BIOMARKERS, VOL. 9, NO. 6 (NOVEMBER–DECEMBER 2004), 479–488

Taylor & Francis
healthsciences

Vitamin K in combination with other biochemical markers to diagnose osteoporosis

C. HEISS[1]*, L. M. HOESEL[1], U. WEHR[2], T. KELLER[3], U. HORAS[1], C. MEYER[1], W. RAMBECK[2] and R. SCHNETTLER[1]

[1] Department of Trauma Surgery, Justus-Liebig-University of Giessen, Giessen, Germany
[2] Institute of Veterinary Physiology, Ludwig-Maximilians-University of Munich, Munich, Germany
[3] pe Diagnostik GmbH, Leipzig-Markkleeberg, Germany

Received 18 August 2004, revised form accepted 18 November 2004

The significance of a multiparametric classification approach of vitamin K is analysed to differentiate premenopausal (CTRL), postmenopausal non-osteoporotic (nOSP) and osteoporotic (OSP) women. Data records of women between 28 and 74 years of age were used for evaluation. Bone mineral density was determined by quantitative computed tomography of the lumbar spine using the *T*-score to diagnose osteoporosis. Vitamin K and biochemical markers of bone formation and resorption — alkaline phosphatase (AP), bone alkaline phosphatase (bAP), osteocalcin (OC), undercarboxylated osteocalcin (ucOC), procollagen type I carboxyterminal propeptide (PICP), pyridinoline (PYD), deoxypyridinoline (DPD), N-terminal cross-linked telopeptide of type I collagen (NTx) and bone sialo protein (BSP) — were analysed in all women on days 1 and 42. Vitamin K was significantly lower in the OSP group versus nOSP and CTRL. The odds ratio results revealed the following: vitamin K, 16.7; PYD, 7.5; NTx, 6.0; DPD, 2.7; and ucOC, 2.7. Vitamin K represented a sensitivity rate of 64% and a specificity rate of 82%. In the receiver operating curve analysis, vitamin K reached the highest area under curve (AUC) score. The combination of vitamin K and AP, bAP and PYD resulted in increased AUC scores (>0.9). The parameter combination of vitamin K/PYD and vitamin K/bAP demonstrated a sensitivity rate of 75–88%, with a specificity rate of more than 82%. The data suggests that a combination of vitamin K with other biochemical bone indices might be a useful tool for assessing bone metabolism, especially in metabolic bone diseases such as osteoporosis.

Keywords: vitamin K, osteoporosis, biochemical marker profiles, receiver operating curve (ROC) analysis, area under curve (AUC) score.

Introduction

Osteoporosis, the most frequent metabolic bone disease, is characterized by a reduced bone quantity and quality; therefore, it is associated with an increased fracture risk (Kanis 1994). Occurring mainly past middle age, approximately 50% of all men and women older than 50 years of age can be considered as being osteoporotic (Kanis 1994, Melton *et al*. 1997). Due to the increase in life expectancy, the costs for treatment of osteoporosis and associated fractures are expected to double in the next 50 years (Chrischilles *et al*. 1994, Schwartz *et al*. 1999). Currently, osteoporosis and its complications burden the European health system with approximately €3.5 billion/year (Norris 1992).

* Corresponding author: Christian Heiss, Department of Trauma Surgery, Justus-Liebig-University of Giessen, Rudolf-Buchheim Strasse 7, D-35385 Giessen, Germany. Tel: 49 641 99 44200; Fax: 49 641 99 44609; e-mail: christian.heiss@chiru.med.uni-giessen.de

Biomarkers ISSN 1354-750X print/ISSN 1366-5804 online © 2004 Taylor & Francis Group Ltd
http://www.tandf.co.uk/journals
DOI: 10.1080/13547500400026862

Biochemical markers are metabolites of bone turnover that can be measured in serum and urine (Delmas 1996, Bikle 1997, Christenson 1997, Dominguez *et al*. 1998). During the past 10–20 years, these bone indices were mainly used for monitoring therapeutic responses in bone diseases. However, their usefulness for the clinical diagnosis of bone diseases, especially osteoporosis, remains to be elucidated. Despite the findings of new biochemical bone indices, e.g. amino-terminal telopeptide (NTx), bone sialo protein (BSP) and vitamin K, their suitability remains highly controversial (Hodges *et al*. 1993, Vermeer *et al*. 1995, 1997). Hence, there is still an urgent need for a suitable biochemical marker profile for prophylaxis and diagnosis of bone diseases, especially osteoporosis.

