more likely in obese participants, as the later analysis by Santoro of the same sub-study showed [10], the adjustment for BMI may have eliminated the effect of anovulation on bone density in this earlier 2006 publication [11].

In a longitudinal study of 53 premenopausal women who collected daily urine samples for an average of 4.1 cycles, Waller et al. could not prove a relation between the luteal phase length or progesterone metabolites on the one hand and either baseline bone density or percent yearly bone density loss (BMD being measured at baseline, 9 and 18 months) on the other hand [19]. Bemben et al. could not provide evidence for a significant difference concerning the bone density of women with vs. without cycle disturbances in a study on young female athletes [20]. De Souza et al. examined the correlation between reduced ovarian progesterone-production and diminished bone density in a very small study on 33 women who were subdivided into three groups and followed for only 3 months, without finding a positive correlation between BMD and P4. Long follicular phases and reduced estrogen production were associated with reduced bone density in this contribution [21]. Lu et al. found no correlation between luteal progesterone concentration and bone density in a cross-sectional study of 242 premenopausal women, taking six blood samples in 2 cycles, all between day 20 and 24 but using only the first three samples for serum hormones, i.e. the means of one cycle [22].

Progesterone receptors have been found in the membranes of osteoblasts and osteoclasts [23,24]. Former experimental studies of our own group have shown a significant rise in osteoblast differentiation after incubation with physiological concentrations of progesterone [25]. Liang et al. also found that progesterone stimulates the proliferation of the osteoblasts and their differentiation in an in vitro study on human normal osteoblasts. This group concluded that progesterone and even more so 18-methyl-levonorgestrel promote osteocalcin gene transcription, resulting in osteoblast proliferation and differentiation in human osteoblasts, while no effect was noted in the osteosarcoma cell line [26]. Overall, finding a correlation between ovulation and progesterone on the one hand and bone density on the other hand depends strongly on the size and duration of the study and on the age and menstrual history of participants, yet in a meta-analysis of six studies in 436 women, Li et al. detected a negative influence of ovulatory disturbances amounting to  $-0.9\%$  per year. The effect seems physiologically possible both through progesterone receptors in osteoblasts directly as well as through indirect influences via corticosteroid receptors which also bind progesterone, leading to a possible inhibition of bone resorptive effects of endogenous cortisone by progesterone. While not affecting cortisol production, progesterone may act as a competitive inhibitor of cortisol effects at the receptor levels, thereby contributing to a decrease in bone resorption. However, no clinical trials with progesterone application and bone density endpoints have been performed in premenopausal women. None of the women in this study took vitamin D regularly, none were immobilized and none were excessively sportive. The well-documented importance of adequate vitamin D intake to prevent osteomalacia and the impact of adequate load on osteoid mechanoreceptors for enhancement of bone formation by medium impact physical activity on solid ground (walking, hiking, dancing) are not in question by our findings. Regarding BMI, a U-shaped correlation exists between fracture risk and BMI, with both extremely low BMI and also very high BMI (often associated with physical inactivity) exhibiting a negative impact on bone health and density. However, since these influences are often stable over many years and usually impact bone over a longer period of time, the within-cycle changes in bone metabolism reported here are unlikely to have been influenced by sudden changes of the other factors in the individual participant. The significance of our findings lies in an expanded association of ideas relating ovaries with bone beyond estrogen alone. The incidence of radial (forearm) fractures – one of the three typical osteoporotic fractures besides vertebral and femoral fractures – rises in the fifth decade, i.e. starting after age 40. This rise in incidence well before the average age of menopause (52 years) is not only observed in women with very early menopause, and it could point to other factors involved in ovarian function. Bone morphogenic proteins, involved both in the process of ovulation and in bone remodeling, could be one connection. Progesterone may be a biomarker of ovarian function in this context or a bone-protective hormone in itself. Importantly, therapeutic administration of progesterone has not been studied in premenopausal women. The anti-resorptive effect of estrogen is estimated to be approximately fourfold higher than the formation-enhancing effect of progesterone. Therefore, despite the inhibition of ovulation by hormonal contraception, estrogen-containing hormonal contraceptives may still be beneficial to bone, particularly in women with both low estrogen and low androgen production.

## Summary

▼ This study highlights the relevance of subclinical ovulatory disturbances in young women: anovulation may not only lead to increased demands of assisted reproduction, but also impact on women's health later in life [27]. Recent data from Norway showed a point prevalence of 30% anovulation in a population-based sample of the epidemiologic HUNT study [3], this may amount to a considerable gap in bone accrual of 9 to 18% over the course of 10–20 years. Since the rate of anovulation in the present study was low (with 10.2% women without evidence of ovulation throughout the study), a confirmatory study has been conducted with more participants and a higher age at entry (> 45 years) in order to record a higher percentage of anovulatory cycles, which will be reported in due time. A study in younger women would be of great interest, but a high percentage of young women is on hormonal contraception and a low rate of spontaneous anovulation is to be expected in this age group. Therefore, a high screening-to-recruiting-ratio would be expected in younger age groups, so that such a study would likely need to be conducted in a multi-center setting.

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## Conflict of Interest

▼ None.

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