# Importance of Biological Variability of bone markers in clinical practice

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## Variability of bone markers

*Laboratory*

**Preanalytical**
Sampling
Preanalytical treatment
Storage

**Analytical**
Imprecision
Inacuraccy
Interferences: lipemia …

**Interpretation of results**

*Individual*

Timing
Conditions of sample collection

Uncontrollable factors: age, gender, ethnicity, recent fracture, pregnancy and lactation, immovilization, treatments and diseases

Controllable Factors: biological rhythms, exercise, fasting

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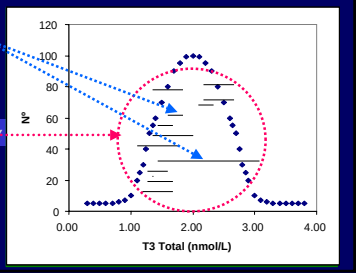
## Biological variability

- **Inherent random fluctuation of constituent concentrations in human fluids around the homeostatic set point**

*Within-individual variability*

*Between-individual variability*




## Variability in the individual

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## Controllable factors

- Circadian rhythms
- Menstrual cycle
- Seasonal variation
- Feeding
- Exercise

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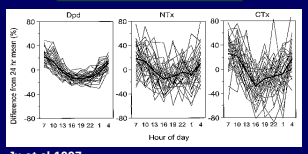
## Effect of circadian rhythms

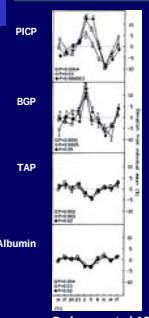
Of all sources of variability that affects the preananlytical phase, circadian rhythm has the most impact

Markers of bone resorption




Ju et al 1997

Markers of bone formation




Pedersen et al 1995

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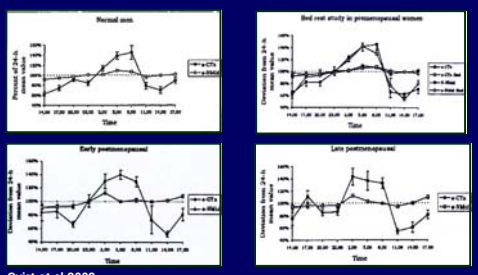
## Effect of circadian rhythms



Qvist et al 2002

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## Effect of menstrual cycle

Discrepancy in the results:

- Serum BGP and BAP are 10 % higher in the luteal phase than in the follicular phase (Nielsen et al 1990) and markers of bone resorption are increased in the follicular phase (Chiu et al 1999, Zitterman et al 2000)
- BAP and CTX increase around the period of ovulation and tend to decrease in the luteal phase (Gorai et al 1998). Urinary NTX higher in women close to ovulation (Glover et al 2008)

The best time for sampling probably is during the first 3-7 days of the menstrual cycle (Seibel 2005)

## Effect of seasonal variation

| | Males | | Females | |
|---|---|---|---|---|
| | Winter | Summer | Winter | Summer |
| BAP (ng/mL) | 12.9 ± 7.1 | 10.2 ± 4.4[c] | 13.6 ± 6.1 | 11.7 ± 4.6[a] |
| BGP (ng/mL) | 8.6 ± 4.3 | 7.5 ± 2.8[a] | 10.8 ± 4.2 | 9.3 ± 3.8[b] |
| PYD (nM/mM Cr) | 25.8 ± 10.6 | 23.6 ± 10.7 | 38.5 ± 15.4 | 28.1 ± 12.2[c] |
| DPD (nM/mM Cr) | 6.3 ± 2.9 | 5.7 ± 2.9 | 10 ± 4.8 | 7.8 ± 3.3[c] |

Results are expressed as the mean ± SD a: p< 0.05; b: p< 0.01; c: p< 0.001 summer vs winter
Woitge et al 1998

- Other studies couldn't find any seasonal change when they measure the same markers

