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Original Research Article

Increased serum osteocalcin levels and vitamin K status by daily cheese intake

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ABSTRACT

Background: Cheese is a major source of long-chained vitamin K2 variants. How intake of vitamin K2 rich cheese affects vitamin K and osteocalcin has not been studied. The aim was to establish a maximum efficacy dose (MED) after daily intake of vitamin K2-rich cheese (Jarlsberg®) based on increase in ratio between carboxylated and undercarboxylated osteocalcin during a five-week diet.
Methods: 20 healthy volunteers (HV) were recruited. The daily intake of Jarlsberg® cheese in the study varied from 20 to 152 g. Clinical investigation was performed initially and after three, four and five weeks with measurement of vital signs, hematological and biochemical variables, carboxylated and undercarboxylated osteocalcin and vitamin K. The ratio OR= carboxylated/undercarboxylated osteocalcin was the main variable.
Results: The MED decreased with treatment duration and was estimated to 57 g/day (95% CI: 47-67) after five weeks diet, resulting in a mean OR increase of 30% (95% CI: 23.8-36.8). Both OR and serum osteocalcin followed a quadratic dose response curve. For osteocalcin, a maximal increase of 46% was estimated at 59 g/day for five weeks. The serum content of long-chained vitamin K2 increased significantly with increasing cheese dose. The increase were mainly obtained the first three weeks and kept unchanged the following two weeks. The cheese doses close to the MED caused nearly significant reductions in total cholesterol, LDL-cholesterol, the LDL/HDL ratio and significant reduction in the blood pressures after five weeks diet ($p \leq 0.05$).
Conclusions: MED of Jarlsberg® cheese was estimated to 57 g/day. Daily intake of Jarlsberg® cheese increased the osteocalcin level, vitamin K2 and positively affected the lipid patterns and blood pressure.

Keywords: Dose-finding study of cheese, Jarlsberg® cheese, Osteocalcin, Osteoporosis, Lipid pattern, Vitamin K2, Vital signs, Women

INTRODUCTION

Osteoporosis remains a huge problem among the elderly and malnourished people. It is well established that dietary calcium and vitamin D are beneficial for skeletal health. Vitamin D stimulates calcium uptake and is important for expression of mineralization-related genes of osteoblast, such as the gene encoding osteocalcin.[1] Osteocalcin is responsible for binding calcium to bone tissue.[2] In addition, osteocalcin is a bone-derived hormone playing important roles in regulation of energy metabolism, physical exercise, testosterone and adiponectin production and is important for brain function.[2-7] A low level of osteocalcin is associated with insulin resistance and diabetes and metabolic syndrome.

Osteocalcin is activated by vitamin K- dependent carboxylation, and for this reason the positive effect of dietary vitamin K on the skeleton is a health claim authorized by the European Food safety Authority (EFSA). The ratio of fully carboxylated to undercarboxylated osteocalcin in serum (OR) reflects the vitamin K status.[8] A low OR in healthy people indicates a subclinical vitamin K deficiency, which is common in Western societies.[9] Very low ORs have been associated with osteoporotic fractures.[10]

The various K vitamins are found in different foods. Vitamin K1 is produced in plants and found at high concentrations in leafy vegetables and is the dominating variant in the Western diet. Vitamin K2, or menaquinone (MK) exists in several variants. The short-chained menaquinone-4 (MK-4) is found in animal products like liver and can be formed from vitamin K1 in humans and animals. The long-chained menaquinones, like MK-7, MK-8, MK-9 and MK-9(4H) are of bacterial origin and present in certain fermented foods. In the Western diet, fermented dairy products like cheese are the main source of vitamin K2.[11]

The long-chained MKs have been found to have greater extra-hepatic activity than vitamin K1 and MK-4, possibly due to more efficient uptake and much longer serum half-life.[12,13] Prospective cohort studies have demonstrated health benefits that can be attributed to intake of vitamin K2, but not to vitamin K1.[14] The main vitamin K2s in cheese are MKs with long side chains, mostly MK-9, but the amounts vary considerably.[15] Some cheeses also contain MK-9(4H), and Jarlsberg® cheese in particular is rich in this variant.[16] This cheese is made with lactic acid bacteria producing MK-8 and MK-9 and *Propionibacterium* producing MK-9(4H).

Although vitamin K2 related health benefits have been associated with cheese, the effects of cheese consumption on bone health has never been studied in intervention studies. Its high calcium content in addition to vitamin K2 indicates that cheese consumption may strengthen bones and reduce the risk of osteoporosis. The idea behind this study was to investigate whether an optimized daily intake of a K2-rich cheese can increase the ratio between carboxylated and undercarboxylated osteocalcin. In case such a dose is achieved within a daily acceptance level, it may be used as a dietary recommendation. Due to its high content of vitamin K2, Jarlsberg® cheese is well suited for clinical studies.

The aim of the present study was to establish a maximum efficacy dose (MED) of daily Jarlsberg® cheese based on increase in the ratio between carboxylated and undercarboxylated osteocalcin during a five-week diet.

Table 1: Changes in intake of Jarlsberg® cheese from one design level to the next with a dose range from 20 to 180 g/day based on the osteocalcin ratio.

