

# Effect on bone anabolic markers of daily cheese intake with and without vitamin K<sub>2</sub>: a randomised clinical trial

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## ABSTRACT

**Background** Daily intake of 57 g Jarlsberg cheese has been shown to increase the total serum osteocalcin (tOC). Is this a general cheese effect or specific for Jarlsberg containing vitamin K<sub>2</sub> and 1,4-dihydroxy-2-naphthoic acid (DHNA)?

**Methods** 66 healthy female volunteers (HV) were recruited. By skewed randomisation (3:2), 41 HV were allocated to daily intake of 57 g Jarlsberg (J-group) and 25–50 g Camembert (C-group) in 6 weeks. After 6 weeks the C-group was switched to Jarlsberg. The study duration was 12 weeks with clinical investigations every 6 weeks. The main variables were procollagen type 1 N-terminal propeptide (PINP), tOC, carboxylated osteocalcin (cOC) and the osteocalcin ratio (R<sub>0</sub>) defined as the ratio between cOC and undercarboxylated osteocalcin (ucOC). Serum cross-linked C-telopeptide type I collagen (CTX), vitamin K<sub>2</sub>, lipids and clinical chemistry were used as secondary variables.

**Results** PINP, tOC, cOC, R<sub>0</sub> and vitamin K<sub>2</sub> increased significantly (p<0.01) after 6 weeks in the J-group. PINP remained unchanged in the C-group. The other variables decreased slightly in the C-group but increased significantly (p≤0.05) after switching to Jarlsberg. No CTX-changes detected in neither of the groups. Serum lipids increased slightly in both groups. Switching to Jarlsberg, total cholesterol and low-density lipoprotein-cholesterol were significantly reduced (p≤0.05). Glycated haemoglobin (HbA1c), Ca<sup>++</sup> and Mg<sup>++</sup> were significantly reduced in the J-group, but unchanged in the C-group. Switching to Jarlsberg, HbA1c and Ca<sup>++</sup> decreased significantly.

**Conclusion** The effect of daily Jarlsberg intake on increased s-osteocalcin level is not a general cheese effect. Jarlsberg contain vitamin K<sub>2</sub> and DHNA which increases PINP, tOC, cOC and R<sub>0</sub> and decreases Ca<sup>++</sup>, Mg<sup>++</sup> and HbA1c. These effects reflect increased bone anabolism and a possible reduced risk of adverse metabolic outcomes.

**Trial registration number** NCT04189796.

## INTRODUCTION

Vitamin K is important for bone health, and low intake of vitamin K has been associated with increased risk of fractures.<sup>1</sup> Supplementation with vitamin K<sub>2</sub>, vitamin D and calcium is a recommended first-line treatment of

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Jarlsberg cheese contains long-chained vitamin K<sub>2</sub> and 1,4-Dihydroxy-naphthoic acid, and the daily optimal efficiency dose of 57 g/day increases the osteocalcin level.

## WHAT THIS STUDY ADDS

⇒ The present study was performed to verify the increase in osteocalcin, the impact on bone turnover markers (BTM) and to verify that the effect is not a general cheese effect.

⇒ Daily Jarlsberg cheese consumption has a positive effect on osteocalcin, other BTM's, glycated haemoglobin and lipids.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ These results indicates that Jarlsberg cheese might have prophylactic effects on osteopenia and metabolic diseases. Further research may reveal the possibility of recommending Jarlsberg cheese as prophylactical treatment or even an addition to antiresorptive treatment in osteoporosis.

osteoporosis.<sup>2</sup> Vitamin K<sub>2</sub> is essential for carboxylation or activation of Gla proteins. Osteocalcin is 1 of the 17 Gla proteins in humans, and carboxylated osteocalcin has a key role in bone formation and maintenance. The ratio between circulating carboxylated and undercarboxylated osteocalcin reflects the extrahepatic vitamin K status.

Vitamin K<sub>2</sub> or menaquinone (MK) has many variants.<sup>3</sup> The short-chained MK-4 can be formed from vitamin K<sub>1</sub> in humans and is found in animal products such as liver. The long-chained vitamin K<sub>2</sub> vitamers like MK-7, MK-8, MK-9 and MK-9(4H) are of bacterial origin, and occur in certain fermented foods such as cheese.

These variants have greater extrahepatic activity than MK-4 and vitamin K<sub>1</sub>; possibly due to more efficient uptake and much longer serum half-life.<sup>4</sup> Prospective cohort

studies have demonstrated health benefits that can be attributed to the intake of vitamin K<sub>2</sub>, but not K<sub>1</sub>. In the Western diet cheese is the major source of long-chained vitamin K<sub>2</sub>.<sup>5</sup>

MK-9 is the most important K<sub>2</sub> vitamer in cheese, but the amount varies considerably.<sup>6</sup> Some cheeses also contain MK-9(4H) and Jarlsberg cheese is particularly rich in both MK-9 and MK-9(4H).<sup>7</sup> This cheese is made with lactic acid bacteria producing MK-8 and MK-9 and *Propionibacterium freudenreichii* producing MK-9(4H).

A previous dose-response study of daily Jarlsberg with healthy premenopausal women was performed with a three-level between patient response surface pathway design.<sup>8,9</sup> The maximum efficacy dose (MED) of Jarlsberg in order to obtain increased vitamin K<sub>2</sub> status, measured as osteocalcin carboxylation and total serum osteocalcin (tOC) level was estimated to 57 g/day.<sup>10</sup> These results were confirmed in a recent clinical study on the same study population.<sup>11</sup> The MED of 57 g/day Jarlsberg cheese was verified to increase the vitamin K<sub>2</sub> status and tOC level.<sup>11</sup> The maintenance dose for this study population was estimated to 45 g/day of Jarlsberg and verified as sufficient to keep the obtained tOC level. Osteocalcin is used as a serum marker of osteoblastic bone formation indicating that Jarlsberg stimulates bone formation.<sup>12</sup> Bone markers like procollagen type 1 N-terminal propeptide (PINP) and serum cross-linked C-telopeptide type I collagen (CTX) were not recorded.<sup>13,14</sup>

Although cheese has been associated with vitamin K<sub>2</sub>-related health benefits, the effect of cheese consumption on bone health has, to the best of our knowledge, never been investigated in human controlled clinical trials. Because of its high vitamin K<sub>2</sub> content, Jarlsberg appears to be a good candidate for such studies.

1,4-dihydroxy-2-naphthoic acid (DHNA) has been shown to have anti-osteoporotic effects and increase bone mineral density in ovariectomised mice.<sup>15</sup> Jarlsberg contains *Propionibacterium freudenreichii*, which produces MK-9(4H), but also secretes DHNA. This might explain the increase in tOC as well as the carboxylation of osteocalcin (cOC) and it has been suggested that DHNA could be used in treatment of postmenopausal osteoporosis.<sup>15</sup> If the raise of tOC and cOC levels is not a general cheese effect, but a Jarlsberg effect, further studies on Jarlsberg as prophylaxis are even more relevant. The aim of this study was to compare the effect of daily Jarlsberg cheese intake with a cheese without vitamin K<sub>2</sub>, but similar fat and protein content, on PINP, tOC, cOC, R<sub>O</sub> and glucose metabolism by glycated haemoglobin (HbA1c).

## MATERIAL AND METHODS

### Population and sample

The study population consisted of healthy Norwegian women (healthy volunteer (HV)) of premenopausal 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 3 weeks or participating in another clinical trial the last 6 weeks before start of the study were not included.

Sixty-eight HVs were recruited by eight general practitioners from Viken County in Norway. Two HVs dropped out from the study for personal reasons during the first week. The study sample consisted of 66 HVs with a mean age of 33.6 years (range: 19.4–51.9 years) and body mass index of 24.3 kg/m<sup>2</sup> (range: 15.0–35.4). By randomisation, 41 HVs were allocated to daily intake of Jarlsberg cheese (J-group) and 25 to Camembert cheese (C-group). The two groups were found comparable on all recorded demographic data, concomitant diseases and treatment (table 1). All ongoing treatments were kept unchanged during the study. Vitamin D deficiency was initially recorded in nine participants, and supplements were provided to obtain normal range.

### Study design and allocation

The study was performed as an open, randomised multicentre trial with semi crossover design.<sup>16</sup> The participants were randomised for either 6 weeks of Jarlsberg or 6 weeks of Camembert cheese. The participants allocated to Camembert were then switched to Jarlsberg for an additional 6 weeks. The results obtained during the first 6 weeks and the 6 weeks after switch in cheese are reported. The study subjects were skewed randomly allocated (2:3) in two treatment groups; 41 in the J-group and 27 in the C-group. The allocation of participants to treatment groups was performed by the data manager prior to the start of the study by stratified randomisation with a fixed block size of 10.<sup>17</sup> Two HVs withdrew, reducing the C-group to 25. The participants were asked not to change their usual diet during the study except for avoiding other cheese than the cheese provided in the study. No diet registration was performed, but the participants daily registered their intake of either Jarlsberg or Camembert during the study. The cheese compliance in the study was 95% and 92% in the J-group and the C-group, respectively. The compliance in the C-group after switching to Jarlsberg was 93%.

### Clinical procedure

The clinical part of the study was performed from March 2020 to August 2020. Clinical status and blood sampling were performed initially and every 6 weeks. In addition to common laboratory variables, the blood samples were used for measurements of osteocalcin; PINP; CTX; lipids and the vitamin K<sub>2</sub> vitamers MK-7, MK-8, MK-9 and MK-9(4H).

### Clinical intervention

The J-group received 57 g/day or six slices Jarlsberg, and the C-group 50 g/day of TINE Camembert cheese for 6 weeks. Participants of the J-group received 100 g packages containing cheese slices of 10 g. The C-group received

**Table 1** Demographics, vital signs, social habits and concomitant diseases or claims

Variable		Jarlsberg (n=41)	Camembert (n=25)
Age (years)	Mean (SD)	34.2 (9.9)	32.6 (7.0)
	Min-max	19.6–51.9	19.4–42.6
Weight (kg)	Mean (SD)	66.8 (9.8)	69.3 (13.4)
	Min-max	37.0–87.1	48.0–102.0
Height (cm)	Mean (SD)	167 (6)	167 (6)
	Min-max	156–185	152–178
Body mass index	Mean (SD)	23.8 (3.0)	25.0 (4.8)
	Min-max	15.0–29.8	17.9–35.4
Systolic blood pressure	Mean (SD)	115 (13)	120 (18)
	Min-max	92–154	93–169
Diastolic blood pressure	Mean (SD)	72 (10)	77 (10)
	Min-max	54–101	56–98
Pulse rate	Mean (SD)	67 (13)	74 (12)
	Min-max	40–120	46–97
Social status	Married/cohabitant	26	15
	Divorced	2	3
	Single	13	7
Smoking	Ex/no	3/38	1/24
Ethnic	Asian/Caucasian	4/37	1/24
Concomitant disease under treatment during the study	D-vitamin deficiency	6	3
	Iron deficiency	1	0
	Allergy	3	3
	Asthma	1	1
	Atopic eczema	2	0
	Hypothyroidism	2	1
	Fibromyalgia	0	1
	Contraceptives	12	2
	Hypertension	6	0
	Tachycardia	1	0
	Dyspepsia	1	1

150 g packages, divided in three equal parts. The corresponding energy and fat intake per day is 680 kJ and 14.0 g in the C-group, and 729 kJ and 15.4 g in the J-group. Both the Jarlsberg and Camembert are produced by TINE SA and cheese from three production batches was used.