The importance of vitamin K, a member of the 2-methyl-1,4-naphthochinons, as a therapeutic target in osteoporosis is well known (Ryan-Harshman and Aldoori 2004). However, its suitability as a diagnostic parameter has not been well established. Recent studies investigated the usefulness, efficacy and power of vitamin K for assessing the bone metabolism and possibility for the diagnosis of osteoporosis (Hodges *et al*. 1993, Vermeer *et al*. 1995, 1997, Kanai *et al*. 1997). To increase the suitability and sensitivity of vitamin K, the combination with several other biochemical bone markers and their multidimensional analysis by adequate mathematical processes (logistic regression, neuronal networks, fuzzy methods) might be useful (Rommelfanger 1994, Keller *et al*. 1998).

The aim of the present study was to evaluate the usefulness and potential of a multidimensional analysis of vitamin K as a biochemical bone marker for the classification of premenopausal, postmenopausal non-osteoporotic and postmenopausal osteoporotic women. By using the fuzzy-logic method, vitamin K-associated classification parameters, and its sensitivity and specificity to distinguish between osteoporotic and non-osteoporotic subjects, were developed and analysed.

Materials and methods

Subjects

Twenty-nine women (age range 28–74 years) were recruited and divided into the following groups: group 1, postmenopausal osteoporotic (OSP) ($n=8$, mean age 68 ± 4 years); group 2, postmenopausal non-osteoporotic (nOSP) ($n=11$, mean age 66 ± 4 years); and group 3, premenopausal controls (CTRL) ($n=10$, mean age 35 ± 3 years). The groups OSP and nOSP were considered as age- and sex-matched groups since only women were recruited and age did not differ significantly between the subgroups. Women in groups 1 and 2 were at least 1 year postmenopausal, whereas females of all groups with premenopausal ovarectomy, known endocrine, inflammatory or malign diseases, as well as subjects under treatment with drugs known to affect bone metabolism (oestrogen, glucocorticoids, anti-convulsants, vitamin D, calcium, phenprocoumon) or a sustained fracture within 2 years before the current study were excluded. Alimentation habits, season or different cultural and social backgrounds were not taken into consideration. Informed consent was obtained from all subjects. The study was performed with permission from and according to the guidelines of the Ethics Commission, Faculty of Medicine, Justus-Liebig-University, Giessen, Germany.

Methods

Sample collection. Fasting blood and first urine spot samples were obtained from all women twice (on days 1 and 42) in clinical standard serum, ethylenediamine tetra-acetic acid (EDTA) and urine tubes. Whole blood was centrifuged at 5000 U min^{-1} at 4°C for 10 min, and aliquots of serum were stored in light-protected conditions at −80°C. Similarly, aliquots of urine were stored in light-protected conditions at −80°C until assayed.

The measurements of whole blood count, calcium, phosphate (blood and urine), sodium, potassium, vitamin D, C-reactive protein (blood), liver, cholestatic, kidney and coagulation parameters as well as urine quick test were performed within 1 h by clinical routine measurements at the main clinical laboratory, Justus-Liebig-University of Giessen. Those measurements served as security parameters to detect any disturbances of the corresponding organ system. Accordingly, parameters of all women were within the normal range and no subject had to be excluded from the study.

In addition to the measurement of vitamin K, all biochemical markers of bone formation and resorption were determined at the Institute of Veterinary Physiology, Ludwig-Maximilians-University, Munich, Germany: the serum concentration of alkaline phosphatase (AP), bone alkaline phosphatase (bAP), osteocalcin (OC), undercarboxylated osteocalcin (ucOC) and procollagen type I carboxyterminal propeptide (PICP), as markers of bone resorption urinary N-terminal cross-linked telopeptide of type I collagen (NTx), cross-linked pyridinoline (PYD) and deoxypyridinoline (DPD) as well as serum BSP.