Intake on the first design level	Response at design level 1 OR level	Intake of cheese design level 2	Response at design level 2 OR level	Intake of cheese design level 3
m=100 g/day	High OR >1.5	m + m/k =152 g/day	High	m + m/k + m/k^2 = 180 g/day
			Moderate high	m + m/k + m/k^3 = 167 g/day
			Suitable	m + m/k = 152 g/day
			Moderate low	m + m/k-m/k^3 = 138 g/day
			Low	m + m/k-m/k^2 = 125 g/day
	Moderate high 1.1≤ OR ≤1.5	m + m/k^2=127 g/day	High	m + m/k^2 +m/k^3 = 142 g/day
			Moderate high	m + m/k^2+m/k^4 = 135 g/day
			Suitable	m + m/k^2= 127 g/day
			Moderate low	m + m/k^2-m/k^4 =120 g/day
			Low	m + m/k^2-m/k^3 =113 g/day
	Suitable 0.9< OR <1.1	m =100 g/day	High	m + m/k = 152 g/day
			Moderate high	m + m/k^2= 127 g/day
			Suitable	m = 100 g/day
			Moderate low	m-m/k2= 72 g /day
			Low	m-m/k = 48 g/day
	Moderate low 0.5≤ OR ≤0.9	m-m/k^2=72 g/day	High	m-m/k^2 + m/k^3= 87 g/day
			Moderate high	m-m/k^2 + m/k^4= 80 g/day
			Suitable	m-m/k^2= 72 g /day
			Moderate low	m-m/k^2-m/k^4 = 65 g/day
			Low	m-m/k^2-m/k^3= 58 g/day
	Low OR <0.5	m-m/k =48 g/day	High	m-m/k + m/k^2 = 75 g/day
			Moderate high	m-m/k + m/k^3 = 62 g/day
			Suitable	m-m/k = 48 g/day
			Moderate low	m-m/k-m/k^3 = 33 g/day
			Low	m-m/k-m/k^2 = 20 g/day

OR=carboxylated/ undercarboxylated osteocalcin.

METHODS

The study population consisted of healthy Norwegian women in pre-menopausal age. Pregnant women and women suffering from known gastrointestinal disorder, abnormal liver or kidney function, lactose intolerance or known milk product allergy, diabetes mellitus or verified cancer were excluded. Women under systemic treatment with corticosteroids or immunosuppressive drugs the last three weeks or participating in another clinical trial the last six weeks before start of the study were not included.

The study material consisted of 20 healthy volunteers (HV) from the study population, recruited at Skjetten Medical Centre and Primary Medicine. The study was approved by the Norwegian regional ethical committee (REK) and performed February 2019 to June 2019. One of the HVs failed to perform the study. The material consisted of 16 Caucasian and three Malaysian Thai women with a mean age of 41.5 years ranging from 22 to 50 years and a body mass index (BMI) of 24.8 kg/m^2 ranging from 18.7 to 31.2 kg/m^2. Fourteen of the HVs were married or in cohabitation, two divorced and three were single. Vitamin D deficiency was recorded in four of the HVs, iron deficiency in two and hypertension in two HVs at the time of inclusion. One HV suffered from Asthmatic disorder and one from bladder cramps. All medical treatments were kept unchanged during the study.

Vitamin K extraction and analysis

Vitamin K in cheese was extracted in heptane after fat hydrolysis.[15] To extract vitamin K from plasma, included 0.75 ml plasma samples were mixed with 1.5 ml ethanol, then 1 ml heptane. The mixtures were vortexed for 30s and centrifuged for 1 min at 1000g. Samples of 0.5 ml from the upper phase were withdrawn, dried under vacuum and re-dissolved in 50 µl isopropanol prior to HPLC. Vitamin K was analyzed by isocratic reverse phase chromatography on a Dionex Ultimate 3000 HPLC system with fluorescence detection at 436 nm and emission at 248 nm with a Shiseido Capcell pak C 18 MGII 100A 3 µm, 2.0×100 mm column coupled to a Shiseido CQ-R 2.0×20 mm for post column reduction. The mobile phase was isopropanol-methanol (1:1) and flow 0.2 ml/min at 50 °C. Injected volumes were 0.5-4 µL. Standards vitamin K1 and MK-7 were from Sigma and MK-9 from Toronto Research Chemicals. Standards MK-8 and MK-9(4H) were extracted from anaerobically grown pure cultures of *Escherichia coli* and *Propionibacterium freudenreichii*, respectively. Carboxylated and undercarboxylated osteocalcin were measured in plasma by immunoassays kits (Takara Bio, Ōtsu, Japan) by Vitas AS.

Jarlsberg® cheese is produced by TINE SA. Cheese from three production batches was used in this study. The participants received the cheese in 100 g packages containing cheese slices of 10g. The average vitamin K content per 100 g of cheese was 3.0 µg vitamin K1, 5.2 µg MK-4, 1.5 µg MK-7, 6.7 µg MK-8, 23.9 µg MK-9 and 40.5 µg MK-9(4H).

Clinical procedure

The HVs were clinically investigated. Vital signs were measured and blood sample collected initially and after three, four and five week of cheese intake. The HVs were asked not to change the usual diet, but replace other cheeses with Jarlsberg® cheese.

Study design

The study was conducted as a randomized between-patient, 3-level response surface pathway (RSP) designed trial with daily intake of Jarlsberg® cheese as the intervention variable.[17-19] The increase in OR from baseline to three weeks with Jarlsberg® cheese was used as the response variable. The dose-window of Jarlsberg® cheese in the study was (20-180 g/day) with a starting mid-dose of m=100 g/day (Table 1). An OR increase < 0.5 was classified as "low"; 0.5 ≤OR ≤0.9 as "moderate low"; 0.9 <OR <1.1 as "suitable"; 1.1≤ or ≤1.5 as "moderate high" and an OR >1.5 classified as "High". The main procedure for changing the daily dose of Jarlsberg® cheese was to reduce the dose in case of low OR increase, repeated the dose for suitable OR result and increase the dose in case of high OR increase. To ensure this procedure at the first design level, a parallel opposite procedure to increase the dose in the second design level was developed.

Three HVs were included in the first design-level and received a daily dose of 100 g/day. The obtained change in OR after the first three weeks of Jarlsberg® cheese recommended the doses to be used for the five HVs in the second design-level in accordance with the main procedure. Additionally, five HVs received doses for the parallel second design level based on the opposite strategy. The OR-results obtained by the five HVs in the second design-level recommended the cheese dose to be used for the seven participants in the third design-level.