The average vitamin K per 100 g of Jarlsberg cheese contained 3.0 µg vitamin K<sub>1</sub>, 5.2 µg MK-4 and 1.5 µg MK-7, 6.7 µg MK-8, 23.9 µg MK-9 and 40.5 µg MK-9(4H). TINE Camembert cheese does not contain vitamin K.

### Vitamin K extraction and osteocalcin analysis

Vitamin K in cheese and blood samples was analysed as described previously.<sup>10 11</sup> The cOC and the undercarboxylated osteocalcin (ucOC) were measured in plasma by immunoassays kits (Takara Bio, Ōtsu, Japan) by Vitas AS. The tOC is defined as the sum of cOC and ucOC.

### Central variables

The three main variables were PINP, tOC and the osteocalcin ratio  $R_o = \text{cOC} / \text{ucOC}$ . The supporting variables were sum of vitamin K<sub>2</sub> and the variants MK-7, MK-8, MK-9 and MK-9(4H). Additionally CTX, Glycated haemoglobin; the lipids; haematological and clinical chemistry were used as secondary variables.

### Randomisation

The participants were skewed allocated (2:3) to the J-group and C-group by block randomisation with fixed block size of 10.<sup>17</sup>

### Statistical analysis

A significance level of 0.05; a power of 90% and a clinical relevant difference between groups of one time SD, a minimum of 23 participants are required in each group.

The assumed continuously distributed variables were expressed by mean values with 95% CI.<sup>18</sup> As an index of dispersion, SD were given. Categorical variables were given in contingency tables.<sup>19</sup> All tests were performed two-tailed with a significance level of 0.05. Analysis of covariance with baseline measurement as covariate was used for comparison of groups.<sup>20</sup>

Changes within groups were tested by using a paired two-tailed t-test.<sup>18</sup>

## Approvals

The study was performed in accordance with the research protocol; 'Effect of Jarlsberg cheese compared with cheese without vitamin K2 (Camembert) regarding increased Osteocalcin level in healthy women', (ClinicalTrials.gov).

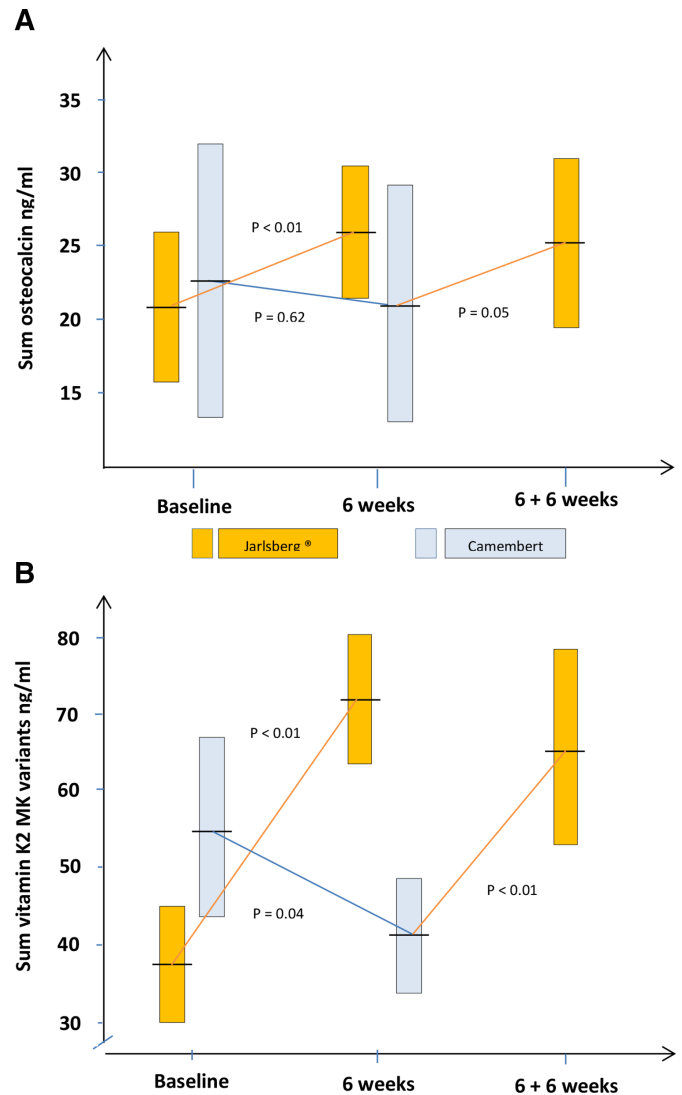
## RESULTS

The tOC level increased significantly ( $p < 0.01$ ) from 21.4 ng/mL (95% CI: 16.6 to 26.2) to 25.8 ng/mL (95% CI: 21.0 to 30.6) after 6 weeks in the J-group (figure 1A). In the C-group this level was slightly reduced from 23.3 ng/mL (95% CI: 13.7 to 32.8) to 21.3 ng/mL (95% CI: 13.3 to 29.3). The difference in tOC increase between groups was 6.4 ng/mL (95% CI: 13.7 to 32.8) and significantly ( $p < 0.01$ ) in favour of Jarlsberg cheese. After switching to daily intake of Jarlsberg in the C-group, the tOC level increased significantly ( $p = 0.05$ ) from 21.3 ng/mL to 25.1 ng/mL (95% CI: 18.8 to 31.4) (figure 1A). A similar pattern was detected for cOC and the  $R_o$  (table 2).

Both variables increased significantly in the J-group, but remained unchanged in the C-group. The increase in cOC and  $R_o$  was significant different in the two groups ( $p < 0.01$ ;  $p = 0.04$ ). The level of ucOC was nearly unchanged in both groups. After switching to Jarlsberg in the C-group, both the cOC and the  $R_o$  increased significantly ( $p < 0.01$ ;  $p = 0.05$ ) whereas the ucOC were unaffected (table 3).

PINP increased significantly ( $p < 0.01$ ) in the J-group, but remained unchanged in the C-group (table 2). The mean difference between the groups was 9.4 ng/mL (95% CI: 1.3 to 9.3) and significant ( $p = 0.01$ ) in favour of Jarlsberg. CTX was unaffected in both groups during the study. After switching to Jarlsberg in the C-group, PINP increased, but not significantly (table 3). However, PINP decreased in 7 of the 25 participants; a proportion significantly ( $p \leq 0.05$ ) below 50%. Four of these seven HVs had a remarkably large PINP reduction, resulting in the lack of significance on the mean level. CTX was unaffected after switching in cheese.

The sum of vitamin K<sub>2</sub> vitamers increased significantly ( $p < 0.01$ ) from 0.38 ng/mL (95% CI: 0.30 to 0.45) to 0.72 ng/mL (95% CI: 0.64 to 0.81) after 6 weeks in the J-group (figure 1A). A significant reduction ( $p = 0.04$ ) was detected in the C-group. The development in sum of vitamin K<sub>2</sub> was significant in favour of the J-group ( $p < 0.01$ ). After switching to Jarlsberg in the C-group, the sum of vitamin K<sub>2</sub> increased significantly ( $p < 0.01$ ) from 0.41 ng/mL (95% CI: 0.34 to 0.48) to 0.65 ng/mL (95% CI: 0.53 to



**Figure 1** Development within- and comparison between cheese groups on (A) total serum osteocalcin and (B) Sum vitamin K<sub>2</sub> vitamers. The yellow boxes show 95% confidence intervals of the mean in the Jarlsberg treated group and the blue similarly in the Camembert group. The mean values are given as horizontal lines crossing the boxes.

0.79) (figure 1B). All the vitamin K<sub>2</sub> vitamers MK-7, MK-8, MK-9 and MK-9(4H) increased significantly ( $p < 0.01$ ) after 6 weeks in the J-group but decreased in the C-group ( $0.02 \leq p \leq 0.21$ ) (table 2). The increases in all MK-variants were significant in favour of Jarlsberg cheese. After switching from Camembert to Jarlsberg, MK-8, MK-9 and MK-9(4H) increased significantly ( $p < 0.01$ ) whereas MK-7 was nearly unchanged (table 3).

Mean HbA1c decreased significantly ( $p < 0.01$ ) after 6 weeks in the J-group (table 4). In the C-group this level was significantly increased ( $p = 0.05$ ). The mean difference in HbA1c development between groups was significantly ( $p < 0.01$ ) in favour of Jarlsberg cheese. After switching to daily intake of Jarlsberg in the C-group, mean HbA1c decreased significantly ( $p < 0.01$ ) (table 5).

Triglycerides, low-density lipoprotein (LDL)-cholesterol, total cholesterol and the cholesterol ratio



**Table 2** Comparison of Jarlsberg and Camembert cheese in development of osteocalcin variables and bone markers and the vitamin K<sub>2</sub> vitamers during 6 weeks of daily cheese intake. The results are expressed by mean values with SD in bracket and 95% CIs