Measurement of vitamin K. Vitamin K belongs to the family of 2-methyl-1,4-naphthochinos, its derivatives possessing anti-haemorrhagic effects. It is a cofactor for the vitamin K-dependent enzyme carboxylase. This microsomal enzyme is responsible for the post-translational carboxylation of glutamyl (Glu) to gammacarboxyglutamyl (Gla) of proteins (Shearer 1995). Serum phyllochinon (vitamin K_1) was extracted from precipitated lipoproteins and cleaned to remove interfering lipids. Analysis with reversed-phase high-performance liquid chromatography (HPLC) and fluorescence detection was then carried out (Shearer 1995, Schmolke 2001). The agents for vitamin K_1 determination (vitamin K_1 analytic assay) and the vitamin K_1 calibrator were purchased from Immundiagnostik GmbH (Bensheim, Germany). Each C18 cartridge for pre-cleaning of the serum samples was conditioned three times with 1 ml methanol and aqua dest. Then, a 1 ml sample was added to the columns and the filtrate collected. The filtrate was mixed with the agent (HPLC assay), vortexed and centrifuged until phase division. The evaporated sample was put into 150 µl eluent (HPLC assay) then injected in the HPLC column. Vitamin K was reduced by connection of a reduction reactor to the column and detected with a fluorescence detector.

Measurement of biochemical markers. The usefulness and methods of quantification of biochemical bone markers have already been described (Withold 1996, Watts 1999, Delmas *et al*. 2000, Schmolke 2001). Serum AP and bAP were determined in the clinical routine laboratory using the Alkaline Phosphatase Optimized Kit and the BONE-ALP KIT (Boehringer, Mannheim, Germany) according to the manufacturer's protocol. Serum OC and ucOC were measured by a competitive coated-tube radio-immunoassay (RIA), according to the manufacturer's protocol (OSCAtest, Osteocalcin BGP, Brahms Diagnostica, Berlin, Germany). The C-terminal propeptide of collagen-type-I (PICP) in the serum was determined quantitatively with the Sandwich-ELISA Prolagene-C-IEMA (Metra Biosystems, Osnabrueck, Germany). Urinary NTx was measured by enzyme-linked immunosorbent assay (ELISA; Osteomark, Ostex International, Seattle, WA, USA) according to the manufacturer's instructions. Determination of urinary PYD and DPD by isocratic, reverse-phase, ion-pair HPLC has already been described (Weile *et al*. 2001). Aliquots of urine were hydrolysed with an equal volume of 37% HCl at 110°C for 24 h to convert all cross-links into the free form. Aliquots of the centrifuged hydrolysates were fractionated by partition column chromatography with a mobile phase of glacial acetic acid, H_2O and butan-1-ol (1:1:4, v/v/v) on CF1 cellulose (Whatman International Ltd., Maidstone, UK). Pyridinium compounds were recovered by elution with H_2O. Subsequently, the aqueous fraction was evaporated to dryness and the residues re-dissolved in 0.2 ml 1% heptafluorobutyric acid. Final separation was performed on a reversed-phase C18 column by HPLC and identified by spectrofluorometry. The concentrations of PYD and DPD were obtained by comparison with an external standard. Serum BSP was quantified by RIA according to the manufacturer's protocol (Bone-Sialo-Protein, Immundiagnostik GmbH, Bensheim, Germany) (Weile *et al*. 2001).

All values measured in urine were corrected for urinary creatinine and are given in nmol $mmol^{-1}$ creatinine. All assays were run blinded and in duplicate; any pair of samples with a coefficient of variation over 5% was re-assayed. All measurements were performed according to good laboratory practice.

Measurement of bone mineral density: osteodensitometry. For measurement of bone mineral density, quantitative computed tomography (qCT) of the lumbar spine was used for groups 1 (OSP) and 2 (nOSP) (SOMATOM PLUS 4, Siemens AG, Erlangen, Germany) (Dambacher and Ruegsegger 1994, Prevrhal and Genant 1999). When the patient was in a supine position, the trabecular density of lumbar vertebrae 1–4 was determined. The obtained results are expressed as mean mg hydroxylapatite/cm^{-3} bone (Siemens AG, Erlangen, Germany). According to the WHO, osteoporosis was diagnosed if a *T*-score (the number of standard deviations (SD) the bone mineral density measurement is above or below the young normal mean bone mineral density) was −2.5 or less (Kanis 1994). Accordingly, the

critical value for the diagnosis of osteoporosis using the SOMATOM PLUS 4 was 89.9 mg/cm^{-3} and below. Subjects with a bone mineral density >89.9 mg/cm^{-3} were considered as being non-osteoporotic.