⇒ It seems not to be an important source of variability (2 % interindividual variability)

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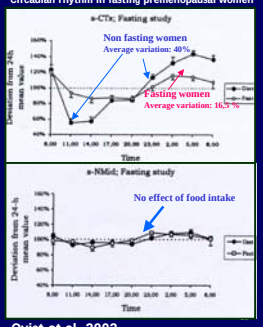
## Effect of feeding

Food intake plays a major role

| | Magnitude of the decrease after a meal (%) mean± SE |
|---|---|
| BAP | - 1.6 ± 1 % |
| BGP | - 4.1 ± 1.1 % |
| PINP | - 3.8 ± 0.9 % |
| DPD | - 7.5 ± 1.5 % |
| u-NTX | - 7.9 ± 3.7 % |
| u-CTX | - 7 ± 2.6 % |
| s-NTX | - 8.5 ± 1.7 % |
| s-CTX | - 17.8 ± 2.6 % |

Clowes et al 2002

Circadian rhythm in fasting premenopausal women




Qvist et al. 2002

## Effect of exercise

It is difficult to quantify as it depends on the age of the subject and the type and intensity of the exercise

- Moderate exercise: increase in bone formation markers and decrease in bone resorption markers
- Acute effect of exercise: increase markers of collagen formation and degradation by 15-40 %. These variations persist for 24 hours and possibly as long as 72 hours

⇒ Ask about regular exercise and tell the subject not to exercise for at least 24 hours before samples are collected

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## Uncontrollable factors

- Age and gender
- Ethnicity
- Geographical differences
- Fractures
- Oral contraceptives
- Bed rest and immobilization
- Smoking
- Diseases
- Treatments

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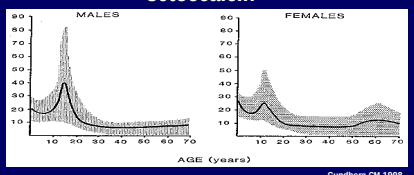
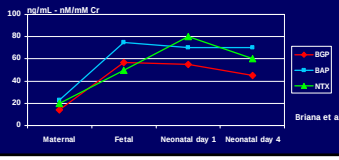
## Age and gender (1)

### Osteocalcin




Gundberg CM 1998

### Perinatal bone turnover




Briana et al 2008

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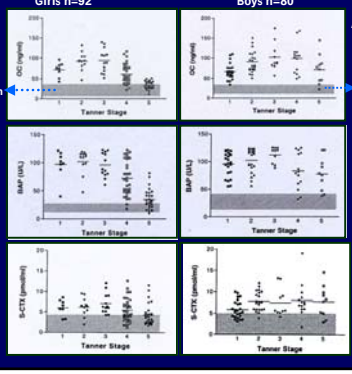
## Age and gender (2)

Girls n=92 | Boys n=80

Age: 10-17 years

Normal range in premenopausal women

Normal range in adult males




Fares et al 2003

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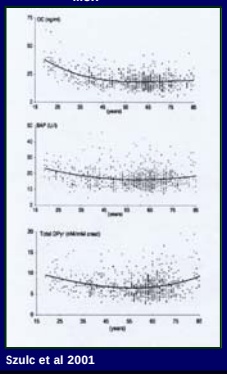
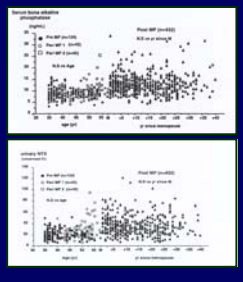
## Age and gender (3)