Variables	Baseline		Week 6		Week 6—baseline		P values between groups
	Jarlsberg (n=41)	Camembert (n=25)	Jarlsberg (n=41)	Camembert (n=25)	Jarlsberg (n=41)	Camembert (n=25)	
cOC=carboxylated osteocalcin (ng/mL)	11.2 (9.0) 8.4 to 14.1	16.1 (18.3) 8.6 to 23.7	15.3 (9.5) 12.3 to 18.3	14.9 (15.0) 8.7 to 21.0	4.1 (6.8) 2.0 to 6.2	-1.2 (9.7) -5.3 to 2.8	p<0.01
ucOC=undercarboxylated osteocalcin (ng/mL)	10.2 (10.4) 6.9 to 13.5	7.2 (5.4) 5.0 to 9.4	10.3 (12.9) 6.3 to 14.4	6.6 (5.0) 4.5 to 8.6	0.2 (6.0) -1.7 to 2.1	-0.6 (1.9) -1.4 to 0.2	p=0.47
R <sub>o</sub> =carboxylated/undercarboxylated	1.47 (0.94) 1.17 to 1.76	1.86 (1.14) 1.39 to 2.33	2.19 (1.31) 1.78 to 2.60	2.09 (1.34) 1.54 to 2.64	0.72 (0.90) 0.44 to 1.01	0.23 (1.09) -0.26 to 0.65	p=0.03
PINP=procollagen (ng/mL)	61.1 (27.5) 52.4 to 69.7	61.4 (24.1)* 51.0 to 71.8	69.1 (34.9) 58.1 to 80.2	58.4 (21.7) 49.4 to 67.4	8.1 (13.9) 3.7 to 12.4	-1.3 (11.4)* -6.2 to 3.6	p<0.01
CTX=collagen I (ng/mL)	0.29 (0.13) 0.25 to 0.33	0.27 (0.17)* 0.19 to 0.34	0.27 (0.15) 0.22 to 0.32	0.28 (0.15) 0.22 to 0.34	-0.02 (0.13) -0.06 to 0.02	0.02 (0.15)* -0.04 to 0.08	p=0.27
MK-7 (ng/mL)	0.12 (0.07) 0.09 to 0.14	0.21 (0.17) 0.14 to 0.28	0.15 (0.06) 0.14 to 0.17	0.18 (0.08) 0.15 to 0.22	0.04 (0.07) 0.02 to 0.06	-0.03 (0.15) -0.09 to 0.04	p=0.02
MK-8 (ng/mL)	0.09 (0.07) 0.07 to 0.12	0.12 (0.08) 0.09 to 0.15	0.22 (0.11) 0.18 to 0.25	0.07 (0.06) 0.04 to 0.09	0.12 (0.10) 0.09 to 0.16	-0.05 (0.06) -0.08 to 0.03	p<0.01
MK-9 (ng/mL)	0.09 (0.09) 0.06 to 0.12	0.11 (0.07) 0.08 to 0.14	0.21 (0.09) 0.18 to 0.24	0.05 (0.04) 0.04 to 0.07	0.12 (0.11) 0.09 to 0.16	-0.06 (0.07) -0.09 to -0.03	p<0.01
MK-9(4H) (ng/mL)	0.08 (0.06) 0.06 to 0.09 1.15 to 1.26	0.10 (0.07) 0.06 to 0.09 1.11 to 1.26	0.14 (0.07) 0.12 to 0.16 1.18 to 1.29	0.10 (0.05) 0.08 to 0.12 1.10 to 1.22	0.06 (0.05) 0.05 to 0.08 -0.02 to 0.09	0.00 (0.05) -0.02 to 0.02 -0.08 to 0.03	p<0.01

\*Two baseline blood samples in the Camembert group were lost and reduced the sample to 23 for estimation and comparison of the development in PINP and CTX.

cOC, carboxylation osteocalcin ; CTX, C-telopeptide type I collagen ; MK, menaquinone; PINP, procollagen type 1 N-terminal propeptide ; R<sub>o</sub>, osteocalcin ratio; ucOC, undercarboxylated osteocalcin.

LDL/high-density lipoprotein (HDL) increased slightly, but significantly ( $p \leq 0.05$ ) in both groups during the first 6 weeks of cheese intake (table 4). No significant difference was detected between groups. After switching cheese in the C-group, total cholesterol and LDL-cholesterol were significantly reduced ( $p \leq 0.05$ ). A slight but not significant reduction was detected in the triglycerides and the cholesterol ratio (table 5).

Ca<sup>++</sup> and Mg<sup>++</sup> were significantly reduced in the J-group ( $p \leq 0.05$ ) but remained unchanged in the C-group (table 4). The differences between the groups were found significant ( $p \leq 0.05$ ). After switching cheese in the C-group, Ca<sup>++</sup> were significantly reduced ( $p \leq 0.05$ ), but Mg<sup>++</sup> was found unchanged (table 5).

Phosphate increased in the J-group, but reduced in the C-group. None of the changes were significant, but the difference between groups was borderline significant (table 4). When switching to Jarlsberg in the C-group, the phosphate increased, but not significantly (table 5).

Urea increased significantly ( $p < 0.01$ ) in the J-group and decreased non-significantly in the C-group (table 4). The difference between groups was found significant ( $p < 0.01$ ). No change in urea was detected in the C-group

after the switch to Jarlsberg (table 5). Creatinine was reduced significantly ( $p < 0.01$ ) in the C-group, but non-significantly in the J-group (table 4). The difference between groups was not significant. No change in creatinine was detected in the C-group after the switch to Jarlsberg cheese (table 5).

## DISCUSSION

In the J-group, the tOC and cOC increased significantly confirming the results in the two previous dose-response studies.<sup>10 11</sup> In the C-group the tOC was slightly, but not significantly reduced after the first 6 weeks. Switching from Camembert cheese to Jarlsberg was led to a significant increase in tOC and cOC. This supports that Jarlsberg has a significant stimulatory effect on serum osteocalcin. A Mediterranean diet enriched with virgin olive oil has been associated with increased tOC in elderly men.<sup>21</sup> The present study is probably the first time dietary intervention has been able to demonstrate a significant increase in the tOC level in a randomised controlled trial.

Measurable changes in Bone Mass Density (BMD) require a minimum of 1 year observation. Like tOC,

**Table 3** Development in osteocalcin, bone markers and the vitamin MK variants after switching from Camembert to 6 weeks daily Jarlsberg cheese intake. The results are expressed by mean values with SD in bracket and 95% CIs

Variables	Baseline Jarlsberg (n=25)	6 weeks on Jarlsberg (n=25)	Week 6—baseline (n=25)	P values within variables
Carboxylated osteocalcin (ng/mL)	14.9 (15.0) 8.7 to 21.0	19.0 (12.2) 14.0 to 24.1	4.2 (8.5) 0.7 to 7.7	p=0.02
Undercarboxylated osteocalcin (ng/mL)	6.6 (5.0) 4.5 to 8.6	6.1 (3.4) 4.6 to 7.5	−0.5 (2.6) −1.6 to 0.6	p=0.32
Ratio: carboxylated/undercarboxylated	2.09 (1.34) 1.54 to 2.64	3.1 (1.28) 2.53 to 3.58	0.96 (1.06) 0.53 to 1.40	p<0.01
PINP: procollagen (ng/mL)	58.4 (21.7) 49.4 to 67.4	61.1 (24.1) 51.2 to 71.1	2.7 (9.6) −1.2 to 6.7	p=0.17
CTX: (ng/mL)	0.28 (0.15) 0.22 to 0.34	0.25 (0.12) 0.20 to 0.30	−0.03 (0.10) −0.07 to 0.01	p=0.07
MK-7 (ng/mL)	0.18 (0.08) 0.15 to 0.22	0.17 (0.10) 0.13 to 0.21	−0.02 (0.07) −0.05 to 0.02	p=0.30
MK-8 (ng/mL)	0.07 (0.06) 0.04 to 0.09	0.17 (0.10) 0.13 to 0.21	0.10 (0.08) 0.07 to 0.14	p<0.01
MK-9 (ng/mL)	0.05 (0.04) 0.04 to 0.07	0.19 (0.12) 0.15 to 0.24	0.14 (0.11) 0.10 to 0.19	p<0.01
MK-9(4H) (ng/mL)	0.10 (0.05) 0.08 to 0.12	0.13 (0.06) 0.10 to 0.15	0.02 (0.04) 0.01 to 0.04	p=0.01

CTX, C-telopeptide type I collagen ; MK, menaquinone; PINP, procollagen type 1 N-terminal propeptide .

PINP is an osteoblast-derived bone formation marker.<sup>13</sup> CTX is a marker of osteoclast activity and used to assess the level of bone resorption.<sup>14</sup> PINP and CTX are used as bone turnover biomarkers and may predict changes in BMD within 1–3 months.<sup>22</sup> The present study included healthy premenopausal women, but detected a significant increase in PINP combined with unchanged CTX level the J-group; reflecting stimulated osteoblast activity and unaltered bone resorption. This indicates that daily use of 57 g Jarlsberg during a period of at least 6 weeks may increase BMD. The decreases in Ca ++ and Mg ++ caused by consumption of Jarlsberg probably also reflect uptake from the blood for net bone formation. Daily intake of Camembert did not show these effects.

The vitamin K<sub>2</sub> vitamers MK-7, MK-8, MK-9 and MK-9(4H) all increased significantly during the 6 weeks in the Jarlsberg group. Significant reductions were recorded in the C-group. After switching to Jarlsberg, the increase in vitamin K<sub>2</sub> in the C-group showed an almost parallel pattern to the increase in the J-group. There was a high degree of covariation between the rise of tOC and vitamin K<sub>2</sub>. The calculated correlation between these two variables is significant, but substantially weaker than the graphical illustration may indicate, and was obvious in the previous studies.<sup>10 11</sup> In the dose-response study, the level of vitamin K<sub>2</sub> increased with increasing cheese dose whereas tOC and cOC showed a maximum at 57 g cheese a day and falling trends above this dose.<sup>10</sup> A similar pattern

was also detected in the dose de-escalation study.<sup>11</sup> This should not be the case if vitamin K<sub>2</sub> was the only factor which increases tOC. Thus the increase in tOC cannot solely be ascribed to the vitamin K<sub>2</sub> content of Jarlsberg.

In vitro, vitamin K<sub>2</sub> has been shown to stimulate osteoblast differentiation and mineralisation, and some human studies have found a BMD increase in postmenopausal women taking 45 mg daily of vitamin K<sub>2</sub>.<sup>23 24</sup> The vitamin K<sub>2</sub> in Jarlsberg is formed by lactic acid bacteria and propionic acid bacteria. These vitamin K<sub>2</sub> vitamers are different from those K<sub>2</sub>-tablets tested in clinical trials. *Propionibacterium freudenreichii* has been found to produce DHNA; an intermediate in the vitamin K<sub>2</sub> biosynthesis.<sup>25</sup> It has been suggested that DHNA could be an alternative or at least an additional treatment of postmenopausal osteoporosis.<sup>16</sup> In ovariectomised mice, this compound was shown to stimulate the production of osteocalcin, while vitamin K<sub>2</sub> could not.<sup>16</sup> DHNA is the possible key to the tOC stimulatory effect of Jarlsberg. DHNA is formed as an intermediate in the biosynthesis of vitamin K<sub>2</sub> in all organisms that produce the vitamin. However, to our knowledge propionic acid bacteria are the only bacteria reported to produce DHNA in substantial excess.

These results may come out additionally as important facts for future pharmaceutical interventions.