Statistics

The distributions of vitamin K and all other biochemical markers were first tested within the groups OSP, nOSP and CTRL, and then compared by Mann–Whitney *U*-test (statistical significance was assigned where $p < 0.05$). For classification of CTRL and OSP versus nOSP, the multidimensional data sets were tested by logistic regression and fuzzy-logic methods (Rommelfanger 1994, Keller *et al*. 1998). Together with the pertinent sensitivities and specificities, the resulting potential of classification was then evaluated in a receiver-operating curve (ROC) analysis. Further, sensitivities for different specificity (95, 90, 80%) and the area under curve (AUC) were calculated (0.5 being no discrimination, 1.0 being ideal discrimination). In addition to sensitivity and specificity, the odds ratio for potential of classification was calculated indicating the possibility of a correct diagnosis if this test is applied. Statistical tests and logistic regression were performed professionally by pe Diagnostik GmbH (Leipzig, Germany) using Statistica® 5.5 (StatSoft Europe, Hamburg, Germany).

Results

Focusing on single biochemical bone indices, vitamin K was significantly lower in OSP women ($p = 0.008$) compared with nOSP women ($p = 0.015$). Likewise, in comparison with premenopausal women (CTRL) ($p = 0.015$) at days 1 and 42 (figure 1). The cross-links PYD and DPD revealed a trend of higher concentration in OSP versus nOSP and controls (PYD $p = 0.028$; DPD $p = 0.08$). However, the

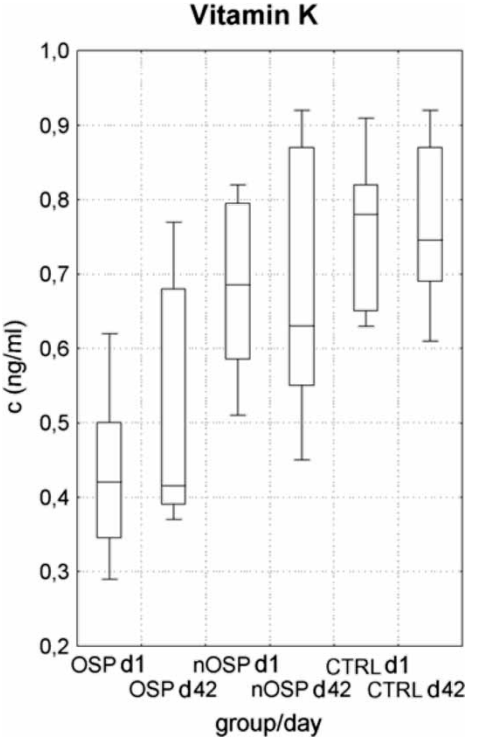

Figure 1. Box plot graph of the concentration of vitamin K in groups postmenopausal osteoporotic (OSP), postmenopausal non-osteoporotic (nOSP) and premenopausal control (CTRL) on days 1 and 42. Bottom to top: 5/25/50/75/95th percentile.

other parameters AP, bAP, OC, ucOC, PICP, NTx and BSP showed no significant differences when comparing the groups (data not shown).

Since the concentration of biochemical markers on day 1 compared with day 42 did not differ significantly, it was possible to use the means of both measurements to compare osteoporotic and non-osteoporotic women. Thereby, significantly lower concentration of vitamin K ($p = 0.01$) and significantly higher concentration of DPD ($p = 0.01$) as well as PYD ($p = 0.04$) were found in OSP. Although statistically not significant, AP, bAP and NTx were also higher in the OSP group, while OC, ucOC, PICP and BSP were lower in osteoporotic women. These results are summarized in table 1.

Taking the results of premenopausal women (CTRL) into consideration, the relationship of concentrations of biochemical markers vitamin K, OC, ucOC, PICP and BSP was CTRL $\geq$ nOSP $\geq$ OSP (figure 1), while that of AP, bAP, PYD, DPD and NTx was CTRL $\leq$ nOSP $\leq$ OSP. The odds ratio for the different biochemical bone markers revealed: 16.7 for vitamin K, 7.5 for PYD, 6.0 for NTx, 2.7 for DPD and 2.7 for ucOC. Vitamin K as the best single biochemical marker reached a sensitivity rate of 64% and specificity rate of 82%, respectively.

ROC analysis

To compare the results of single parameters and combinations of parameters, a ROC analysis was performed and the corresponding AUC calculated. Logistic regressions were used for the combination of single biochemical markers.