Men



Szulc et al 2001

**Women:**
**Bone markers are elevated under 35 and remain stable between the ages of 35 and 45**
*Glover et al 2008*



Garnero et al 1996

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## Ethnicity

- There is a trend for markers of bone resorption to be lower in black children (Pratt J et al 1996)
- In young men and women there are no consistent differences (Kleerekoper 1994, Bikle 1999, Dibba 1999)

| | Blacks (african-caribbean) | | Whites (caucasian) | |
|---|---|---|---|---|
| | Men (n=16) | Women (n=28) | Men (n=28) | Women (n=31) |
| BAP (U/L) | 44.4 (3.4) | 34.5 (2.7) | 53.5 (2.5) | 33.7 (2.5) |
| BGP (ng/mL) Intact +N-Mid | 29.9 (2.7) | 21.6 (2.1) | 33.2 (2.0) | 24.2 (2.0) |
| ifDPD outpout (nM/day) | 60.3 (5.7) | 50.3 (4.2) | 58.8 (4.3) | 47.9 (4.1) |
| NTX outpout (nM BCE / day) | 564 (75) | 338 (55) | 729 (56) | 282 (53) |

Age: 25- 35 years. Results are expressed as the mean (SE) Henry and Eastell 2000

- In peri- and postmenopausal women markers are 5-15 % lower in black (Kleerekoper 1994, Han 1997)

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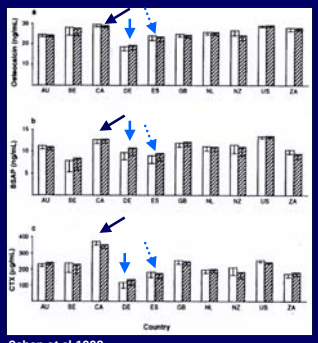
## Geographical differences



n= 619 caucasian women

Values before (open bars) and after (hatched bars) multivariate adjustment for age, total serum cholesterol level, FSH level, vit D level and years posthysterectomy

Cohen et al 1998

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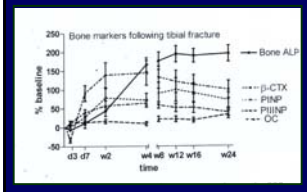
## Fractures

n= 18 Range of age: 18 -78 years, mean: 34




Veitch et al 2006

* Bone turnover increases after fracture but the timing and magnitude of this increase varied for the different markers

n= 85 women out of 1044 (Malmo OPRA study)
Age, mean (SD): 77.9 (1.8) years

| | Before fracture | Immediately after fracture | 4 months after fracture |
|---|---|---|---|
| BGP (ng/mL) | 3.33 (2.4-4.6) | 2.77 (1.7-4.2) | 4.83 * 83.4-6.6) |
| PINP (ng/mL) | 52.2 (41-60) | 43.8 (34-60) | 67.1 * (45-92) |
| TRAP5b (U/L) | 3.47 (2.9-4.1) | 3.36 (2.7-4.1) | 3.83 * (3.1-4.5) |
| s-CTX (nM) | 300 (236-420) | 342 (192-471) | 390 * (257-499) |
| u-total DPD (nM/mM Cr) | 9.21 (6.8-12) | 8.3 (6-10) | 10.23 * (8-15) |

Results are expressed as mean and interquartile range
* p<0.001 Ivaska et al 2007

•Immediate postfracture sampling may provide information on the baseline state of a fractured patient
• At least 12 months are needed to eliminate the effect of a recent fracture

## Effect of oral contraceptives

| Age, mean years (range) | Pill users (n=119) 36.3 (30-45) | Pill non-users (n=118) 38.3 (28-45) |
|---|---|---|
| *Bone formation* | | |
| BAP (ng/mL) | 8 (2.8) | 9.8 (2.8)* |
| PINP (ng/mL) | 32.6 (13.7) | 45.1 (16.6)* |
| *Bone resorption* | | |
| s-CTX (pg/mL) | 251 (128) | 304 (137)** |
| u-NTX (nM/mM Cr) | 19.3 (14.3) | 29.8 (16.2)* |

Values are the mean (SD) *p<0.0001; **p=0.009 vs users De Papp et al 2007

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## Effect of bed rest and immobility