The participants were asked to maintain their usual diet during the study except for any cheese provided in the study. The cheese compliances were found satisfactory,

**Table 4** Comparison of Jarlsberg and Camembert cheese in development of biochemical variables during 6 weeks of daily cheese intake. The results are expressed by mean values with SD in bracket and 95% CIs

Variables	Baseline		Week 6		Week 6—baseline		P values between groups
	Jarlsberg (n=41)	Camembert (n=25)	Jarlsberg (n=41)	Camembert (n=25)	Jarlsberg (n=41)	Camembert (n=25)	
Triglyceride (mmol/L)	1.0 (0.6) 0.9 to 1.1	1.2 (0.6) 1.0 to 1.5	1.1 (0.5) 0.9 to 1.3	1.4 (0.8) 1.1 to 1.7	0.1 (0.5) 0.0 to 0.3	0.2 (0.6) −0.1 to 0.4	p=0.41
LDL-cholesterol (mmol/L)	2.7 (0.7) 2.5 to 2.9	3.2 (0.8) 2.9 to 3.6	2.9 (0.7) 2.6 to 3.2	3.4 (0.9) 3.1 to 3.8	0.2 (0.4) 0.0 to 0.4	0.2 (0.6) 0.0 to 0.4	p=0.67
HDL-cholesterol (mmol/L)	1.7 (0.4) 1.6 to 1.8	1.6 (0.4) 1.4 to 1.7	1.7 (0.4) 1.6 to 1.8	1.6 (0.3) 1.5 to 1.8	0.0 (0.2) −0.1 to 0.1	0.0 (0.2) −0.1 to 0.1	p=0.99
Total cholesterol (mmol/L)	4.6 (0.7) 4.4 to 4.8	4.9 (0.6) 4.6 to 5.1	4.8 (0.7) 4.6 to 5.0	5.1 (0.7) 4.8 to 5.4	0.2 (0.4) 0.1 to 0.4	0.2 (0.5) 0.0 to 0.5	p=0.52
LDL/HDL	1.7 (0.6) 1.5 to 1.9	2.2 (1.0) 1.8 to 2.6	1.85 (0.62) 1.6 to 2.0	2.3 (0.9) 1.9 to 2.7	0.1 (0.3) 0.0 to 0.2	0.1 (0.4) −0.1 to 0.2	p=0.94
Creatinine (μmol/L)	60.9 (10.0) 57.8 to 64.0	64.1 (7.8) 60.9 to 67.3	59.9 (8.7) 57.1 to 62.6	61.2 (8.9) 57.5 to 64.9	−1.0 (6.5) −3.1 to 1.0	−2.9 (4.0) −4.5 to −1.2	p=0.43
Urea (mmol/L)	5.15 (1.25) 4.76 to 5.55	5.67 (1.61) 5.01 to 6.34	5.78 (1.06) 5.45 to 6.12	5.39 (1.62) 4.72 to 6.06	0.63 (0.95) 0.33 to 0.93	−0.28 (1.25) −0.80 to 0.23	p<0.01
Glycated haemoglobin (mmol/L)	34.2 (2.8) 33.3 to 35.1	33.1 (2.4) 32.1 to 34.1	33.3 (3.1) 32.4 to 34.3	33.8 (3.1) 32.6 to 35.1	−1.0 (1.5) −1.4 to −0.5	0.7 (1.5) 0.0 to 1.4	p<0.01
Calcium (Ca <sup>+</sup> ) (mmol/L)	2.34 (0.09) 2.31 to 2.37	2.31 (0.09) 2.28 to 2.35	2.31 (0.08) 2.29 to 2.34	2.33 (0.09) 2.29 to 2.37	−0.03 (0.08) −0.06 to 0.00	0.02 (0.08) −0.01 to 0.05	p=0.05
Magnesium (Mg <sup>+</sup> ) (mmol/L)	0.86 (0.06) 0.81 to 0.85	0.86 (0.05) 0.83 to 0.88	0.83 (0.07) 0.81 to 0.85	0.85 (0.05) 0.83 to 0.87	−0.03 (0.06) −0.05 to −0.01	0.00 (0.04) −0.02 to 0.01	p=0.05
Phosphate (mmol/L)	1.20 (0.16) 1.15 to 1.26	1.18 (0.18) 1.11 to 1.26	1.24 (0.18) 1.18 to 1.29	1.16 (0.14) 1.10 to 1.22	0.04 (0.15) −0.02 to 0.09	−0.02 (0.14) −0.08 to 0.03	p=0.06

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

but the daily diet or possible change in diet was not recorded. This might reduce the value of the obtained results to some extent. However, the participants were properly followed-up during the study and a systematic significant change in the diet it is not likely.

Some studies on Asian women have reported that high doses of vitamin K<sub>2</sub> gave an increase in BMD, but this has not been detected in Europeans.<sup>24</sup> The bone anabolic effects of daily Jarlsberg intake is more possibly caused by its content of DHNA with stronger osteoblast stimulation compared with vitamin K<sub>2</sub>.<sup>16</sup>

A possible explanation is that the increased tOC is mainly caused by DHNA and carboxylated by the long-chained vitamin K<sub>2</sub> in Jarlsberg. In this process, calcium and additionally magnesium is absorbed from serum and increases BMD. Further studies should be performed to investigate if daily intake of Jarlsberg cheese could affect bone mass and structure in humans. Such studies could be performed on borderline, untreated osteoporotic patients or on endurance athletes. Several of such athletes suffer from decreased BMD due to energy deficiency;

reduced resistance to infections and lack of nutritional factors.<sup>26</sup>

During the 6 weeks daily intake of 57 g Jarlsberg, the mean HbA1c was significantly reduced with 3% and increased with 2% in the C-group. Switching from Camembert cheese to Jarlsberg led to a significant 3% decrease in HbA1c. The inverse association of tOC with blood glucose, HbA1c, the risk of type 2 diabetes and metabolic syndrome is previously reported.<sup>27 28</sup> The mechanisms underlying these effects in humans are unknown, but the role of osteocalcin as a hormone controlling energy metabolism has been suggested.<sup>29</sup> The obtained reduction of HbA1c during daily Jarlsberg intake indicates that these associations are maintained even in the healthy population, and suggests that daily intake of Jarlsberg can be an aid in controlling major lifestyle-related diseases.

In the previous dose-response study of Jarlsberg a significantly reduction of blood lipids was observed.<sup>10</sup> All the results obtained in this dose-response study were verified in the de-escalation study except for the reduction in blood lipids. This may possibly be due to temporary

**Table 5** Development in lipid and biochemical variables after switching from Camembert to 6 weeks daily intake of Jarlsberg cheese. The results are expressed by mean values with SD in bracket and 95% CIs

Variables	Baseline Jarlsberg (n=25)	6 weeks on Jarlsberg (n=25)	Week 6—baseline (n=25)	P values within variables
Triglyceride (mmol/L)	1.4 (0.8) 1.1 to 1.7	1.3 (0.6) 1.0 to 1.5	−0.1 (0.7) −0.4 to 0.1	p=0.32
LDL-cholesterol (mmol/L)	3.4 (0.9) 3.1 to 3.8	3.1 (0.8) 2.8 to 3.5	−0.3 (0.5) −0.5 to −0.1	p<0.01
HDL-cholesterol (mmol/L)	1.6 (0.3) 1.5 to 1.8	1.5 (0.3) 1.4 to 1.7	−0.1 (0.2) −0.2 to 0.0	p=0.05
Total cholesterol (mmol/L)	5.1 (0.7) 4.8 to 5.4	4.7 (0.6) 4.5 to 5.0	−0.4 (0.4) −0.6 to −0.2	p<0.01
LDL/HDL cholesterol	2.3 (0.9) 1.9 to 2.7	2.2 (0.9) 1.8 to 2.6	−0.1 (0.3) −0.2 to 0.1	p=0.20
Creatinine (μmol/L)	61.2 (8.9) 57.5 to 64.9	61.3 (6.2) 58.8 to 63.9	0.2 (6.4) −2.5 to 2.8	p=0.90
Urea (mmol/L)	5.39 (1.62) 4.72 to 6.06	5.42 (1.63) 4.76 to 6.08	0.0 (1.5) −0.6 to 0.7	p=0.92
Glycated haemoglobin (mmol/L)	33.8 (3.1) 32.6 to 35.1	32.8 (2.4) 31.9 to 33.8	−1.0 (1.2) −1.5 to −0.5	p<0.01
Calcium (Ca <sup>++</sup> ) (mmol/L)	2.33 (0.09) 2.29 to 2.37	2.29 (0.07) 2.26 to 2.32	−0.04 (0.08) −0.07 to 0.00	p=0.03
Magnesium (Mg <sup>++</sup> ) (mmol/L)	0.85 (0.05) 0.83 to 0.87	0.86 (0.07) 0.83 to 0.88	0.00 (0.06) −0.02 to 0.03	p=0.72
Phosphate (mmol/L)	1.16 (0.14) 1.10 to 1.22	1.20 (0.19) 1.12 to 1.28	0.04 (0.15) −0.02 to 0.10	p=0.19

HDL, high-density lipoprotein; LDL, low-density lipoprotein.

changes in dietary habits and physical activity caused by measures to limit the spread of SARS-CoV2.<sup>30</sup> In the present study, such factors might explain the lack of blood lipid reduction during the first 6 weeks. After switching to Jarlsberg in the C-group, a significant reduction of the lipids was observed. This is in line with the inverse association between tOC and body fat reported in prospective studies.<sup>23</sup> The present study detected an increase in s-phosphate connected with daily Jarlsberg intake which might indicate that this cheese is not suitable for nutrition in end stage renal disease.

## CONCLUSION

The effect of daily Jarlsberg intake on increased s-osteocalcin level is not a general cheese effect. Jarlsberg containing vitamin K<sub>2</sub> and DHNA increases tOC, cOC, R<sub>O</sub> and PINP and decreases s-Ca<sup>++</sup>, s-Mg<sup>++</sup> and HbA1c. These effects reflect increased bone anabolism and a possible reduced risk of adverse metabolic outcomes.

**Contributors** All the authors participated in construction of the trial protocol, manuscript writing and approved the manuscript. The author acting as guarantor is HEL. TINE SA provided Jarlsberg and Camembert cheese, along with financial support, but did not play any role in the design, implementation, analysis,

interpretation or manuscript writing. MSc Vivy Liang Larsen, Meddoc was in charge of Data Management and BSc Natharat Thiendilokkul, Meddoc in charge of all clinical monitoring. Vitas Laboratory performed all the osteocalcin analyses.

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**Competing interests** None declared.

**Patient consent for publication** Not applicable.

**Ethics approval** This study involves human participants and was approved by Norwegian Regional Ethical Committee South-East, project nr.84845. Participants gave informed consent to participate in the study before taking part.

**Provenance and peer review** Not commissioned; externally peer reviewed by Joerg Saupe, Germany.

**Data availability statement** Data are available upon reasonable request. All data is saved in a SAS database at Meddoc Research.

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## RESEARCH PROTOCOL

Final version 4 12<sup>th</sup> February 2020

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### Title

**Effect of Jarlsberg cheese compared to cheese without vitamin K2  
(Camembert) regarding increased Osteocalcin level in healthy women**

**Protocol number: HV-Jarlsberg/III**

**EudraCT number: 2019-004593-26**

**ClinicalTrial.gov: NCT04189796**

### Sponsor

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#### CONFIDENTIAL

The information in this document is confidential and will not be disclosed to others without written authorization from TINE A/S except to the extent necessary to obtain informed consent or for discussions with regulatory authorities, Independent Ethics Committees (IECs) or persons participating in the conduct of the study.