Randomization procedure

Assume a_1 represent the number of HVs calculated by the RSP-procedure to receive the intervention A_1 at the second level based on the response obtained at first design level; a_2 the number of HVs calculated to receive dose A_2 and a_3 to receive dose A_3 (Table 1). In general, a weighted randomization (a_1:a_2:a_3) will be performed. If two cheese doses are equal (1=2) and one is different, the HVs on the second level will be randomized (2:1). This means that the probability for a participant to be allocated to intake A_1=A_2 is 2/3 and 1/3 for A_3.

Based on responses obtained in the five HVs in the second design level, theoretically five new cheese doses (B_1, B_2, B_3, B_4, and B_5) will be allocated to the seven

patients at the third design level. Assume that b_1 participants on the second design level recommended by the RSP-procedure to receive cheese dose B_1, b_2 to receive B_2, b_3 to receive B_3, b_4 to receive B_4 and b_5 to receive B_5. The cheese doses used by the seven new participants in the third design level will be allocated by weighted randomization (b_1:b_2:b_3:b_4:b_5) following the same procedure as explained for the second design level.

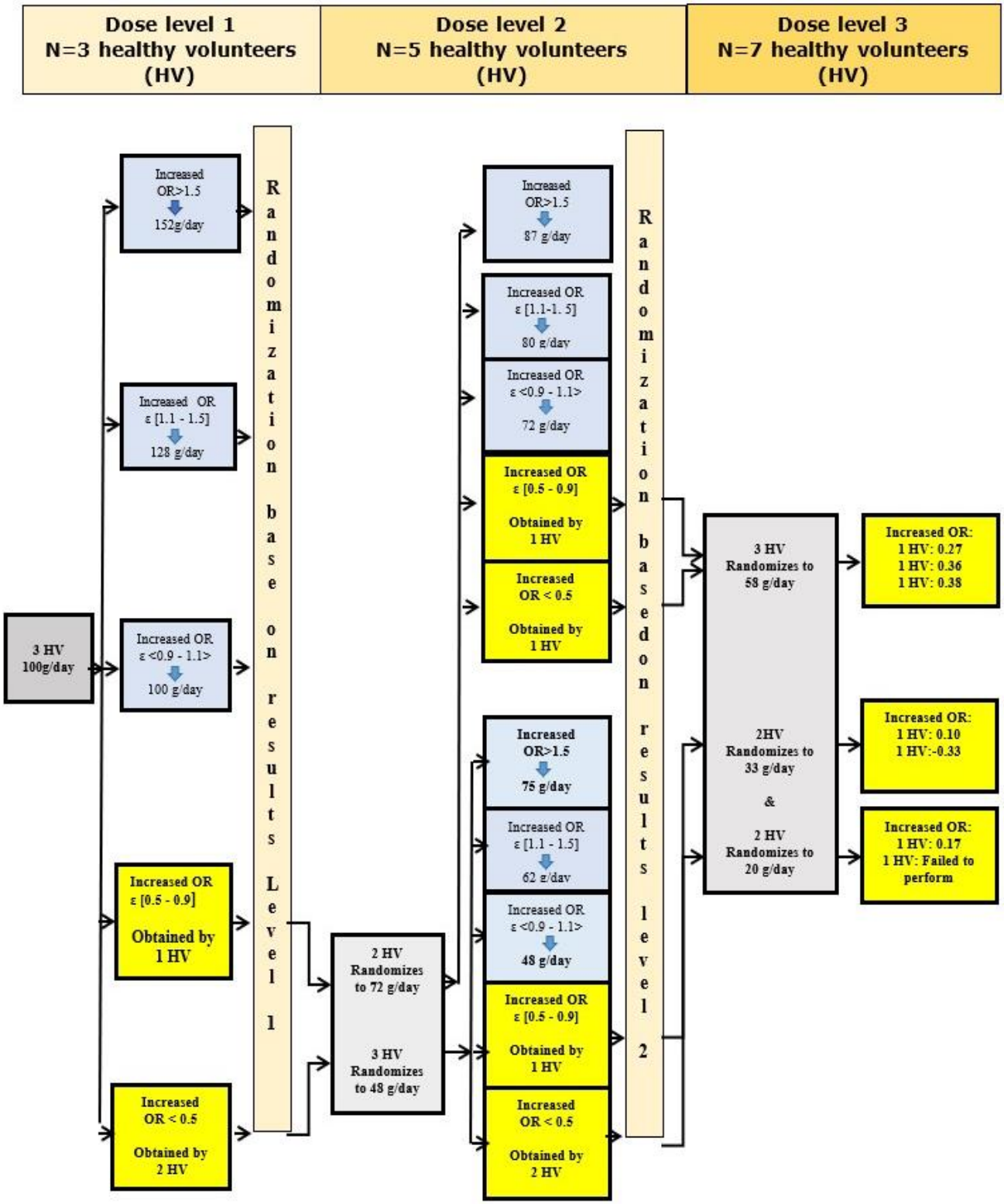

Figure 1: The results obtained in accordance with the response surface pathway design. The yellow boxes give number of participants with obtained results and recommended cheese dose for the next design level. The Grey boxes give results of randomization based on results from previous design level.

Table 2: Increase in osteocalcin, carboxylated-and undercarboxylated osteocalcin presented and the osteocalcin ratio in Jarlsberg® cheese dose groups.

