One-year B and D vitamins supplementation improves metabolic bone markers and bone metabolism

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Osteoporosis

Multifactorial pathologic condition which affects the entire skeleton and is characterized by a low bone mass in combination with a poor bone quality:

- Reduced bone mineral density (BMD)
- Deteriorated bone architecture (e.g. cross-links of collagen)
- Altered bone metabolism

→ Higher bone fracture risk
Bone remodeling

Risk factors for osteoporosis:

Higher Age
↓ 1,25(OH)$_2$ vitamin D$_3$
↑ PTH (secondary hyperparathyroidism)

↓ Estrogen

Genetic factors

Hyperhomocysteinemia

...
Experimental Hyperhomocysteinemia

35 Wistar rats (age 3 month)

Blood sampling

Blood sampling

12 weeks diet

Controls (n=17)
Methionine 2.4% (n=10)
Homocystin 2% (n=8)

Plasma Homocysteine (µmol/l)

Ko Meth Homo

Homocysteine and Osteoporosis - In Vivo Studies

HHCY reduces
- Bone strength
- Trabecular bone mass

Tissue Specific Enrichment of Homocysteine in bone collagen

65% of homocysteine in bone is bound to collagen !!!

Herrmann M et al. Bone. 2009 Mar;44(3):467-75
Hyperhomocysteinemia disrupts nanostructure of the extracellular bone matrix
Homocysteine accumulates especially in bone tissue.

Herrmann M et al. Bone. 2009 Mar;44(3):467-75
Homocysteine: a risk factor for osteoporosis

- **Interference with normal collagen cross-link formation**
- **Reduction of bone toughness**, independent from BMD
- **Accumulation of Hcy in bone tissue** (mostly bound to collagen)
- **Induction of oxidative stress**, leading to endothelial dysfunction, decreased bone blood flow, and eventually to osteoporosis

Hcy lowering therapy (supplementation with folic acid, vitamins B<sub>6</sub>, and B<sub>12</sub>) can favorably influence the course in osteoporotic patients!

One Year Vitamin Therapy with Subjects of Rehabilitation Sports

Volunteer randomization (n=111)

Blood and urine samples (n=96)

Group A
Calcium, vitamin D, and B-vitamins
Daily:
456 mg Ca, 1200 IE Vit D, 500 µg FA, 50 mg B₆, 500 µg B₁₂

n=50

Blood and urine samples (n=65)

Group B
Calcium and vitamin D
Daily:
456 mg Ca, 1200 IE Vit D

n=46

Supplementation for 1 year

n=34

n=31

Blood and urine samples (n=65)

Herrmann W et al.
2013 Mar 1;51(3):639-47
Analysis of metabolites and vitamins

25-(OH)-VitD₃

Parathyroid hormone (PTH)

- Bone formation markers:
  - Osteocalcin (OC)
  - Bone-specific alkaline phosphatase (BAP)

- Bone resorption markers:
  - Tartrate-resistant acid phosphatase 5b (TRAP5b)
  - Deoxypyridinoline (DPD) cross-links (urine)

- Bone mineralization
  - Sclerostin (SCL)

- Folate and B₁² markers:
  - 5-MTHF
  - total B₁², holoTC, MMA
  - tHcy

Participant characteristics (n=96)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A Ca + D + B</th>
<th>Group B Ca + D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females, n</td>
<td>15/35</td>
<td>24/22</td>
</tr>
<tr>
<td>Median age, y</td>
<td>68</td>
<td>71</td>
</tr>
</tbody>
</table>