*Research protocol HV-Jarlsberg/III***0: Preface**

The following steering committee will administer the study:

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Prof. Stig Larsen has written the trial protocol with support from the steering committee. The statistical analysis will be performed by Hans Fagertun and together with Stig Larsen. They are responsible for writing the integrated clinical and statistical report. The result aims to be published in an international medical journal and the manuscript prepared by the steering committee. The study will be monitored by Natharat Thiendilokkul (BSc) and Data Management by Vivy Liang Larsen (MSc) from the Meddoc Biometric group in Norway.

*Research protocol HV-Jarlsberg/III***0.1: Signature Sheet*****Protocol Authorized:***Stig Larsen<sup>1</sup> and Helge Holo<sup>2</sup>***Qualifications:***

- 1) Professor: Faculty of Veterinary Medicine; NMBU
- 2) Professor: Faculty of Biotechnology and Food Science; NMBU

Signature

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*Research protocol HV-Jarlsberg/III***0.3: List of Abbreviations**

Abbreviation	Explanation
AE	Adverse Event
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BSC	Bachelor of Science
CPK	Creatinine Phosphokinase
CRF	Case Report Form
CRP	C-Reactive Protein
CTCAE	Common Terminology Criteria for Adverse Events
CV	Curriculum Vita
DM	Data Management
FNB	Food and Nutrition Board
GCP	Good Clinical Practice
IP	Investigational Product
ITT	Intention to treat
LDH	Lactate Dehydrogenase
LOCF	Last Observation Carried Forward
MEDD	Minimum efficacy daily dose
MSC	Master of Science
OR	Osteocalcin Ratio
PP	Per Protocol
REK	Regional Ethical Committee
RSP	Response Surface Pathway
SAE	Serious Adverse Event
SD	Standard Deviation
SOC	System Organ Class

*Research protocol HV-Jarlsberg/III***0.4: Distribution of Clinical trial Protocols**

Complete Version	Date	Writer	Receivers	Internal review	External review
Version 01	22.03.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Version 02	01.04.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Version 03	05.04.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Version 04	15.08.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Version 05	06.09.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Version 06	11.09.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Final version 1	24.09.2019	SL	HF,VLL,NT,TH,HH,HEL	Yes	No
Final version 2	25.11.2019	SL	HF,VLL,NT,TH,HH,HEL,TA,KH,ZS,SI,AS,RC	Yes	Yes
Final version 3	10.12.2019	SL	HF,VLL,NT,TH,HH,HEL,TA,KH,ZS,SI,AS,RC,MG	Yes	Yes
Final version 3	12.02.2020	SL	HF,VLL,NT,TH,HH,HEL,TA,KH,ZS,SI,AS,RC,MG	Yes	Yes

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*Research protocol HV-Jarlsberg/III***0.5: Participating Institutions**

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*Research protocol HV-Jarlsberg/III*

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*Research protocol HV-Jarlsberg/III***I: Protocol Synopsis****1.1: Project title**

Effect of Jarlsberg cheese compared to Camembert cheese without vitamin K<sub>2</sub> regarding increased Osteocalcin levels in healthy women

**1.2: Protocol numbers**

Protocol number: HV-Jarlsberg/III

Regional Ethical Committee number:

EudraCT number: 2019-004593-26

ClinicalTrial.gov: NCT04189796

**1.3: Objectives**

The study objective consists of the following three aims:

1. To compare the effect of daily intake of Jarlsberg cheese and cheese without content of Vitamin K<sub>2</sub> (Camembert) in change of the Osteocalcin level in healthy women after 6 weeks.
2. To estimate the long-term increase of the osteocalcin level, change in the lipid pattern and the vital signs caused by optimized daily intake of Jarlsberg cheese.
3. To verify the estimated maintaining dose of Jarlsberg cheese related to stabilized osteocalcin level

**1.4: Population and sampling**

The study population consists of Healthy Voluntary (HV) women between 20 years and pre-menopausal age.

**1.5: Design and randomization**

The study will be performed as a randomized Norwegian multicenter study with a semi-cross over design in which the participants randomized to Camembert cheese will be switched to Jarlsberg cheese after 6 weeks.

**1.6: Study procedure**

The recruited HV women fulfilled the inclusion without the exclusion criteria for the study will undergo a screening clinical investigation. The participants will be asked to avoid use of other cheese than the one allocated to in the study but eat as usual. One week later, the first clinical investigation in the study will take place including blood sampling and measurement of vital signs. The HV women verified to fulfil the criteria for participation and signed the informed consent form will be included in the study. During this first clinical investigation in the study denoted as Day 0, the participants receive a study identification number. The HV will randomly be allocated to either daily intake of Jarlsberg cheese or Camembert cheese without content of vitamin K<sub>2</sub>. The daily intake of Jarlsberg cheese will be 57g/day and 50g/day Camembert cheese. The two cheese doses are nearly equal with regard to total energy, protein carbohydrate and fat. The trial cheese can be consumed with other food at breakfast, lunch or other meals during the day.



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The participants meet for new clinical investigations every third week with measurement of vital signs and blood sampling. Osteocalcin and vitamin K will be analysed every third week whereas the haematological and biochemical analysis will be performed every six week. The HVs allocated to Camembert cheese will after 6 weeks be switched to daily intake of Jarlsberg cheese in additional 6 weeks with clinical investigations after 3 and 6 weeks. The participants performed the 6 weeks of daily intake of Jarlsberg cheese either by randomization or switching to Jarlsberg cheese will either be offered participation in the cheese de-escalation study (HV-Jarlsberg/IB) or an extended study of 6 weeks with unchanged Jarlsberg cheese dose. The first 12 HVs finalized 6 weeks with daily intake of Jarlsberg cheese obtaining an increase in the osteocalcin level from baseline  $\geq 10\%$  will be allocated to the de-escalation study HV-Jarlsberg/IB (separate protocol). The HVs included in the extended part of this study will receive an unchanged daily dose of Jarlsberg cheese for additional 6 weeks with clinical investigation every third week. The HVs switched to Jarlsberg cheese may be offered participation in a study part aiming to verify the maintaining dose obtained in HV-Jarlsberg/IB study. The duration of this part will be 6 weeks with clinical investigation every third week.

1.7: Main variables

The main variable in this study will be osteocalcin measured in blood serum. Additionally, carboxylated and under carboxylated Osteocalcin and the ratio  $OR = [Carboxylated / Under Carboxylated]$  osteocalcin in serum will be central together the K2 variants MK-7, 8, 9, 9(4H) and vitamin K1. Triglyceride, LDL- and HDL cholesterol, vitamin D and vital signs will be secondary variables. As safety variables, haematological- and biochemical variables and adverse events (AE) will be recorded at each visit.

1.8: Sample size

With a significant level of 5%, a power of 90% and a clinical relevant difference in total osteocalcin increase of one-time SD between the two groups, at least 24 HVs in each group have to be included. By correcting for the number of participating General Practitioners (GP) and drop-outs during the first part of the study, 32 HVs will be included in each group. Totally 64 HVs will be recruited from the eight participating GP sites.

1.9: Study duration

The duration of the first comparative part of the study will vary from 6 to 12 weeks depending on allocation to Jarlsberg cheese or the alternative cheese. The duration of the expending Jarlsberg part or the maintaining dose part will be 6 weeks. The total duration of the study will be maximum 18 weeks:

Inclusion of the first participant	13 <sup>th</sup> January	2020
Inclusion of the last participant	24 <sup>th</sup> February	2020
Last participant finalized comparative study part	14 <sup>th</sup> April	2020
Last patient finalized extended part	6 <sup>th</sup> July	2020
Final statistical reports	18 <sup>th</sup> September	2020

*Research protocol HV-Jarlsberg/III***1.10: Flow Chart**

Trial procedure	Screening	Baseline & Day0	Comparative study part				Extended	
			Week 3	Week 6	Week 9	Week 12	Week 15	Week 18
Jarlsberg cheese group		x	x	x	x	x		
Camembert cheese group		x	x	x				
Switched to Jarlsberg cheese					x	x	x	x
Inclusion / Exclusion criteria.	x							
Patient factors	x							
history of disease	x							
Concomitant treatment	x	x	x	x	x	x	x	x
Vital signs								
-Systolic BP		x	x	x	x	x	x	x
-Diastolic BP		x	x	x	x	x	x	x
-Pulse rate		x	x	x	x	x	x	x
Blood sampling								
-Osteocalcin		x	x	x	x	x		x
-Vitamin K <sub>1</sub>		x	x	x	x	x		x
-Vitamin K <sub>2</sub>		x	x	x	x	x		x
-Vitamin D		x		x		x		x
-Triglyceride		x		x		x		x
-LDL Cholesterol		x		x		x		x
-HDL Cholesterol		x		x		x		x
-Hematology		x		x		x		x
-Biochemical		x		x		x		x
Adverse Events [CTCAE]		x	x	x	x	x	x	x
Cheese compliance			x	x	x	x	x	x

x) Indicate visits in the Camembert group after switching to Jarlsberg

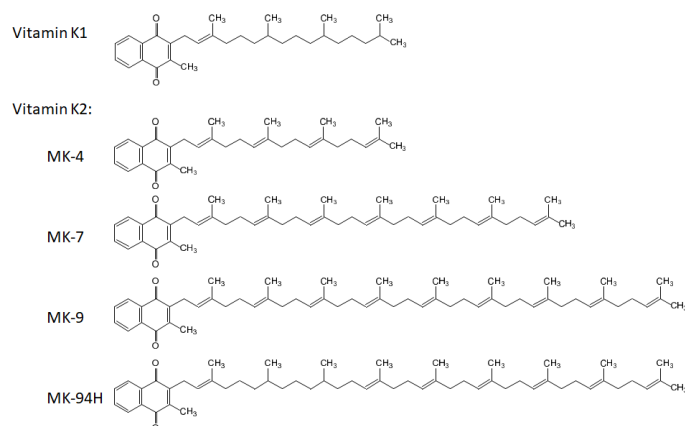
*Research protocol HV-Jarlsberg/III***II: Introduction****2.1: Background**

Bone loss remains a huge problem among the elderly. It is well established that dietary calcium and vitamin D are beneficial for skeletal health, but more recently research clearly demonstrates the importance of vitamin K and health claims stating the positive effects on the skeleton have been authorized by the European Food Safety Authority (EFSA). Dairy products are good calcium sources, important for bone formation. But cheese is also an important vitamin K (especially vitamin K2) source, indicating that cheese consumption may strengthen bones and reduce the risk of osteoporosis. The effect of eating vitamin K2 rich cheese on bone health has not been studied.

**2.2: Osteocalcin and Vitamin K**

Activated osteocalcin has a key role in bone formation and maintenance. It is one of the body's 17 so called GLA proteins, all of which being activated by carboxylation in a process involving vitamin K. While vitamin K dependent coagulation factors are practically fully carboxylated under normal conditions, osteocalcin is not. The ratio of fully carboxylated to under carboxylated osteocalcin in the blood (OR) reflects a person's vitamin K status. The high levels of under carboxylated osteocalcin in healthy people indicate that suboptimal vitamin K status or subclinical vitamin K deficiency is common in Western societies [1]. Very low ORs have been associated with osteoporotic fractures [2].