Variables	Dose group [g/day]	Baseline	3 weeks-baseline	4 weeks-baseline	5 weeks-baseline
Carboxylated osteocalcin (ng/ml)	1: (20-40) (n=3)**	7.0 (5.6) 3.5-13.4	0.6 (0.2) 0.3-1.0	0.8 (2.5) -5.3-7.0	1.7 (2.8) -5.2-8.6
	2: (40-60) (n=6)	14.6 (8.9) 5.3-23.9	1.7 (2.9) -1.3-4.8	2.2 (5.7) -3.8-8.2	4.4 (5.7) -1.6-10.4
	3: (60-100) (n=5)	16.4 (2.1) 13.8-18.9	0.8 (2.2) -2.0-3.5	-0.7 (5.1) -7.0-5.6	0.3 (3.7) -4.3-4.9
	4:>100 (n=5)	13.1 (4.9) 6.9-19.2	-2.6 (1.8)* -4.9-0.3	-4.4 (3.0)* -8.1--0.6	Not performed
	Group 1+2+3 (N=14)	13.6 (7.1) 10.4-16.6	1.2 (2.2)* -0.1-2.4	0.9 (4.8) -1.9-3.6	2.4 (4.7)* -0.3-5.1
Under-carboxylated osteocalcin (ng/ml)	1: (20-40) (n=3)**	2.6 (2.2) 1.2-5.1	0.2 (0.2) -0.2-0.6	0.2 (0.3) -0.6-1.0	0.1 (0.7) -1.6-1.8
	2: (40-60) (n=6)	5.3 (2.0) 3.1-7.4	0.8 (0.9)* -0.1-1.8	0.2 (1.6) -1.4-1.9	0.6 (1.7) -1.2-2.5
	3: (60-100) (n=5)	6.0 (1.0) 4.8-7.2	0.1 (1.2) -1.4-1.6	0.0 (1.5) -1.9-1.9	0.2 (1.3) -1.4-1.8
	4:>100 (n=5)	6.2 (4.3) 1.0-11.5	-0.3 (1.7) -2.4-1.9	-1.6 (1.7) -3.7-0.5	Not performed
	Group1+2+3 (N=14)	5.0 (2.1) 3.8-6.2	0.4 (0.9)* -0.1-1.0	0.1 (1.3) -0.6-0.9	0.4 (1.34) -0.4-1.1
Osteocalcin ratio OR = carboxylated/ under-carboxylated	1: (20-40) (n=3)**	2.76 (0.51) 1.49-4.04	-0.03 (0.26) -0.69-0.62	-0.23 (0.83) -2.28-1.82	0.38 (0.45) -0.73-1.49
	2: (40-60) (n=6)	2.77 (1.10) 1.62-3.93	0.00 (0.40) -0.43-0.43	0.41 (0.51)* 0.03-0.79	0.63 (0.55)* 0.19-1.07
	3: (60-100) (n=5)	2.77 (0.43) 2.24-3.30	0.17 (0.35)* 0.04-0.30	-0.09 (0.24) -0.38-0.21	0.00 (0.14) -0.17-0.17
	4:>100 (n=5)	2.56 (1.06) 1.24-3.89	-0.49 (0.48) -1.09-0.10	-0.26 (0.31) -0.63-0.12	Not performed
	Group1+2+3 (N=14)	2.77 (0.75) 2.33-3.20	0.05 (0.26)* 0.00-0.10	0.09 (0.39) -0.11-0.20	0.35 (0.43)* 0.12-0.58
Total osteocalcin (ng/ml)	1: (20-40) (n=3)**	9.6 (7.7) 5.0-18.5	0.8 (0.3)* 0.0-1.6	1.0 (2.7) -5.7-7.7	1.8 (3.4) -6.7-10.3
	2: (40-60) (n=6)	19.9 (10.4) 9.0-30.8	2.6 (3.5) -1.1-6.2	2.8 (6.3) -3.9-9.4	5.1 (7.1) -2.4-12.5
	3: (60-100) (n=5)	22.3 (2.6) 19.2-25.5	0.9 (3.0) -2.8-4.5	-0.7 (6.6) -8.9-7.4	0.5 (4.9) -5.7-6.6
	4:>100 (n=5)	19.3 (7.3) 10.2-28.4	-2.9 (3.3) -7.0-1.3	-5.9 (4.5)* -11.6--0.3	Not performed
	Group1+2+3 (N=14)	18.6 (8.8) 13.5-23.6	1.6 (2.9)* 0.0-3.2	1.1 (5.7) -2.1-4.4	2.7 (5.8)* 0.0-5.4

*Indicate significant ($p \leq 0.05$) change from baseline. **The total range is given due to the sample size. The results given in the shadowed line are not included in the main analysis. The results are expressed by mean values, standard deviation in brackets and 95% confidence intervals.

Statistical analysis

The assumed continuously distributed variables were expressed by mean values with 95% confidence interval (CI).[20] As index of dispersion, standard deviations (SD) were given. Categorized variables were given in contingency tables.[21] Changes in mean were tested by using a paired two-tailed T-test with a significant level of 5%. The response variables as a function of cheese dose fit a quadratic function and analyzed by using polynomial regression analysis.[22] The sample space of the dose in the present studies may be expressed as $\Omega_D = \{D_L \leq \ldots . \leq D_U\}$. Let μ be the MED and assume μ is contained in Ω_D. The increase in OR assumed to be ordinal and the probability increases monotonically in a limited cheese dose interval. Isotonic regression was used for estimation of MED.[23]

RESULTS

On the first design-level three HVs received 100 g cheese/day. After three weeks one had an increase in OR of 0.5 and two HVs a reduction. In accordance with the original RSP procedure (Table 1), two participants in the second design-level were randomized to a cheese-dose of 72 g/day and three to 48 g/day (Figure 1). Based on the parallel opposite procedure, two HVs were randomized to 127 g/day and three to 152 g/day. One of the two HVs receiving 72 g/day had an increase in OR between 0.5 and 0.9 and one obtained an increase below 0.5. Of the three HVs with a cheese intake of 48 g/day; one obtained an OR increase between 0.5 and 0.9 and two an increase below 0.5. Based on these results, three of the seven HVs included in the third design-level were randomized to 58 g/day, two to 33 g/day and two to 20 g/day. The three participants receiving 58 g/day obtained an OR increase of 0.27, 0.36 and 0.38. The two HVs receiving 33 g/day obtained an OR increase of 10.1 and 0.33. One of the two HVs receiving 20 g/day failed to finalize the study and the other obtained an OR increase of 0.17. In the parallel and opposite procedure, two HVs randomized to 127 g/day obtained an OR-reduction of 0.64 and 1.11. Two participants receiving 152 g/day obtained an OR-reduction of 0.19 and 0.67 and the third HV an OR-increase of 0.13.