Table 1. Mean and 5/95th percentile of concentration of the biochemical bone markers on days 1 and 42. A Mann–Whitney *U*-test was used to compare groups postmenopausal osteoporotic (OSP) versus postmenopausal non-osteoporotic (nOSP).

	Mean concentration on days 1 and 42 (5/95th percentile)		
Marker	OSP	nOSP	Difference
AP	110 51–128	98.8 74–127	n.s.
bAP	20.5 12–22.7	18.3 14.6–26.6	n.s.
OC	7.6 3.5–9.2	7.8 5.6–13.4	n.s.
ucOC	4.0 1.9–6.6	4.9 2.5–6.3	n.s.
PICP	74.2 61–107	81.2 63–142	n.s.
PYD	110 99–137	99 83–116	$p = 0.04$
DPD	29.8 28.0–41.1	25.1 19.3–34.1	$p = 0.01$
NTx	53.3 38–98	51.3 36–93	n.s.
BSP	6.2 5.0–16.4	8.0 5.2–10.5	n.s.
Vitamin K	0.408 0.36–0.65	0.633 0.52–0.86	$p = 0.01$

For abbreviations of biochemical markers, see the text.

Table 2. ROC analysis for single biochemical bone markers arranged according to increasing AUC score. Sensitivities are given at variant specificities (80, 90 and 95% to nOSP).

	Sensitivity (%) with a specificity rate of:			
Marker	95%	90%	80%	AUC
bAP	16	16	19	0.56
BSP	0	0	0	0.57
AP	11	28	33	0.57
PICP	10	30	38	0.58
NTx	0	14	43	0.58
ucOC	0	19	44	0.61
OC	16	31	31	0.63
PYD	50	50	54	0.74
DPD	20	25	61	0.75
Vitamin K	38	38	66	0.86

Table 2 summarizes the obtained results displaying the biochemical markers arranged by increasing AUC. Vitamin K reached the highest AUC (0.86) with the highest sensitivities (38/38/66%) followed by DPD, PYD and OC. In addition, figure 2 shows the ROC analysis as well as the sensitivity and specificity of the promising marker vitamin K. Further ROC analysis for single bone markers and different combinations of any two parameters revealed that a combination of vitamin K and AP, bAP or PYD could even increase the AUC (e.g. vitamin K/AP 0.99, vitamin K/PYD 0.91). Likewise, combinations of OC (AUC = 0.63) with

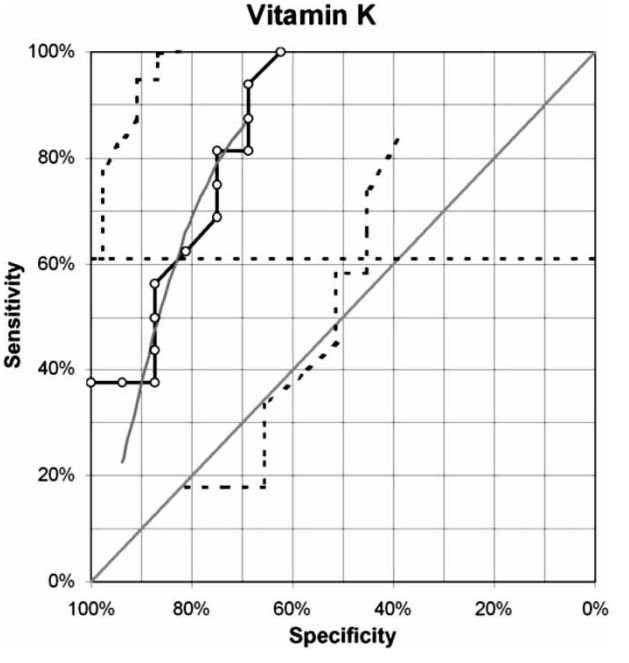

Figure 2. ROC analysis for vitamin K (black solid line). Grey line in ROC curve: trend line. Space within dashed lines: 90% confidence interval. Grey diagonal: ROC curve of lacking discrimination.

other markers also increased the AUC (OC/AP = 0.86; OC/PYD = 0.85; OC/NTx = 0.77).