Figure 1 shows the structure of common forms of vitamin K. Vitamin K1 is produced in plants and found at high concentrations in leafy vegetables and is the dominating variant in the Western diet.



*Figure 1: Structures of some vitamin K variants*

Vitamin K2 (menaquinone, MK) is found in several variants (Fig 1). The short-chained MK-4 can be formed from vitamin K1 in humans and is found in animal products like liver. The K<sub>2</sub> variants with longer side chains, like MK-7, MK-8, MK-9 and MK-9(4H) are of bacterial origin. They can be found in certain fermented foods. In the Western diet fermented dairy products like cheese are the main source of vitamin K2.

The long chained MKs have been found to have greater extra-hepatic activity than K1 and MK-4, possibly due to more efficient uptake and much longer serum half-life [3, 4].

Prospective cohort studies have demonstrated health benefits that can be attributed the intake

*Research protocol HV-Jarlsberg/III*

of vitamin K2 but not K1, and the main contributor to the vitamin K2 is cheese containing MKs with long side chains [5, 6].

**2.3: Jarlsberg Cheese**

The dominating vitamin K2 variant is MK-9 in most cheeses, but the amount varies considerably [7]. However, some cheeses also contain MK-9(4H). Jarlsberg cheese in particular is rich in this compound [8]. This cheese is made with lactic acid bacteria producing MK-8 and MK-9 and *Propionibacterium freudenreichii* 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. Because of its high vitamin K2 content Jarlsberg cheese is well suited for such a study.

**Table I: Typical vitamin K2 content of 100g Jarlsberg cheese**

Component	
MK-8	3 µg
MK-9	13 µg
MK-9(4H)	75 µg

Recently, a dose-response study in healthy Norwegian women was performed with a 3-level between patient Response Surface Pathway design [9, 10, 11]. The study was performed in 19 women with daily intake of Jarlsberg cheese during a period of 5 weeks [12].

**2.4: Comparative Cheese**

As comparable cheese, Camembert in a daily dose of 50 g was chosen.

**Table II: Nutritional content of 100g of the comparative cheese**

Nutritional content	Jarlsberg cheese	Camembert cheese
Energy	1458 kJ	1359 kJ
Fat	27 g	28 g
Saturated fatty acids	17 g	18 g
Carbohydrate	0 g	0 g
Sugars	0 g	0 g
Protein	27 g	19 g
Salt	1.1 g	1.5 g
Vitamin A	270 µg (34%)	280 µg (35%)
Riboflavin	0.32 mg (23%)	0.33 mg (24%)
Folic Acid	36 µg (18%)	51 µg (26%)
Vitamin B12	2.2 µg (88%)	1.7 µg (67%)
Calcium	770 mg (96%)	540 mg (68%)
Phosphorus	550 mg (79%)	390 mg (56%)
Zinc	4.2 mg (42%)	3.0 mg (30%)
Selena	12 µg (22%)	11 µg (20%)
Iodine	32 µg (21%)	45 µg (30%)



*Research protocol HV-Jarlsberg/III*

Camembert cheese is without vitamin K and the daily comparative dose of 50 gram Camembert represents 680 kJ and 14.0 gram fat. The daily dose with Jarlsberg cheese is 57 gram, representing 729 kJ and 15.4 gram fat.

**2.5: Objectives**

The study objective consists of the following three aims:

1. To compare the effect of daily intake of Jarlsberg cheese and cheese without content of Vitamin K<sub>2</sub> (Camembert) in change of the Osteocalcin level in healthy women after 6 weeks.
2. To estimate the long-term increase of the osteocalcin level, change in the lipid pattern and the vital signs caused by optimized daily intake of Jarlsberg cheese.
3. To verify the estimated maintaining dose of Jarlsberg cheese related to stabilized osteocalcin level

*Research protocol HV-Jarlsberg/III***III: Population and sampling****3.1: Reference population**

The reference population consists of healthy women (HV) in pre-menopausal age.

**3.2: Study population***3.2.1: Inclusion criteria*

HV from 20 years of age and within pre-menopausal age

*3.2.2: Exclusion criteria.*

Health volunteers fulfil at least one of the following criteria will be excluded from participation in the study:

- Pregnant women
- Known gastrointestinal disorder
- Abnormal liver or kidney function.
- Diabetes
- Suffering from verified cancer
- Under systemic treatment with corticosteroids or other immunosuppressive drugs the last 3 weeks before start of the trial treatment.
- LDL-cholesterol > 3 mmol/L or Triglyceride > 2 mmol/l
- Participating in another clinical trial with pharmaceuticals the last six weeks before start of this trial treatment.
- Lactose intolerance or known milk product allergy
- Not able to understand information.
- Do not want or not able to give written consent to participate in the study.

**3.3: Recruitment of patients**

The volunteers will be recruited by the participating General Practitioner (GP) centers. HV fulfils the inclusion and none of the exclusion criteria will be asked to participate. The HV has to give written consent for participation.

Research protocol HV-Jarlsberg/III

IV: Design

4.1: Project design

This project consists of one comparative study HV-Jarlsberg/III and one dose de-escalation study HV-Jarlsberg/IB.

The HVs to be included in the de-escalation study will be recruited from HV-Jarlsberg/III after finalized 6 weeks with daily use of Jarlsberg cheese

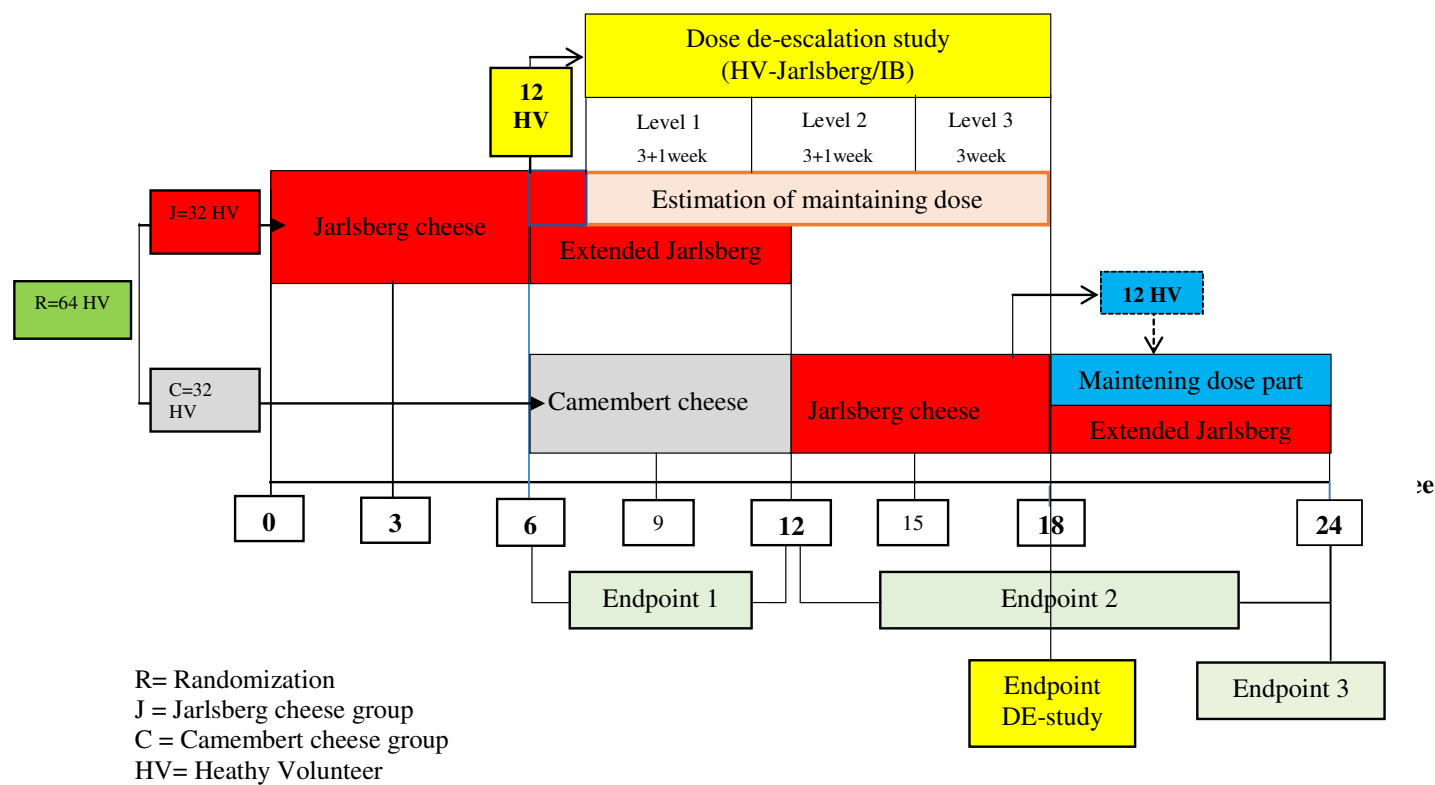


Figure 2: Overall project design; HV-Jarlsberg/III & HV-Jarlsberg/IB

4.2: Comparative study design

The comparative study will be performed with a semi-cross design. The HV will be randomly allocated to either daily intake of Jarlsberg cheese or Camembert cheese without content of vitamin K2. The daily intake of cheese will be 57 g Jarlsberg cheese and 50g Camembert cheese. These doses are nearly equal regarding total energy, protein, carbohydrate and fat. The dose to be used is based on the previous dose-response study of Jarlsberg cheese performed in HV [12].