Based on the OR increase after three and four weeks cheese intake, the MED was estimated at 70 g/day (95% CI: 47-93 g/day) and 61 g/day (95% CI: 50-82 g/day), respectively (Figure 2). The MED after five weeks was estimated at 57 g/day (95% CI: 47-67), with an OR increase from baseline of 30% (95% CI: 23.8%-36.8%). Cheese intake also affected total serum osteocalcin levels, and the dose response curves after three, four and five weeks showed maximal increases in total osteocalcin of 20.0% on 56 g/day, 21.8% on 61 g/day and 46.1% on 59 g/day, respectively (Figure 3). The development of carboxylated osteocalcin follows almost the previously described pattern of total osteocalcin (Table 2). Like OR, osteocalcin levels decreased from baseline with the highest doses.

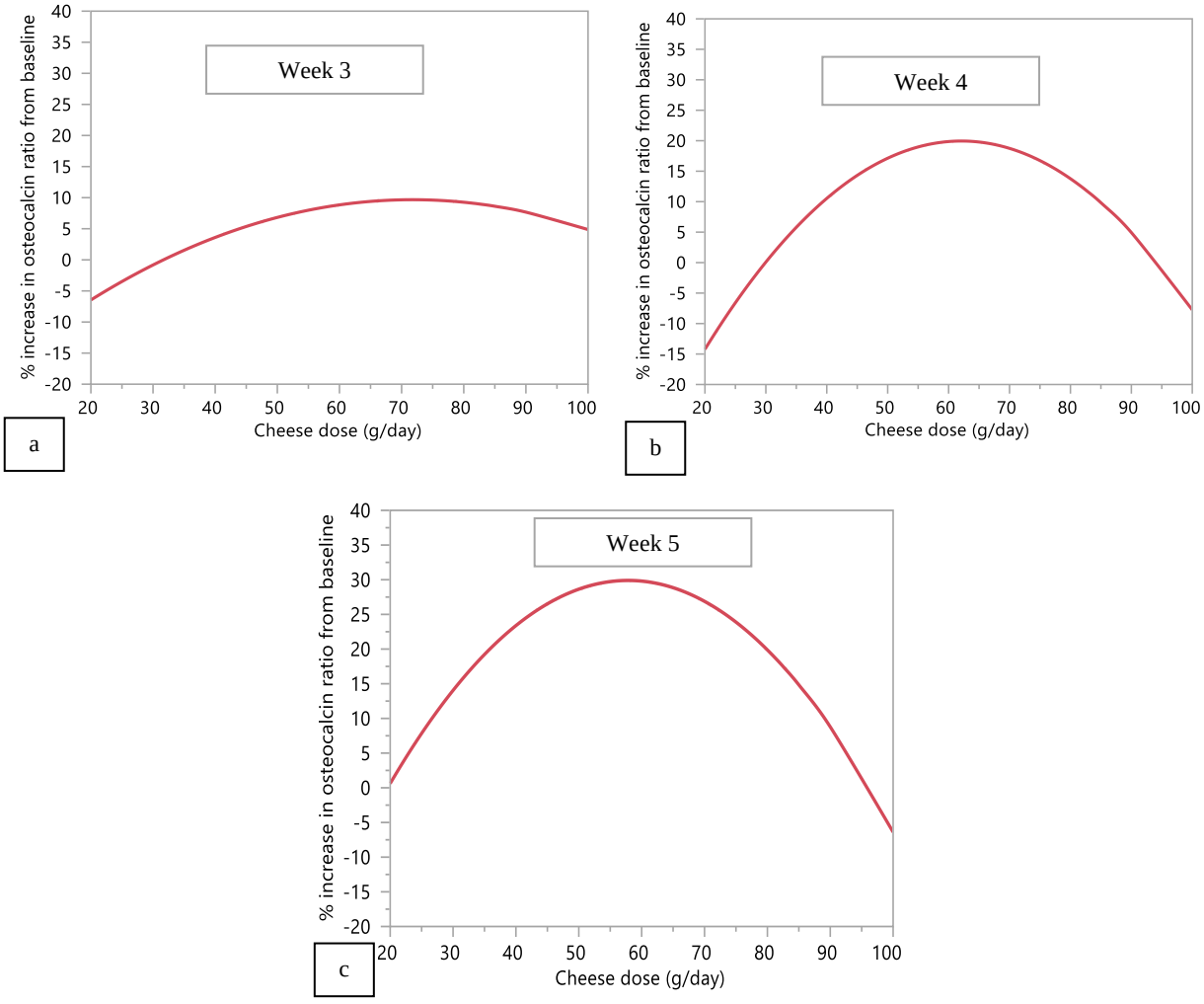

Figure 2: The predicted development of increased ratio between carboxylated and under-carboxylated osteocalcin from baseline as a function of cheese dose after a) three weeks, b) four weeks and c) five weeks. The horizontal lines indicate the maximum increase obtained as indicated by the vertical lines.

Table 3: Development in the vitamin K_2 long chain variants MK-7, MK-8, MK-9 and MK-9/4H as a total and within cheese-dose groups.