Classification approach

For calculation of potential of classification by means of logistic regression, data from days 1 and 42 were considered as independent measurements (doubling of data). By using the results obtained at day 1 as learning data and those obtained at day 42 as test data, the two- and three-dimensional classification approaches reached high sensitivities and specificities. The combinations of vitamin K/PYD, vitamin K/bAP (sensitivity 75–88%) and vitamin K/ucOC (sensitivity 75–90%) showed higher sensitivities at a specificity rate higher than 82% (table 3). Accordingly, the following conclusions can be drawn: high vitamin K and low PYD (ucOC) may indicate a normal (premenopausal) bone status (CTRL); high vitamin K and high PYD (ucOC) may indicate a postmenopausal, but non-osteoporotic status (nOSP); low vitamin K and high PYD (ucOC) may indicate OSP conditions (figure 3).

Several models of classification (exact modelling, logistic regression) using the results of day 1 as developing data and the results of day 42 as test data reached sensitivities and specificities higher than 80%. Thereby, combinations of either two or three biochemical bone markers (AP, bAP, ucOC, PICP, PYD, DPD, NTx, BSP, vitamin K) can be used.

Further analysis of the data revealed that a combination of PICP/NTx/BSP reached a potential of classification similar to measurements of the bone mineral density (*T*-score of trabecular bone). However, this similarity was not found when the same combination of parameters where compared with the *T*-score of cortical bone. Interestingly, some of the biochemical markers, especially PICP, showed marked changes of the concentration in a time-dependent manner. Thus, the combination of PICP/AP and taking the time-dependent change in concentration of PICP (day 42 versus day 1) into account showed promising results.

Discussion

According to the ROC analysis, vitamin K was the best single parameter to classify nOSP and osteoporotic women with a sensitivity rate of 64% (66%) and specificity rate of 82% (80%), followed by DPD (sensitivity rate of 61%) and PYD (54%). Due to the rather small number of subjects in this study, the confidence intervals (dashed line in figure 2) include the ROC curve of lacking discrimination for vitamin K (grey diagonal in figure 2). However, the ROC analysis showed

Table 3. Sensitivities and specificities of combinations of parameters with vitamin K.

Combination of parameters	*n*	Sensitivity	95% Confidence interval	Specificity	95% Confidence interval
Vitamin K/PYD	21	day 1 7/8 88%	53%	day 1 9/11 82%	53%
		day 42 6/8 75%	40%	day 42 9/11 82%	53%
Vitamin K/bAP	21	day 1 7/8 88%	53%	day 1 10/11 91%	64%
		day 42 6/8 75%	40%	day 42 9/10 90%	53%

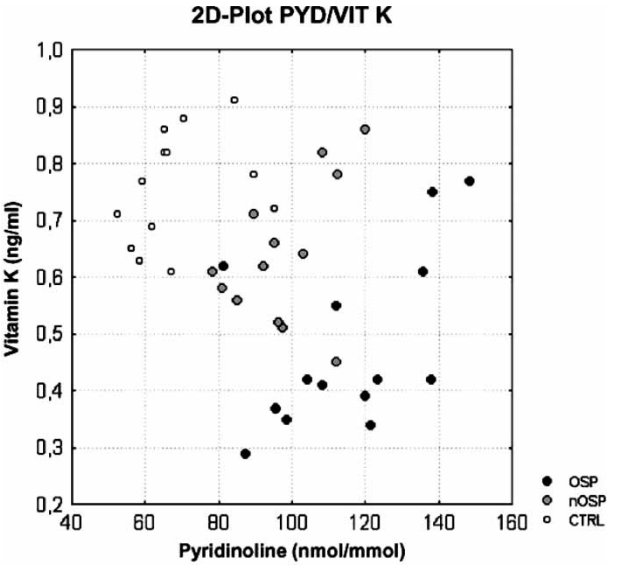

Figure 3. Two-dimensional graph of the combination vitamin K/pyridinoline. OSP, postmenopausal osteoporotic; nOSP, postmenopausal non-osteoporotic; CTRL, premenopausal control.

qualitative differences in the characteristics of biochemical bone markers. Some markers have flat, some have steep curves indicating a higher sensitivity or a higher specificity, accordingly. This may be the starting point of promising two- or three-dimensional combinations of vitamin K with other parameters. Concentrations of vitamin K were found to arrange as CTRL $\geq$ nOSP $\geq$ OSP (figure 1). The classification approaches (two- and three-dimensional) reached high sensitivities and specificities, while the combination of vitamin K/PYD and vitamin K/bAP showed the highest sensitivity (75–88%) with a specificity rate of more than 82% (table 3).