### Characteristics of participants at baseline and 1-year after

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A Median</th>
<th>Group B Median</th>
<th>Group A Median</th>
<th>Group B Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH, pg/ml</td>
<td>63,7</td>
<td>77,9</td>
<td>45,7</td>
<td>45,8</td>
</tr>
<tr>
<td>Vitamin D ng/ml</td>
<td>16,0</td>
<td>15,8</td>
<td>32,0</td>
<td>28,6</td>
</tr>
<tr>
<td>5-MTHF serum, nmol/l</td>
<td>18,8</td>
<td>21,1</td>
<td>47,0</td>
<td>16,3</td>
</tr>
<tr>
<td>5-MTHF whole blood, nmol/l</td>
<td>545</td>
<td>489</td>
<td>1329</td>
<td>534</td>
</tr>
<tr>
<td>Vitamin B12, pg/ml</td>
<td>379</td>
<td>390</td>
<td>622*</td>
<td>354*</td>
</tr>
<tr>
<td>HoloTC, pmol/l</td>
<td>53</td>
<td>47</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tHcy, µmol/l</td>
<td>12,9</td>
<td>12,3</td>
<td>8,9*</td>
<td>13,8*</td>
</tr>
<tr>
<td>Cys, nmol/l</td>
<td>232</td>
<td>225</td>
<td>152*</td>
<td>283*</td>
</tr>
<tr>
<td>MMA, nmol/l</td>
<td>212</td>
<td>212</td>
<td>224</td>
<td>274</td>
</tr>
<tr>
<td>SAH, nmol/l</td>
<td>17,6</td>
<td>18,3</td>
<td>20,2</td>
<td>22,5</td>
</tr>
<tr>
<td>SAM, nmol/l</td>
<td>121</td>
<td>116</td>
<td>128</td>
<td>118</td>
</tr>
<tr>
<td>Betain, µmol/l</td>
<td>33,2</td>
<td>32,0</td>
<td>36,9</td>
<td>32,8</td>
</tr>
<tr>
<td>Cholin, µmol/l</td>
<td>8,9</td>
<td>8,9</td>
<td>12,0</td>
<td>11,1</td>
</tr>
<tr>
<td>DMG, µmol/l</td>
<td>2,84</td>
<td>2,96</td>
<td>3,00*</td>
<td>3,76*</td>
</tr>
</tbody>
</table>

Normal ranges in plasma: Betaine 19 – 47 µmol/L, Choline 7 – 12,3 µmol/L, DMG 1,5 – 4,3 µmol/L, SAH 3,3 – 23 nmol/l, SAM 63 – 107 nmol/l

*p < 0.05 between Group A and B

5-MethylTHF (nmol/L) in serum at baseline and after 6 and 12 months

Data are presented as means (95% CI). p values (baseline vs. 6 and 12 months) according to repeated measure ANOVA test.

5-MethylTHF (nmol/L) in whole blood at baseline and after 6 and 12 months

Group A
Ca + D + B
n=19

Group B
Ca + D
n=22

Baseline
6 months
12 months

Sufficient
Deficient
<567 nmol/L TFOL

Data are presented as means (95% CI).

p values (baseline vs. 6 and 12 months) according to repeated measure ANOVA test

Vitamin B$_{12}$ (ng/mL) at baseline, after 6, and after 12 months

- **Baseline**: 549 pg/mL  
- **6 months**: 622 pg/mL  
- **12 months**: 352 pg/mL

**Group A**: Ca + D + B  
- **Baseline**: 379 pg/mL  
- **6 months**: 549 pg/mL  
- **12 months**: 390 pg/mL

**Group B**: Ca + D  
- **Baseline**: 354 pg/mL  
- **6 months**: 352 pg/mL  
- **12 months**: 354 pg/mL

**p values (group A vs. Group B)** according to Mann-Whitney-U test; **p values (baseline vs. 6 and 12 months)** according to repeated measure ANOVA test.