Variables	Dose group [g/day]	Baseline	2 weeks-baseline	4 weeks-baseline	5 weeks-baseline
MK-7 (ng/ml)	1: [20-40] (n=3) **	0.16 (0.08) 0.10-0.26	0.01 (0.02) -0.03-0.05	0.00 (0.04) -0.09-0.10	-0.02 (0.03) -0.16-0.12
	2: (40-60] (n=6)	0.13 (0.06) 0.07-0.19	0.01 (0.06) -0.05-0.07	0.02 (0.07) -0.06-0.09	-0.03 (0.04) -0.06-0.01
	3: (60-100] (n=5)	0.26 (0.28) 0.00-0.52	-0.07 (0.25) -0.39-0.25	-0,08 (0.26) -0.40-0.025	-009 (0.27) -0.42-0.25
	4: >100 (n=5)	0.16 (0.10) 0.04-0.29	004 (0.09) -0.07-0.15	0,05 (0.16) -0.14-0.25	Not performed
	Group 1+2+3+4 (N=19)	0.18 (0.18) 0.09-0.26	0.00 (0.14) -0.07-0.06	0.00 (0.16) -0.07-0.07	-0.05 (0.16) -0.14-0.04
MK-8 (ng/ml)	1: [20-40] (n=3) **	0.09 (0.02) 0.08-0.11	0.03 (0.04) -0.08-0.13	0.02 (0.05) -0.10-0.13	0,02 (0.05) -0.11-0.15
	2: (40-60] (n=6)	0.07 (0.04) 0.02-0.11	0.06 (0.08) -0.02-0.15	0.04 (0.06) -0.02-0.10	0,03 (0.05) -0.03-0.08
	3: (60-100] (n=5)	0.09 (0.05) 0.03-0.14	0.23 (0.17)* 0.02-0.44	0.21 (0.17)* 0.00-0.42	0.22 (0.20)* -0.02-0.47
	4: >100 (n=5)	0.09 (0.09) 0.0-0.18	0.32 (0.27)* -002-0.65	0.32(0.34) -0.10-0.73	Not performed
	Group 1+2+3+4 (N=19)	0.08 (0.05) 0.06-0.11	0.17 (0.20)* 0.07-0.26	0.16 (0.22)* 0.5-0.26	0.10 (0.15)* 0.01-0.18
MK-9 (ng/ml)	1: [20-40] (n=3) **	0.07 (0.05) 0.02-0.11	0.03 (0.07) -0.16-0.21	0,02 (0.07) -0.15-0.18	0,00 (0.05) -0.13-0.14
	2: (40-60] (n=6)	0.07 (0.05) 0.02-0.13	0.06 (0.11) -0.05-0.17	0.03 (0.08) -0.05-0.11	0.02 (0.07) -0.06-0.09
	3: (60-100] (n=5)	0.06 (0.03) 0.02-0.10	0.19 (0.15)* 0.00-0.37	0.19 (0.15)* 0.00-0.38	0.18 (013)* 0.03-0.34
	4: >100 (n=5)	0.05 (0.05) 0.00-0.10	0.24 (0.18)* 0.01-0.47	0.21 (0.21) -0.05-0.47	Not performed
	Group 1+2+3+4 (N=19)	0.06 (0.05) 0.04-0.09	0.14 (0.15)* 0.06-0.21	0.12 (0.16)* 0.04-0.19	0.07 (0.12)* 0.00-0.14
MK-9(4H)* (ng/ml)**	1: [20-40] (n=3) **	0.08 (0.04) 0.05-0.13	0.02 (0.03) -0.05-0.08	0.02 (0.01)* -0.01-0.05	0.01 (0.02) -0.04-0.06
	2: (40-60] (n=6)	0.08 (0.04) 0.04-0.11	0.04 (0.04)* 0.00-0.08	0,03 (0.04)* -0.01-0.07	0,01 (0.03) -0.02-0.05
	3: (60-100] (n=5)	0.10 (0.06) 0.03-0.18	0.04 (0.04)* -0.01-0.09	0.05 (0.03)* 0.01-0-09	0.05 (0.04)* 0.00-0.10
	4: >100 (n=5)	0.06 (0.03) 0.03-0.09	0.07 (0.04)* 0.02-0.12	007 (0.09) -0.03-0.18	Not performed
	Group 1+2+3+4 (N=19)	0.08 (0.04) 0.06-0.10	0.04 (0.04)* 0.02-0.06	0.04 (0.05)* 0.02-0.07	0.03 (0.04)* 0.00-0.05

*Indicate significant ($p \leq 0.05$) change from baseline; **the total range is given due to the sample size. ***May include other MK variants such as MK-10. The results are expressed by mean values, Standard Deviation in brackets and 95% confidence intervals.

The serum content of long-chained vitamin K2 variants MK-8, MK-9 and MK-9(4H) increased significantly and linearly with increasing cheese dose ($p<0.01$) and plateaued after three weeks. No changes were detected for MK-7 (Table 3). The cheese dose explains 36.5%, 33.7% and 50.6% of the variation in the K2 level after 3, 4 and 5 weeks, respectively. No significant change in the vitamin K1 level related to cheese dose was detected.

The lipid profile was measured in 11 of the HVs receiving a mean cheese dose of 50 g/day, ranging from 20 to 72 g/day (Table 4). Total cholesterol, LDL cholesterol and the ratios to HDL cholesterol were significantly reduced after the five weeks of cheese intake ($p \leq 0.05$). The HDL cholesterol increased slightly and non-significantly. The systolic and diastolic blood pressure were both significantly reduced ($p<0.05$). None of the recorded hematological and biochemical variables changed significantly during the study.

One participant reported cheese-related nausea and abdominal pain which was ongoing from baseline and up to week 4, but was resolved at week 5. Additionally, the HVs receiving 100 g/day or more claimed that the cheese-dose was too large and interfered with their usual daily food intake.