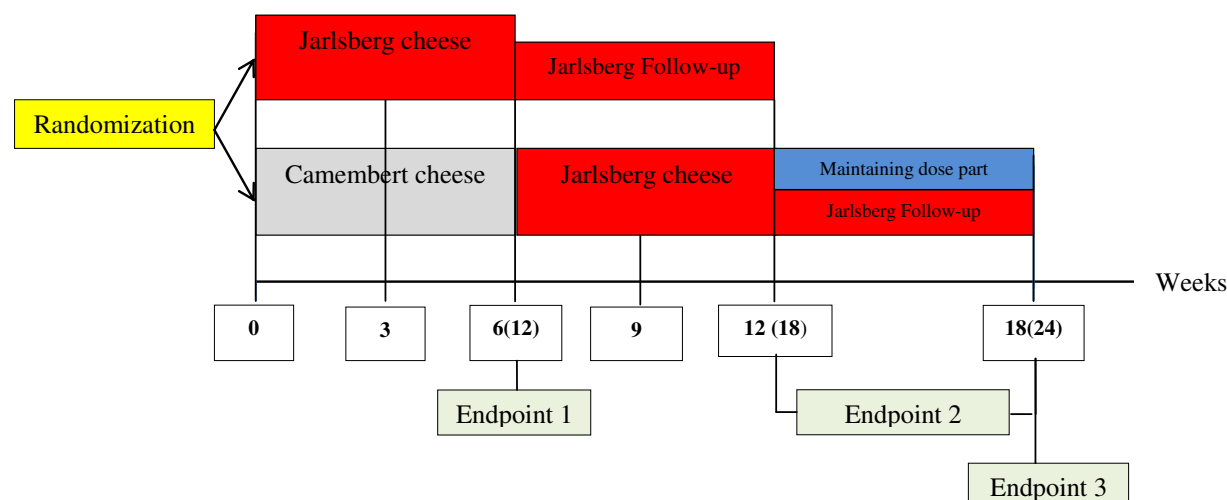
*Research protocol HV-Jarlsberg/III*

Figure 3: Comparative study design; HV-Jarlsberg/III

The participants meet for clinical investigations at screening Day 0 and after 3 and 6 weeks (Fig 3). At the end of this 6<sup>th</sup> week, the HV allocated to Camembert cheese will be switched to daily intake of Jarlsberg cheese for additional 6 weeks with clinical investigations every third weeks. The participants allocated to Jarlsberg cheese and had performed the 6 weeks of daily intake of Jarlsberg cheese will be offered participation either in the dose de-escalating study HV-Jarlsberg/IB (separate protocol) or in the extended part with unchanged daily dose of Jarlsberg cheese.

#### 4.3: Extended Jarlsberg period

The participants performed the 6 weeks of daily intake of Jarlsberg cheese either by randomization or switching to Jarlsberg cheese will be offered participation in either:

- 1) an extended study of 6 weeks with unchanged dose of Jarlsberg
- 2) a de-escalation dose study of 12 weeks (HV-Jarlsberg/IB protocol)
- 3) a maintaining dose study of 6 weeks duration to verify the results from the de-escalation study

HVs with an increase <10% in the osteocalcin level from screening to 6 weeks of Jarlsberg cheese intake will not be offered inclusion in the de-escalation study.

The first 12 HVs finalized 6 weeks with daily intake of Jarlsberg cheese and obtained an increase in osteocalcin from baseline  $\geq 10\%$  in the comparative study will be allocated to the de-escalation study HV-Jarlsberg/IB.

The remaining HVs from the comparative parts will be offered participation in the extended part of this study receiving an unchanged daily dose of Jarlsberg cheese (Fig 3). The duration of this part is 6 weeks with clinical investigation at the end of the study.

#### 4.4: Verification of the estimated maintaining dose

The 12 first HVs switched from Camembert to Jarlsberg cheese; finalized 6 weeks with daily intake of 57g Jarlsberg and obtained an Osteocalcin increase  $\geq 10\%$  will be offered participation in the study part aiming to verify the maintaining dose obtained in HV-

*Research protocol HV-Jarlsberg/III*

Jarlsberg/IB study (Fig 3). The duration of this part will be 6 weeks with clinical investigation at the end of the study.

**4.5: Randomization**

The volunteers will be randomized 1:1 to either daily intake of Jarlsberg cheese or a Camembert cheese by using block randomization with random block size between 2 and 10 [13].

**4.6: Identification of Volunteers**

All the participants will be given one study identification number of five digits constructed as follows:

*Digit 1 & 2:* Indicate the GP-site [01=site 1; 02 site= 2 etc.]

*Digit 3 & 5:* Indicate the number of the patient within each GP-site [001, 0 02 etc.]

*Research protocol HV-Jarlsberg/III***V: Evaluation****5.1: Main variables**

The main variables in this study are total osteocalcin, carboxylated and under carboxylated osteocalcin and the ratio OR= [carboxylated / under carboxylated] osteocalcin, measured in blood serum. Additionally, vitamin K1 and the different fractions MK 7, 8, 9 and 9/4H will be recorded as part of Vitamin K2.

**5.2: Laboratory variables**

Blood sample for measurement of the hematological and the clinical biochemical variables will be taken every sixth week. The list of variables to be measured is given below.

*5.2.1: Clinical chemistry*

The following variables will be measured in serum:

- Lactate dehydrogenase (LDH)
- Alkaline phosphatase (ALP)
- Amylase
- Creatinine
- Albumin
- Urea
- Total Bilirubin
- ALAT
- HbA1c
- K<sup>+</sup>
- Na<sup>+</sup>
- Ca<sup>++</sup>
- Magnesium
- Phosphate
- Vitamin D
- Total Cholesterol
- HDL cholesterol
- LDL cholesterol

*5.2.2: Hematology*

The following hematological variables will be measured:

- Hemoglobin (Hgb)
- Erythrocytes
- Hematocrit
- Mean Cell Volume (MCV)
- Ferritin
- Thrombocytes
- Leucocytes
- Diff. count:
- Neutrophils
- Eosinophils
- Basophils
- Monocytes
- Lymphocytes



*Research protocol HV-Jarlsberg/III*

Blood samples for measurement of osteocalcin and vitamin K will be taken every third week

**5.3: Common Terminology Criteria for Adverse Events version 4.0**

The CTCAE is divided in 26 System Organ Class (SOC) in accordance with the MedDRA classification [14]. Within each SOC, adverse events (AE) are listed and accompanied by descriptions of severity or grade:

- Blood and lymphatic system disorders (11 Items)
- Cardiac disorders (36 Items)
- Congenital, familial and genetic disorders (1 Items)
- Ear and labyrinth disorders (9 Items)
- Endocrine disorders (11 Items)
- Eye disorders (25 Items)
- Gastrointestinal disorders (117 Items)
- General disorders and administration site conditions (24 Items)
- Hepatobiliary disorder (16 Items)
- Immune system disorders (6 Items)
- Infections and infestations (76 Items)
- Injury, poisoning and procedural complications (78 Items)
- Investigations (38 Items)
- Metabolism and nutrition disorders (24 Items)
- Musculoskeletal and connective disorders (41 Items)
- Neoplasms benign, malignant and unspecified incl. cysts and polyps (5 Items)
- Nervous system disorders (63 Items)
- Pregnancy, puerperium and perinatal conditions (5 Items)
- Psychiatric disorders (20 Items)
- Renal and urinary disorders (20 Items)
- Reproductive system and breast disorders (51 Items)
- Respiratory, thoracic and mediastinal disorders (59 Items)
- Skin and subcutaneous tissue disorders (34 Items)
- Social circumstances (2 Items)
- Surgical and medical procedures (1 Item)
- Vascular disorders (17 Items)

*5.3.1: Grading and classification of Items*

Grade refers to the severity of the AE. The CTCAE displays Grade 1 through 5 with unique clinical descriptions of severity for each AE based on this general guideline:

- |           |   |
|-----------|---|
| Grade 0 = | None  |
| Grade 1 = | Mild; asymptomatic or mild symptoms, clinical or diagnostic<br>Observations only, intervention not indicated.   |
| Grade 2 = | Moderate; minimal, local or non-invasive intervention indicated.<br>Limiting age-appropriate instrumental Activity of Daily Living (ADL)                                    |
| Grade 3 = | Severe or medically significant but not immediately life-threatening;<br>Hospitalization or prolongation of hospitalization indicated; disabling;<br>Limiting self-care ADL |

*Research protocol HV-Jarlsberg/III*

Grade 4 = Life-threatening consequences; urgent intervention indicated  
Grade 5 = Death related to AE.

Relation to trial medication is shown as: “Definitely”, “Probably”, “Possibly” or “Unrelated”.

Action taken as: “None”, “Interruption”, “Modified” or “Discontinued”

AE treatment as: “None”, “Continue Medication”, “Procedure” or “Hospitalization”

Outcome at last visit as “Resolved”, “Ongoing” or “Fatal”

Definitions of relationship to study medication are as follows:

- |             |  |
|-------------|--|
| Unrelated:  | bears no relation to timing of medication, similar to symptoms or signs expected in the disease process, does not recur on re-challenge.       |
| Possibly:   | bears relation to timing of medication, similar to symptoms or signs expected in the disease process, does not recur on re-challenge.          |
| Probably:   | bears clear relation to timing of medication, distinct from symptoms or signs expected in the disease process, does not recur on re-challenge. |
| Definitely: | clear relation to timing of medication, distinct from symptoms or signs expected in the disease process, recurs on re-challenge.               |

*5.3.2: Serious Adverse Event (SAE)*

An adverse event (AE) is any untoward symptom or sign befalling a patient in a clinical trial regardless of its relationship to the study medications. All AEs have to be described in detail and their severity and putative relationship to the study medication noted.

AEs may be considered serious. The definition of this is as follows:

- Death
- Life threatening
- Leads to or prolongs hospitalization
- Results in persistent of significant disability

**5.4: Factor and vital sign**

The participant factors recorded in the study will be age in days from birth to the screening visit calculated in the database, height in cm, and body weight in kg. Additionally, concomitant disease and treatments will be recorded. The vital signs defined as systolic and diastolic blood pressure in mmHg and heart rate in beats/min will be recorded in sitting position after five minutes rest.

*Research protocol HV-Jarlsberg/III***VI: Study procedure****6.1: Trial treatment**

In the first part of the study, half of the participants will have a daily intake of either marketed common Jarlsberg cheese with the composition specified in Table I. The second half of the participants will have a daily intake of a cheese with none or strongly reduced contents of vitamin K<sub>2</sub> with the composition specified in section 2.3.

*6.1.2 Administration and doses*

The daily intake of cheese to be taken is based on the results obtained in the previous dose-response study [12]. The recommended Jarlsberg dose is 57 gram/day representing 5 slices and 50 gram/day of Camembert cheese. The duration of the comparative study part will be six weeks. The duration of the expanded Jarlsberg part or the maintaining dose verification part will be minimum 6 weeks and maximum 12 weeks. In the expanding Jarlsberg part, the participating HVs will use the same dose as in the comparative part. The dose used for the HVs participating in maintaining dose will be determined from the de-escalation study HV-Jarlsberg/IB.

*6.1.3 Cheese supply, packing and storage*

The Jarlsberg cheese will be delivered in 100-gram packages and the Camembert cheese in 3x50-gram pieces free of costs from Tine. The participants will receive the cheese at the GP-sites at every 3-week or 6-week visits. Both the Jarlsberg- and the Camembert cheese used in the study will be given free of charge to the participating volunteers

Each package is labeled in accordance with the procedure for clinical trials. Additionally, the participants will be informed on how to perform the intake of the cheese and to store the cheese in a refrigerator at a temperature from 4° to 10°C. Expiry date will be printed on the label.

**Table III: Label on each package**

Description	For use only in clinical trial
Trial substance	Type of cheese
Expire date	
Administration	Oral intake
Investigator	
Name of exporter	TINE A/S:
Phone number	+47 908 67088
Study	Comparative study in healthy volunteers
EudraCT number	2019-
Protocol id	HV-Jarlsberg/III
Storage	Refrigerator between 4° to 10°C

*Research protocol HV-Jarlsberg/III***6.2: Inclusion and start-up visit**

The recruited HV fulfilling the inclusion and avoided the exclusion criteria will be registered and asked to participate in the study. The participants who are willing to sign the informed consent form will be enrolled in the study. The participants will undergo a clinical investigation and an appointment for the starting visit in the study one week later. All the voluntaries will be instructed not to change their usual intake of food during the study, but change the usual used cheese with the study cheese.