**Herrmann W et al.**  
*Clin Chem Lab Med.*  
2013 Mar 1;51(3):639-47
Total Hcy (µmol/L) at baseline and after 6 and 12 months

5-MethylTHF (nmol/L) in serum and whole blood according to tHcy

**25-(OH)-VitD<sub>3</sub> and PTH concentrations at baseline and after 12 months**

**Correction of vitamin D insufficiency in both groups**

25-(OH)-VitD<sub>3</sub>, ng/mL

- **Group A**: Ca + D + B (Baseline: 16.0, 15.8; 12 months: 32.0, 28.6)
- **Group B**: Ca + D (Baseline: 25.0, 74.0; 12 months: 28.6, 46.2)

**Correction of secondary hyperparathyroidism in both groups**

PTH (median), pg/mL

- **Group A**: Ca + D + B (Baseline: 66.5, 10.4; 12 months: 66.7, 46.0)
- **Group B**: Ca + D (Baseline: 72.7, 46.2; 12 months: 72.7, 46.2)

**Baselines**

- **Group A**: Ca + D + B
- **Group B**: Ca + D

**12 months**

- **Group A**: Ca + D + B
- **Group B**: Ca + D

**Significance Levels**

- **Group A**: p < 0.001, p < 0.001, p = 0.050
- **Group B**: p < 0.001

## Bone turnover markers at baseline and after 12 months

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group A Ca + D + B (n=34)</th>
<th>Group B Ca + D (n=31)</th>
<th>Group A Ca + D + B (n=34)</th>
<th>Group B Ca + D (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bone formation markers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OC, ng/mL</td>
<td>8.8</td>
<td>8.3</td>
<td>5.5§</td>
<td>4.2§</td>
</tr>
<tr>
<td>BAP, U/L</td>
<td>24.8</td>
<td>29.6</td>
<td>24.1§</td>
<td>24.8§</td>
</tr>
<tr>
<td><strong>Bone resorption markers</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>TRAP5b, U/L</td>
<td>2.1↓</td>
<td>2.4</td>
<td>2.0↓</td>
<td>1.6§↓</td>
</tr>
<tr>
<td>DPD, nmol/mmol cr</td>
<td>6.8</td>
<td>3.8</td>
<td>7.0</td>
<td>5.6</td>
</tr>
<tr>
<td>Sclerostin, ng/mL</td>
<td>0.56</td>
<td>0.53</td>
<td>0.68§</td>
<td>0.73§</td>
</tr>
</tbody>
</table>

* p < 0.05 group A vs. group B (Mann-Whitney-U test)

§ p < 0.05 baseline vs. 12 months (repeated measure ANOVA test)

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Osteocalcin (ng/mL) at baseline and after 6 and 12 months

-36%  -45%

Group A  
Ca + D + B  
n=30

Group B  
Ca + D  
n=30

Baseline  6 months  12 months

p values (baseline vs. 6 and 12 months) according to repeated measure ANOVA test
Comparable changes of bone resorption and bone formation markers in the same direction in both groups which indicate the effect of vitamin D supplementation

Down regulation of bone turnover reflects bone improvement

<table>
<thead>
<tr>
<th>Bone formation markers</th>
<th>BAP</th>
<th>OC</th>
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<tbody>
<tr>
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</table>

<table>
<thead>
<tr>
<th>Bone resorption markers</th>
<th>TRAP5b</th>
<th>Sclerostin</th>
<th>DPD/Crea</th>
<th>PTH</th>
</tr>
</thead>
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</table>

<table>
<thead>
<tr>
<th>Median difference % of baseline (baseline - 12 months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.1</td>
</tr>
<tr>
<td>45.1</td>
</tr>
<tr>
<td>41.2</td>
</tr>
<tr>
<td>41.2</td>
</tr>
<tr>
<td>34.8</td>
</tr>
<tr>
<td>34.8</td>
</tr>
<tr>
<td>30.0</td>
</tr>
<tr>
<td>28.3</td>
</tr>
</tbody>
</table>

Correlation of SAM and Sclerostin at baseline

- After supplementation in group A, Sclerostin correlated with tHcy, SAH and SAM.
- Sclerostin inhibits differentiation and activity of osteoblasts, and promotes their apoptosis.
- Sclerostin expression is inhibited by PTH.