The two-dimensional graph indicates that an additional dimension, i.e. an additional biochemical marker, may decrease the overlap of OSP and nOSP for vitamin K (figure 1). Therefore, even standard statistical procedures (e.g. logistic regression) should reach discriminations of a high quality. Especially the combinations of vitamin K and AP or bAP as well as the combinations of OC and PYD, AP or NTx were able to increase the discriminatory power.

From a methodical standpoint, note that these results should be considered as preliminary data and need to be confirmed in larger studies with a higher number of subjects included. However, with confidence intervals on the other side of the grey diagonal (figure 2), these results indicated that the found combinations of biochemical bone markers improved sensitivity and specificity. In addition, this was confirmed by the obtained results from the CTRL group (figure 3) as well as the high ability of vitamin K to distinguish between OSP and CTRL.

The results underscore the importance of vitamin K for bone metabolism in postmenopausal osteoporosis. Accordingly, studies showed that the administration of vitamin K led to an increase of bone mineral density in osteoporotic patients (Plantalech *et al*. 1991, Vermeer *et al*. 1995). Further, administration of vitamin K

was shown to prevent bone loss and effectively to reduce the incidence of fractures (Shiraki *et al*. 2000). In contrast, a very recent study failed to confirm this protective effect of vitamin K (Ishida and Kawai 2004). The cellular mechanisms underlying the effects of vitamin K on bone cells have been reviewed by Iwamoto *et al*. (2004). However, in the light of controversial clinical results of vitamin K administration further studies and investigations will be necessary to evaluate its usefulness for the prophylaxis, diagnosis and therapy of metabolic bone diseases, especially osteoporosis.

In conclusion, there is an increasing interest in biochemical parameters of bone metabolism for the diagnosis of osteoporosis. They may be, in contrast to osteodensitometric imaging, a valuable additional tool for the short-term evaluation of 'bone status'. Those markers, and especially the here presented 'new marker' combination of vitamin K, might be helpful to screen and diagnose osteoporosis as well as to evaluate the associated bone loss and future fracture risk. Additionally, vitamin K might be useful to monitor therapeutic responses in osteoporotic patients.

Acknowledgements

The authors appreciate the technical assistance of the collaborators from the Clinical Laboratory, Justus-Liebig-University, Giessen, and the Institute of Veterinary Physiology, Ludwig-Maximilians-University, Munich, Germany. They thank the Department of Diagnostic Radiology, Justus-Liebig-University, Giessen, for performing the measurement of bone mineral density by quantitative computed tomography. The study was supported by the Justus-Liebig-University and the Ludwig-Maximilians-University.

References

Bikle, D. D. 1997, Biochemical markers in the assessment of bone disease. *American Journal of Medicine*, **103**, 427–436.

Chrischilles, E., Shireman, T. and Wallace, R. 1994, Costs and health effects of osteoporotic fractures. *Bone*, **15**, 377–386.

Christenson, R. H. 1997, Biochemical markers of bone metabolism: an overview. *Clinical Biochemistry*, **30**, 573–593.

Dambacher, M. A. and Ruegsegger, P. 1994, Bone density measurements and their indications. *Orthopade*, **23**, 38–44.

Delmas, P. D. 1996, Biochemical markers for the assessment of bone turnover. In *Osteoporosis: Etiology, Diagnosis and Management*, edited by B. L. Riggs and L. J. Melton III (Philadelphia: Lippincott-Raven), pp. 319–333.

Delmas, P. D., Eastell, R., Garnero, P., Seibel, M. J. and Stepan, J. 2000, The use of biochemical markers of bone turnover in osteoporosis. Committee of Scientific Advisors of the International Osteoporosis Foundation. *Osteoporosis International*, **11** (Suppl. 6), S2–S17.

Dominguez, C. C., Sosa, H. M., Traba, M. L., Alvarez, V. E. and De La Piedra, C. 1998, Biochemical markers of bone formation in the study of postmenopausal osteoporosis. *Osteoporosis International*, **8**, 147–151.

Hodges, S. J., Akesson, K., Vergnaud, P., Obrant, K. and Delmas, P. D. 1993, Circulating levels of vitamins K1 and K2 decreased in elderly women with hip fracture. *Journal of Bone and Mineral Research*, **8**, 1241–1245.