Increase in bone resorption and little or no effect on bone formation

*After 10 days, values of u-PYD and u-DPD increase from 20 % to 44 %

* Once remobilization occurs, resorption markers gradually return to initial levels

Lueken et al 1993; Zerwekh et al 1998; Smith et al 1998; Uebelhart et al 2000

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## Effect of smoking

**Men**

| | Current smokers | p | Former smokers | p | Never smokers |
|---|---|---|---|---|---|
| BGP (nM/L) | 3.5±1.3 | 0.59 | 3.5±1.2 | 0.11 | 3.6±1.1 |
| BAP (U/L) | 16±5 | 0.37 | 16±5 | 0.82 | 16±5 |
| PINP (ng/mL) | 38±17 | 0.17 | 34±15 | 0.68 | 35±13 |
| Total DPD (nM/mM Cr) | 7.3±2.7 | <0.01 | 6.7±2.3 | 0.91 | 6.8±2.5 |
| Free-DPD (nM/mM Cr) | 3.6±1.2 | <0.03 | 3.4±1 | 0.79 | 3.4±1.1 |
| u-CTX (µM/ mM Cr) | 134±68 | <0.005 | 115±62 | 0.39 | 123±58 |

Values were adjusted for age, body weight, caffeine and ethanol intake — Szulc et al 2002

Conflicting results in women
* Higher circulating levels of BAP (32 %) and NTX (33 %) in current smokers (*Glover at al 2008*)
* Reduced levels of BAP, PICP, BGP, u-PYD, u-DPD in current smokers (*Woitge et al; Bjarnason et al 1999*)

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## Effect of diseases

Related to the endocrine system
- Estrogen deficiency
- Hypercortisolism, hypogonadism, hyperparathyroidism and hyperthyroidism
- Acromegaly, growth hormone / receptor deficiencies and other growth disorders

Related to other diseases
- Multiple myeloma, hypercalcemia of malignancy
- Breast and prostatic cancer
- Renal insufficiency or failure
- Liver diseases
- Rheumatoid arthritis and other connective disorders

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## Effect of treatments and other factors

Chronic therapy with:
- Corticosteroids (glucocorticoids)
- Anticonvulsants
- Excess thyroid hormone
- Gonadotropin-releasing hormone agonists
- Heparin

Related to nutrition:
- High sodium intake
- Alcohol consumption
- Anorexia and bulimia

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## Variability in the laboratory

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## Preanalytical phase (1)

**Before the analysis:**

- *Type of sample to analyse*: **serum / plasma (EDTA, heparin anticoagulant) or urine (24-hour collection, spot, first or second morning void)**
- *Protocols describing patient preparation*
- *Best time for obtaining samples*: **For both serum and urine sampling is always done between 8 and 9 in the morning after a fast of at least 6 hours**

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## Within-individual variability of bone markers

**Choosing the sample to analyse**

| | CV |
|---|---|
| s-BAP | 3.4 % |
| s-PINP | 6.2 % |
| s-CTX | 9.3 % |
| u-CTX | 25.6 % |
| u-HYP | 19.3 % |
| u-NTX | 17.2 % |

Alvarez et al 2000

Serum and second morning void urine collected every two months during 1 year in 11 healthy women

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## Preanalitical phase (2)

**Handling**

- **Enzymatic hydrolysis in vitro (BGP)**
- **Instability of PINP at 37 °C for more than 4 hours**
- **Effect of intensive ultraviolet irradiation on aqueous solutions of pyridinium crosslinks. No effect of fluorescent light and filtered daylight**

**Storage**

- **In general, a storage temperature of at least -40°C for serum samples and of -30°C for urine samples, is recommended.**

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## Analytical phase

- **Instability of PINP at 37°C**
- **Ultraviolet irradiation (DPD and PYD)**
- **Haemolysis, lipemia and bilirubin present in the sample**
- **Imprecision**
  - **Repeatability (intra-assay, intra-day)**
  - **Reproducibility (inter-assay, inter-day)**
- **Inaccuracy**