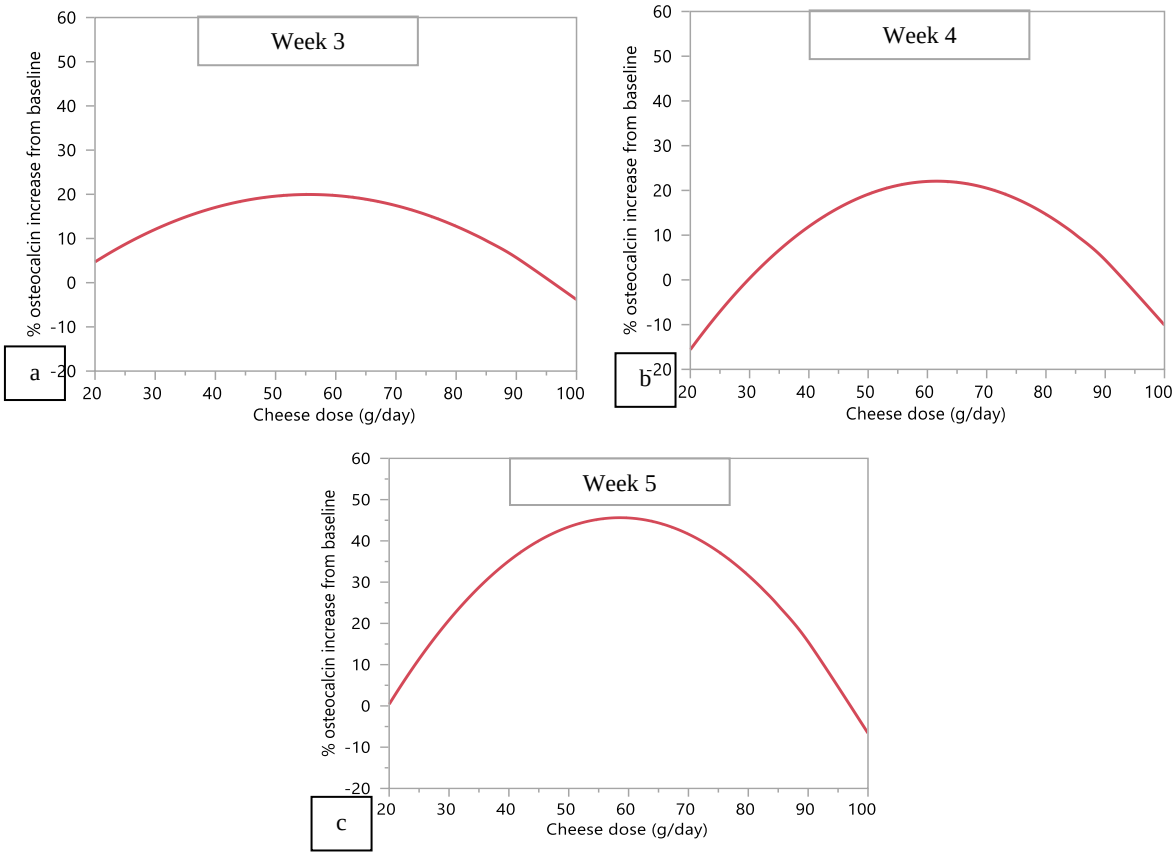

Figure 3: The predicted development of increased osteocalcin level from baseline as a function of cheese dose after (a) three weeks, (b) four weeks and (c) five weeks. The horizontal lines indicate the maximum increase obtained as indicated by the vertical lines.

DISCUSSION

The OR increased monotonically within a duration-dependent dose-window of daily cheese intake. For cheese-doses passing the upper limit of the dose-window, the OR decreased with increasing dose. This upper limit of the dose-window will then be the MED of Jarlsberg® cheese. The dose-response curve in OR seem to fit a quadratic function dependent of the treatment duration. The maximal OR increased and MED appeared slightly reduced with increasing treatment duration, and the recommendation will be a daily Jarlsberg® cheese intake around 60 g/day. Several questions related to this result may be raised. Is it possible to reduce the dose without losing the effect? If the dose is maintained, will the OR continue to increase and in case how much and for how long? In order to answer these questions, larger placebo controlled clinical long-term studies are needed.

In dose-response studies, it is common to assume a monotonically increasing effect or toxicity with increasing dose. This assumption was not fulfilled in this study. Cheese is a part of the daily diet. If the amount of cheese gets too high, it may influence the usual food intake. The chosen dose-window in the present study was 20 g/day to 180 g/day with a starting dose of 100 g/day. This large starting dose might have a major impact on the nutritional status. In order to control for this situation, the chosen RSP-procedure was to reduce the daily dose in case the given cheese dose resulted in reduced OR compared to baseline. In a common dose-response study, the opposite procedure would have been the logical choice. It is not common to perform dose-response studies in cheese. It was assumed that the response curve will follow a quadratic function. The RSP-design has been developed for such situation in which all the results at one design level recommended the same dose.[24] In clinical trials this is not always the case.

In order to ensure the assumption, a parallel and opposite RSP-procedure was used based on the results obtained at the first design level. The starting dose resulted in a reduced OR-level. In accordance with the parallel and opposite RSP-procedure five HVs were randomized to higher cheese doses in the parallel second design level. All these HVs obtained a reduced OR after three weeks. These reductions were even larger after four weeks and the treatment was terminated. The results from this parallel and opposite RSP procedure showed that a daily dose above 100 g/day is too large, possibly because such high intakes affect the nutritional status. The overall result concludes that MED of Jarlsberg® cheese is slightly below 60 g/day during a period of five weeks.

Table 4: Development in the lipid profile and vital signs.