P-values were calculated using the Spearman’s rho test.
Telomeres and Osteoporosis

✧ They protect the ends of the chromosomes and conserve their length

✧ Separation of the chromosomes within the DNA-sequence

✧ Without telomeres the ends of the chromosomes would be “repaired” leading to end to end fusions with genomic instability

✧ They are the biological “clock” which determines how many times a cell can divide
Telomere influencing factors and age-associated diseases

Physical activity / Inflammatory status / Socio-economic status

Dietary factors (vitamins)  Smoke  Stress  Ethics  Gender  Paternal age

Oxidative stress – Telomerase activity – Epigentics – Sheltrin expression

TELOMERES

DNA damage – Senescence – Genomic instability – etc.

Cardiovascular diseases  Osteoporosis  Neurological disorders
  Diabetes  Cancer  Frailty
Telomere length correlates with BMD and is shorter in women with osteoporosis.

Telomere restriction fragment (TRF) length among healthy women and women with clinical osteoporosis (OP) at one and two or more sites. Women with OP at two or more sites had 206 bp shorter TRF length.

HCY and age-corrected RTL - South Tyrolean Study

$r = -0.151; p = 0.007; n = 317$

$R^2$ Linear = 0.023

- RTL (log-transformed) (n=317)
  - Vitamin B12 (log-transformed) $r=0.097; p=0.085$
  - Folic acid (log-transformed) $r=0.018; p=0.757$

Herrmann, Pusceddu et al. unpublished
Correlations between T/S at baseline and Methyl-THF in whole blood

N = 53
R = 0.357
P = 0.007

T/S after 1 year of supplementation according to tertiles of MMA

**Group A**
- Lowest tertile: 118-196.3 nmol/l
- Middle tertile: 196.3-219.7 nmol/l
- Highest tertile: 219.7-406 nmol/l

**Group B**
- Lowest tertile: 112-200 nmol/l
- Middle tertile: 200-260 nmol/l
- Highest tertile: 260-585 nmol/l

**Pusceddu, Herrmann Eur J Nutr. 2016 Jul 5. [Epub ahead of print]**
T/S after 1 year of supplementation according to tertiles of Choline

**Group A**

- **Lowest tertile**: (-36.7) - 17.9%
- **Middle tertile**: 17.9-49.9%
- **Highest tertile**: 49.9-154.1%

**Group B**

- **Lowest tertile**: (-18.5) – 11.1%
- **Middle tertile**: 11.1-33.5%
- **Highest tertile**: 33.5-98.9%

Choline percentage change Group A

**Choline percentage change Group B**

**Pusceddu, Herrmann Eur J Nutr. 2016 Jul 5. [Epub ahead of print]**
After 1 year, LINE-1 methylation increased in Group A but decreased Group B.
In group B after supplementation, subjects with tHcy >12 µmol/l had compared with <12 µmol/l reduced LINE-1 methylation, in a metabolic picture in agreement with an inhibited transmethylation because high tHcy leads to higher SAH which inhibits the demethylation of SAM to SAH.

T/S after 1-year of vitamin D and B supplementation (Group A)

This metabolic picture after vitamin supplementation indicates an improved methylation status and LINE-methylation and is related to telomere length.

MMA is a sensitive biomarker for B12 status; low MMA indicates a better functioning of the folate cycle which is directly related to the telomere length.

High choline is an expression of a preserving effect that means 5M-THF is preferentially used for re-methylation of HCY to methionine.