Ishida, Y. and Kawai, S. 2004, Comparative efficacy of hormone replacement therapy, etidronate, calcitonin, alfacalcidol, and vitamin K in postmenopausal women with osteoporosis: The Yamaguchi Osteoporosis Prevention Study. *American Journal of Medicine*, **117**, 549–555.

Iwamoto, J., Takeda, T. and Sato, Y. 2004, Effects of vitamin K2 on osteoporosis. *Current Pharmaceutical Design*, **10**, 2557–2576.

KANAI, T., TAKAGI, T., MASUHIRO, K., NAKAMURA, M., IWATA, M. and SAJI, F. 1997, Serum vitamin K level and bone mineral density in post-menopausal women. *International Journal of Gynaecology and Obstetrics*, **56**, 25–30.

KANIS, J. A. 1994, Assessment of fracture risk and its application to screening for postmenopausal osteoporosis: synopsis of a WHO report. WHO Study Group. *Osteoporosis International*, **4**, 368–381.

KELLER, T., BITTERLICH, N., HILFENHAUS, S., BIGL, H., LOSER, T. and LEONHARDT, P. 1998, Tumour markers in the diagnosis of bronchial carcinoma: new options using fuzzy logic-based tumour marker profiles. *Journal of Cancer Research and Clinical Oncology*, **124**, 565–574.

MELTON, L. J. III, KHOSLA, S., ATKINSON, E. J., O'FALLON, W. M. and RIGGS, B. L. 1997, Relationship of bone turnover to bone density and fractures. *Journal of Bone and Mineral Research*, **12**, 1083–1091.

NORRIS, R. J. 1992, Medical costs of osteoporosis. *Bone*, **13** (Suppl. 2), S11–S16.

PLANTALECH, L., GUILLAUMONT, M., VERGNAUD, P., LECLERCQ, M. and DELMAS, P. D. 1991, Impairment of gamma carboxylation of circulating osteocalcin (bone gla protein) in elderly women. *Journal of Bone and Mineral Research*, **6**, 1211–1216.

PREVRHAL, S. and GENANT, H. K. 1999, Quantitative computer tomography. *Radiologe*, **39**, 194–202.

ROMMELFANGER, H. 1994, *Fuzzy Decision Support Systems* (Heidelberg: Springer).

RYAN-HARSHMAN, M. and ALDOORI, W. 2004, Bone health. New role for vitamin K? *Canadian Family Physician*, **50**, 993–997.

SCHMOLKE, B. 2001, Laboratory diagnosis of osteoporosis. *Orthopade*, **30**, 425–436.

SCHWARTZ, A. V., KELSEY, J. L., MAGGI, S., TUTTLEMAN, M., HO, S. C., JONSSON, P. V., POOR, G., SISSON DE CASTRO, J. A., XU, L., MATKIN, C. C., NELSON, L. M. and HEYSE, S. P. 1999, International variation in the incidence of hip fractures: cross-national project on osteoporosis for the World Health Organization Program for Research on Aging. *Osteoporosis International*, **9**, 242–253.

SHEARER, M. J. 1995, Vitamin K. *Lancet*, **345**, 229–234.

SHIRAKI, M., SHIRAKI, Y., AOKI, C. and MIURA, M. 2000, Vitamin K2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. *Journal of Bone and Mineral Research*, **15**, 515–521.

VERMEER, C., JIE, K. S. and KNAPEN, M. H. 1995, Role of vitamin K in bone metabolism. *Annual Reviews of Nutrition*, **15**, 1–22.

VERMEER, C., THIJSSEN, H. H. W. and HAMULYAK, K. 2001, Vitamin K and tissue mineralization. *Bibl Nutr Dieta*, **55**, 159–170.

WATTS, N. B. 1999, Clinical utility of biochemical markers of bone remodeling. *Clinical Chemistry*, **45**, 1359–1368.

WEILE, U., FINSLER, S., WEHR, U. and CLAUS, R. 2001, Comparison of blood markers for the longitudinal monitoring of osteoclastic activity in the pig. *Journal of Veterinarian Medicine Series A Physiology Pathology Clinical Medicine*, **48**, 609–618.

WITHOLD, W. 1996, Monitoring of bone turnover biological, preanalytical and technical criteria in the assessment of biochemical markers. *European Journal of Clinical Chemistry and Clinical Biochemistry*, **34**, 785–799.