**There are no reference methods to assess inaccuracy of the assays used in analysing bone markers.**

→ **Use of interlaboratory comparison studies**

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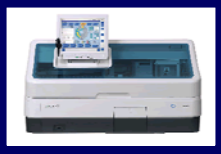
## Automated methods



### Advantages

- No specimen manipulation: less biological risk
- No identification errors
- Less technical complexity
- Improved turnaround time
- Lower intra- and interday variability

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## Analytical imprecision

| | Method | Intra-assay CV % | Inter-assay CV % |
|---|---|---|---|
| TAP | automated | 0.75 | 3.1 |
| | | | |
| BAP | IRMA | 3.5 | 8.3 |
| BAP | automated | 2.3 | 3.7 |
| | | | |
| PINP | RIA | 3 | 5.6 |
| PINP* | automated | 1.7 | 4.4 |
| | | | |
| s-β-CTX | EIA | 6 | 9.2 |
| s-β-CTX | automated | 2 | 2.6 |

* Garnero et al 2008

Alvarez et al 2000

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## Interlaboratory comparability

| | Method | Minimum | Maximum | Max/ Min | Average | SD | CV % |
|---|---|---|---|---|---|---|---|
| BAP | IRMA (ng/mL) | 21 | 39.9 | 1.9 | 29 | 4.6 | 16 |
| | EIA (U/L) | 27.5 | 63.6 | 2.3 | 46 | 8.7 | 20 |
| BGP | LIA (ng/mL) | 10.2 | 16 | 1.6 | 13 | 1.9 | 16 |
| | EIA | 14.9 | 24.6 | 1.7 | 17.3 | 4.1 | 24 |
| | all RIAs (18) | 2.6 | 79.6 | 30 | 20.7 | 17.1 | 71 |
| f-DPD | EIA (nmol) | 61 | 73 | 1.2 | 67.3 | 4.3 | 6.4 |
| | CLIA | 69 | 100 | 1.5 | 79.1 | 8.8 | 11 |
| NTX | EIA (nmol) | 410 | 1564 | 3.8 | 820 | 320 | 39 |

Seibel et al 2001

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## Interpreting results

We should take into consideration:

All sources of uncontrollable variability and analytical variability of bone markers in our laboratory

→ To know if the result is normal or not, we must use adequate reference values

→ For monitoring response to treatments, we need to know if the change between two consecutive values is due to treatment itself

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## Establishing reference ranges

Use the same conditions for reference individuals as for patients: preparation before sampling, time of sampling, analytical methods

**Partitioning factors**
- Age
- Gender
- Ethnicity (Race)
- Geographical location

**Exclusion criteria**
- Oral contraceptive use
- Antiosteoporotic treatment
- Diseases, treatments and conditions before shown

Goal of antiresorptive therapy in postmenopausal women at risk of fragility fractures: reduce bone turnover markers to within the lower half of the reference range for healthy young premenopausal women

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## Monitoring changes in individual patients

$$LSC: 1{,}96 * \sqrt{2} * \sqrt{(CVbi^2 + CVa^2)}$$

| | |
|---|---|
| TAP | 15,2 % |
| BAP | 26,3 % |
| PINP | 23,1 % |
| s-CTX | 36,2 % |
| u-HYP | 55,1 % |
| u-DPD | 24,4 % |
| u-NTX | 54,6 % |
| u-CTX | 73,1 % |

LSC: Least significant change

Alvarez et al 2000

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## Summary

*** Biological variability:**
- Controllable factors: use of strict protocols for patient preparation, time of sampling, handling and storage
- Uncontrollable factors: use of appropriate reference ranges and take into account all possible variations that have been published when interpreting results

***Analytical Variability:**
- Analyze bone markers in automated analysers
- Use of internal quality control program that warns us in advance if results don't fulfil specifications predefined by the laboratory
- Participate in external Quality Programs Assurance

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