### Summary of the stepwise regression analyses with backward variable selection

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variables</th>
<th>Estimated effect</th>
<th>two-sided p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) T/S at baseline(^1)</td>
<td>5-methylTHF, tHcy</td>
<td>0.584</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.540</td>
<td>0.006</td>
</tr>
<tr>
<td>B) T/S percentage change in Group B (Vit. D and Ca) after supplementation(^2)</td>
<td>LINE-1 site 317 percentage change</td>
<td>-0.388</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>LINE-1 site 327 percentage change</td>
<td>-0.385</td>
<td>0.028</td>
</tr>
<tr>
<td>C) Mean LINE-1 in Group A (Vit. B, Vit. D, Ca) after supplementation(^3)</td>
<td>5-methylTHF</td>
<td>-0.398</td>
<td>0.06</td>
</tr>
<tr>
<td>D) Mean LINE-1 in Group B (Vit. D, Ca) after supplementation(^3)</td>
<td>tHcy</td>
<td>-0.68</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^1\) Variables included in the analysis were as follows: 5-methylTHF, tHcy, vitamin B\(_{12}\), vitamin D, mean LINE-1 methylation and LINE-1 methylation at sites 305, 317, 320, 327. 
\(^2\) Variables included in the analysis were as follows: percentage change of LINE-1 methylation at sites 305, 317, 320, 327 and mean LINE-1. 
\(^3\) Variables included in the analysis were as follows: 5-methylTHF, tHcy, vitamin B\(_{12}\) and vitamin D. Statistically significant parameters are in bold.
Summary / Conclusion

- Vitamin D supplementation improves bone metabolism by lowering bone turnover.
- B vit. supplementation lowers HCY modulating bone stiffness and osteoprotic risk.
- Telomeres are related to cellular aging and osteoporosis.
- B vitamins supplementation leads to higher telomere stability and telomere maintenance.
- Low B vit. status and hyperhomocysteinemia alter DNA methylation and telomere length.
- Subjects with high tHcy (>12µmol/l) showed reduced LINE-1 methylation which is in agreement with an inhibited transmethylation by Hcy.
- Long-term supplementation is needed to look further into whether vitamins D and B significantly influence the bone quality.
Thank you for your attention!

Acknowledgments:

Department of Clinical Chemistry, Saarland University, Homburg / Germany
• Dr. Susanne Kirsch-Dahmen
• Prof. Dr. Rima Obeid
• Vera Kruse
• Dr. Ulrich Hübner
• Marion Bodis
• Prof. Dr. Jürgen Geisel

Department of Clinical Pathology District Hospital Bolzano Bolzano, Italy
• Dipl.-Biol. Irene Pusceddu
• Prof. Dr. Markus Herrmann

Institute for Medical Biometry, Epidemiology and Informatics, Saarland University
• Dr. Stefan Gräber

Geriatric Center, Marienkrankenhaus, St. Wendel, Germany
• Dr. Rudolf Eckert
Vitamin D supplementation normalizes PTH and lowers bone turnover (decrease of TRAP, osteocalcin, and BAP) which is seen as improvement of bone metabolism.

B vitamin supplementation normalizes HCY which may increase bone stiffness (the osteoprototic risk).

Telomeres are related to cellular aging and osteoporosis. **We show for the first time that vitamin B supplementation improves telomere stability and maintenance.**

The supplementation with B vitamins causes a saturation of the folate dependent pathways leading to an higher stability of the telomeres and telomere maintenance.

The effect of folate metabolism on telomere length appears to be complex.

Suboptimal B vitamins status and hyperhomocysteinemia are associated with altered DNA methylation and telomere length.

Subjects with tHcy >12µmol/l showed compared with <12µmol/l reduced LINE-1 methylation (surrogate marker for global DNA methylation), in a metabolic picture in agreement with an inhibited transmethylation by Hcy.