Area	Variables	Baseline (week 0)	5 weeks	5 weeks-baseline
Lipid profile (n=11)	Total cholesterol (mmol/l)	4.16 (0.55)	3.95 (0.55)	-0.21 (0.35)*
		3.79-4.53	3.58-4.33	-0.45-0.03
	HDL cholesterol (mmol/l)	1.75 (0.48)	1.78 (0.49)	0.04 (0.23)
		1.42-2.07	1.46-2.11	-0.12-0.19
	LDL cholesterol (mmol/l)	2.10 (0.74)	1.86 (0.71)	-0.24 (0.36)*
		1.61-2.59	1.39-2.34	-0.48-0.01
	Ratio: Total/HDL	2.60 (0.96)	2.37 (0.70)	-0.23 (0.42)*
		1.96-3.24	1.89-2.84	-0.52-0.05
	Ratio: LDL/HDL	1.39 (0.86)	1.18 (0.63)	-0.22 (0.38)*
		0.82-1.97	0.75-1.60	-0.48-0.04
Vital signs (n=19)	Systolic blood	112 (13)	108 (13)	-4 (9)**
	Pressure (mmhg)	106-119	102-115	-8-0
	Diastolic blood	72 (8)	67 (9)	-5 (7)**
	Pressure (mmhg)	68-76	63-71	-8--2
	Heart rate	68 (9)	68 (10)	0 (6)
	(Beats/min)	64-72	63-73	-3-3
	Respiratory rate	13 (1)	12 (2)	-1 (1)**
	(Breath/min)	12-13	11-13	-1-0

*Indicate significant ($p \leq 0.05$) one-tailed reduction and **indicate significant two-tailed from baseline. The results are expressed by mean values, standard deviation in brackets and 95% confidence intervals.

Vitamin K is necessary to carboxylate osteocalcin and deficiency results in increased risk of fracture.[2,3,7,25] An analysis of blood samples from 896 persons suggested that most people do not receive enough vitamin K from their diet (32).[26] The best vitamin K to improve vitamin K status is long-chained MKs.[12,13] Our results showed significant increases in serum levels of the long-chained MK-8, MK-9 and MK-9(4H). These increases were detected after three weeks of cheese intake, but probably occurred much earlier.

A most surprising finding in the present study was the increase in serum osteocalcin. To our knowledge no food has previously been shown to increase osteocalcin. Intervention studies with isolated vitamin K2, as MK-4 or MK-7, have demonstrated increases in OR, but the increase in carboxylated osteocalcin has been at the expense of undercarboxylated osteocalcin and total osteocalcin has not been affected.[27] These studies employed much higher vitamin K2 doses than in the MED in the present study. *In vitro*, very high vitamin K2 levels have been shown to enhance osteocalcin expression.[28] Thus, the increase seen in the present study might be ascribed to vitamin K2. However, the negative impact of the highest cheese doses demonstrates that regulation of osteocalcin is complex. The reduction of OR at the high doses also show that osteocalcin carboxylation is affected by factors additional to vitamin K.

Osteocalcin is responsible for binding calcium to bone tissue, giving the bones strength and flexibility. For this process to occur, osteocalcin needs to be carboxylated by vitamin K (2). The level of circulating osteocalcin is high during rapid growth, notably in children during the first years of life and during puberty and falls during adulthood.[29] From the age of 30 to 40 years, the osteocalcin level is assumed unchanged for young and middle-aged humans. Except for one HV, the participants in the present study were between 30 and 50 years of age. The osteocalcin levels varied a lot between persons, possibly influenced by nutritional factors, lifestyle and genetics.[5] Three of the participants in the present study were Malaysian Thai, but have lived in Norway for more than 10 years. These HVs had an initial osteocalcin level below 1/3 of the remaining sample. However, the ORs were on the same level as the others. All the HVs had a normal nutritional status and osteocalcin level.

Beside its role in bone tissue osteocalcin is a hormone affecting diverse functions such as metabolism, adaptation to exercise and brain function.[2,3,5,7] In mice, these effects have been attributed to undercarboxylated osteocalcin, but in several human studies osteocalcin or only carboxylated osteocalcin rather than the undercarboxylated have shown effects.[30-33] The present results indicate intake of Jarlsberg® cheese to cause both carboxylated and under carboxylated osteocalcin to increase. In old people low levels of osteocalcin have been associated with metabolic syndrome and poor cognitive performance.[34,35]

Both positive and negative associations have been found between circulating osteocalcin and aortic stiffness and calcification, but most studies have reported non-significant association.[3,36] One study including 1691 men

and 1913 women found that the osteocalcin levels and stiffness in the arteries were related in an "inverse J-shaped curve".[37] That study did not report on the extent of osteocalcin carboxylation, an indicator of vitamin K status. The vitamin K status has been found to be negatively associated with cardiovascular disease and mortality in controlled clinical trials and longitudinal studies.[8] Of note, the present study detected significant reductions of blood pressure and total cholesterol in HVs taking between 20 and 73g of Jarlsberg® per day. Blood pressure lowering effects of cheese have been ascribed to casein derived peptides with angiotensin-converting enzyme-inhibiting activity formed in the cheese fermentation.[37] Previous studies have shown that cheese does not cause the same cholesterol increase as a diet with an equal amount of fat given as butter.[39] The cause of this difference is unknown and has tentatively been ascribed to the cheese matrix itself.[40] Neither has vitamin K2 nor osteocalcin been associated with cholesterol reduction, but in persons receiving 500 µg K1 daily over 3 years the study participants experienced 5% reductions in LDL, suggesting that this effect can be ascribed to the vitamin K.

CONCLUSION

The MED of Jarlsberg® cheese was estimated to 57g/day. Daily intake of Jarlsberg® cheese increased the osteocalcin level, vitamin K2, and positively affected the lipid patterns and blood pressure.

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Conflict of interest: In addition to be Professor at the University, Helge Holo hold the possition as researcher at TINE SA. None of the other authors have any conflict of interest

Ethical approval: The study was approved by the Norwegian Regional Ethical Committee

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