**Long-term supplementation is needed to look further whether vitamin D and B significantly influence the bone quality.**
RTL and LINE-1 methylation changes after 1 year

A) Mean LINE-1 methylation

Group A

B) LINE-1 methylation Site317

Group A

C) LINE-1 methylation Site327

Group A

Group B

Group B

Group B

Group B

$r=-0.19; p=0.33; n=26$

$r=-0.14; p=0.48; n=26$

$r=-0.1; p=0.61; n=25$

$r=-0.5; p=0.008; n=25$

$r=-0.43; p=0.028; n=24$

$r=-0.41; p=0.034; n=25$
Subjects with 5-methylTHF >10nmol/l had compared with <10nmol/l at baseline lower LINE-1 methylation, due to a reduced methyl group transfer caused by a lower SAM formation.

Pusceddu et al. EJN 2015
### T/S after 1-year of supplementation

<table>
<thead>
<tr>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T/S correlates:</strong></td>
<td><strong>T/S correlates:</strong></td>
</tr>
<tr>
<td><strong>Negatively with MMA</strong></td>
<td><strong>Negatively with THF in serum</strong></td>
</tr>
<tr>
<td>– T/S is higher in the lowest tertile of MMA</td>
<td>– T/S is higher in the lowest tertile of THF</td>
</tr>
<tr>
<td><strong>Positively with choline percentage change</strong></td>
<td><strong>Negatively with folic acid in whole blood percentage change</strong></td>
</tr>
<tr>
<td>– T/S percentage change is higher in the highest tertile of choline percentage change</td>
<td>– T/S percentage change is lower in the highest tertile of folic acid percentage change</td>
</tr>
<tr>
<td><strong>Negatively with cystathionine difference</strong></td>
<td><strong>Negatively with cystathionine difference</strong></td>
</tr>
<tr>
<td><strong>Positively with 5,10 Methenyl-THF in serum percentage change</strong></td>
<td><strong>Positively with 5,10 Methenyl-THF in serum percentage change</strong></td>
</tr>
</tbody>
</table>
Conclusion

- **Vitamin D deficiency and secondary hyperparathyroidism:**
  → normalization of vitamin D deficiency and normalization of PTH (secondary hyperparathyroidism) in both groups

- **Bone turnover markers:**
  → significant reduction of bone resorption (TRAP5b)
  → significant reduction of bone formation (osteocalcin, BAP)
  → lowering of bone turnover

- **Hyperhomocysteinemia:**
  → corrected by vitamin B supplementation in group A (Ca + D + B)
  → normalization of bone cross-linking, reduction of fracture risk?

→ Can be interpreted as an improvement of bone metabolism.

→ Long-term supplementation is needed to look further whether vitamin D and B significantly influence the bone quality.
Summary

In this study we investigated telomere length in elderly subjects in relation to B and D vitamins, because telomeres are related to cellular aging.

Our findings show that telomere biology is influenced by vitamin B status.

The effect of folate metabolism on telomere length appears to be complex, higher folate status is associated with higher telomere length, which confirms data from the literature.

We showed for the first time that one year vitamin B supplementation improves telomere stability and maintenance.

Low vitamin B status leads to DNA hypomethylation results in an increased genomic instability and altered increased telomere length.

Telomere length is regulated by environmental and epigenetic factor like availability of folate derivatives and nucleotides.

In contrast to data from literature we did not find any significant influence of vitamin D status on telomere length.
B vitamins

- Vitamin B1 Thiamine
- Vitamin B2 Riboflavin
- Vitamin B3 Niacin
- Vitamin B12 Cyanocobalamin
- Vitamin B9 Folic acid
- Vitamin B6 Pyridoxine
- Vitamin B7 Biotin

Water-soluble vitamins
Defects in telomere maintenance molecules impair osteoblast differentiation and promote osteoporosis in mice

Pignolo R J et al.  
Defects in telomere maintenance molecules impair osteoblast differentiation and promote osteoporosis in mice.

Pignolo R J et al.  
Telomeres: the Hayflick limit

Telomeres shorten from 50-200 bp at every cycle

Tumor cell: activation of hTERT

hTERT positive cells: stem cells, germ line cells

hTERT negative cells: somatic cells

Hayflick limit

Cell divisions

Senescence
Cell death