A new clinical investigation with blood sampling will be performed at the start-up denoted as Day 0. During this visit, vital signs, concomitant diseases and treatment will be recorded. Blood samples will be handled in accordance to the GP center procedures, and the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 registered. In addition to hematological and biochemical variables, the blood samples will be used for measurements of vitamin D, triglycerides, LDL- and HDL cholesterol, osteocalcin, vitamin K1 and vitamin K2.

**6.3: Comparative part of the study**

The intake of cheese starts the following day named Day 1. New visit will be performed after 3- and 6-weeks intake of the study cheese. During every visit, vital signs will be measured, adverse events (AE) and concomitant medication recorded. Blood sampling for measurement of osteocalcin and vitamin K will be taken every third week, but a complete measurement including both hematology and biochemistry will be performed after every 6 weeks of cheese intake. CTCAE version 4.0 will be used for registration of AE. In case of AE of grade 3 or 4 according to CTCAE occur for more than three days, the responsible investigator will take action. The intake of cheese will be stopped and the HV will be followed up until disappearance of the symptoms. The total duration from the last intake of the cheese to the disappearance of the symptoms will be recorded in days together with the treatment procedure.

The duration of the first part of the study will be 6 weeks for the participants randomized to Jarlsberg cheese and 12 weeks for those allocated to the comparative group. Within one week after the last clinical investigation in this comparative part of the study, the participants will be informed about the obtained results related to change in the Osteocalcin level. The HVs will be offered to continue in one of the following study parts:

- 1) Expanded Jarlsberg cheese part with unchanged cheese dose
- 2) De-escalation dose study (HV-Jarlsberg/IB; separate protocol)
- 3) Verification of the estimated maintaining Jarlsberg dose

HVs with an increase  $\leq 10\%$  in the Osteocalcin level from screening to 6 weeks of Jarlsberg cheese intake will not be offered included in the de-escalation study. The 12 first HVs completed 6 weeks of Jarlsberg cheese intake obtaining an increase  $\geq 10\%$  in the osteocalcin level will be offered participation in the de-escalation study HV-Jarlsberg/IB.

*Research protocol HV-Jarlsberg/III***6.4: Expanded Jarlsberg cheese part**

The remaining participants fulfilling 6 weeks of Jarlsberg cheese intake will be offered participation in the expanded Jarlsberg cheese part of this study HV-Jarlsberg/III or verification of the maintaining daily Jarlsberg dose. The HVs participating in the expanded part will continue the intake of Jarlsberg cheese in additionally 6 weeks with unchanged daily dose. New visit in the study will be performed at the end of the study. During this final visit, vital signs will be measured, CTCAE and concomitant medication recorded and blood sampling performed.

**6.5: Verification of the estimated maintaining dose**

The HVs switched from Camembert to Jarlsberg cheese; finalized 6 weeks with daily intake of 57g Jarlsberg cheese and obtained an increase  $\geq 10\%$  in the Osteocalcin level may be offered participation in study part aiming to verify the maintaining dose obtained in HV-Jarlsberg/IB study. The included HVs will receive the daily maintaining dose recommended from the de-escalation study HV-Jarlsberg/IB for additionally 6 weeks with clinical investigation at the end of the study. During this final visit, vital signs will be measured, CTCAE and concomitant medication recorded, and blood sampling performed.

**6.6: Stopping rule**

In case of life-threatening AE or Serious Adverse Events (SAE) occurs, the cheese intake has to stop, and the participant treated and followed up in accordance with GP-center routines.

**6.7: Procedures for Blood sampling and analysis.**

Blood will be drawn via a cubital vein and separated into serum by centrifugation. Two ml serum will be sending Vitas for Osteocalcin analysis and 2 ml send for K2 analysis at the biochemical laboratory of NMBU. The blood samples for measurements of the hematological and biochemical variables will be handled in accordance with the standard procedures at the GP-center and send to Fürst laboratory in Oslo for analysis.

**6.8: Report of serious adverse effect (SAE)**

The participant will be advised to contact the investigator if she suffers from severe AE or any other annoying conditions.

In case of SAE, the investigator has to complete the SAE form and send it to the health authorities with copy to TINE and the project manager Prof. Stig Larsen within 24 hours.

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Research protocol HV-Jarlsberg/III

6.9: Risk and benefit of participation

This study includes only products which is for sale in food stores in the country and proven not to cause any risk of disease except for person with lactose intolerance, allergy against milk product or suffering from a disease for which cheese product has to be avoided. During the comparative part of the study, blood sampling will be performed between three and five times. In the expending Jarlsberg part of the study and the verification of the recommended maintenance dose, blood sampling will be performed two times. Penetration like this will always include a risk of complication. However, all the blood sampling in this study will be performed by certificated and highly qualified bioengineers with long experiences in such work. The risk for failure still exists but classified as very low. The HV women will receive an economical compensation, free daily use of cheese, coverage of travel expenses related to participation in the study and free medical investigation. Additionally, they will be informed of their osteocalcin level and possible bone density during this study. This might be an important knowledge in case of osteoporosis symptoms later.

6.8: Study duration

The duration of the first comparative part of the study will vary from 6 to 12 weeks depending on allocation to Jarlsberg cheese or the alternative cheese. The duration of the expending Jarlsberg part or the maintaining dose part will be 6 weeks. The total duration of the study will be maximum 18 weeks:

Inclusion of the first participant	13 <sup>th</sup> January	2020
Inclusion of the last participant	24 <sup>th</sup> February	2020
Last participant finalized comparative study part	14 <sup>th</sup> April	2020
Last patient finalized extended part	6 <sup>th</sup> July	2020
Final statistical reports	18 <sup>th</sup> September	2020



*Research protocol HV-Jarlsberg/III***VII: Project management and Monitoring****7.1: Project management**

The study will be administered by a steering committee consisting of:

- Prof. Stig Larsen; DSc, Clinical Research Methodology and Statistics  
Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo and  
Meddoc AS, Hvamstubben 14, 2013 Skjetten, Norway
- Dr Helge Lundberg; MD, General Practitioner (GP)  
Skjetten Medical Center, 2013 Skjetten Norway
- Prof. Helge Holo; PhD, Biochemistry  
Faculty of Biotechnology and Food Science, Norwegian University of Life Sciences,  
Ås Norway
- Hans E Fagertun; MSc Statistician  
Meddoc Research AS, Hvamstubben 14, 2013 Skjetten Norway
- Dr. Trond Holand; PhD fellow  
Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo Norway

Prof. Stig Larsen supported by Dr. Trond Holand will administer the study. Natharat Thiendilokkul (BSc) will perform the clinical monitoring and Vivy L Larsen (MSc) the Data Management. Hans E Fagertun (MSc) and Prof. Larsen will be in charge of the statistical analysis.

**7.2: Publication of the results**

The results will be published in international medical journals. The steering committee and one investigator from each participating GP-centers will be represented in the list of authors. The rules stated in the Vancouver recommendation will be followed. Prof. Stig Larsen is responsible for the manuscript supported by the committee.

**7.3: Quality assurance demands**

The study will be performed with electronic data entry by using the InCRF database system. The monitoring part will be daily performed by two monitors at Meddoc and each of the participating GP-centers will be on-site monitored at least two times during the study. Additionally, blood samples for osteocalcin and vitamin K analysis will be collected every third week. The main monitoring will be performed electronically from Meddoc Research and DM will generate queries sending regularly to the sites. The monitor will receive the copy of queries in order to perform the routine monitoring follow up check. Paper CRF's printed eCRF with investigator's signature and copies of the laboratory analysis will be sending by e-

### *Research protocol HV-Jarlsberg/III*

mail to both the DM for performing inhouse data entry and monitor from the GP sites as source documents.

It is the duty of the investigator to provide open access to the monitor to all study related records at previously agreed times and locations.

In conducting the trial, the investigator accepts that the Sponsor, the regional Ethics Committee, the regulatory body and monitor may, at any time, by appointment, conduct an audit of the study site.

In conducting the trial, the Sponsor accepts that the Ethics Committee or the regulatory body may, at any time, by appointment, conduct an audit of the study site, the laboratories conducting any clinical testing or the Good Manufacturing Practice (GMP) manufacturing facilities.

#### **7.4: Investigator meeting**

Before application of the trial protocol to the regional ethical committee, a meeting with all the participating investigators will be arranged. The agenda for this meeting will be:

- 1) Go through the protocol chapter by chapter in order to correct the protocol in accordance with input from the investigators
- 2) To synchronize the clinical part of the study

Before start of the study and inclusion of the first HV, all the investigators and the responsible persons for data handling at each participating GP-center will be given specified information about the electronic data monitoring system InCRF.

#### **7.5: Electronic data monitoring and training.**

The validated data management (DM) system InCRF will be used for electronically collecting the data. The system selected is compliance with GCP guidelines and subject to 21 CFR (Code of federal regulations) FDA part 11 requirements. All the data created in this study will be entered at site, stored and monitored electronically. The study case record forms (CRF) will be available at the DM-system and the data entered directly into the system at the site. Monitoring will be performed electronically and copies of the laboratory results and CRFs will be the source-data.

#### **7.6: Training course**

In order to ensure the accuracy of data entry in the InCRF at the sites, one person on each site will be invited to participate in a half-day course organized and performed by the data manager, monitor and the responsible statistician. Additionally, the data manager will visit each GP-center when data-entry of the first HV will be performed.

#### **7.7: Start-up and closing visit**

The project manager and the clinical monitor will perform the start-up visit at each of the participating sites.

The visit will consist of a site inspection, information, instruction and handing the CRFs.

*Research protocol HV-Jarlsberg/III*

The project manager and the clinical monitor will perform the closing visit within one month after the last participant has finalized the study. All the trial material will be removed from the site.

**7.8: Monitoring procedure**

Essential demographic data will be documented with the participants' record notes as the source data and send to the monitor by e-mail after entering the InCRF-system. Source data will also include the date of written consent, times and dates of blood sampling and physical examinations.

It is the responsibility of the investigator to maintain accurate and up to date records of all clinical trial related activities, which should be legibly entered onto the eCRFs provided. The CRFs should be made available in the event of a formal investigator site audit.

Each site will be monitored at site two times during the study. Monitoring will be performed mainly electronically. In case of unclear or missing data in InCRF, a list of suggested corrections will be sent to site by Meddoc study monitor.

**7.9: Curriculum vitae**

The investigators have to submit an updated CV documenting their expertise. The CV has to be signed and dated by the physician and a copy has to be attached to the protocol if required according to international rules. Another copy must be kept in the Trial Master File and a third copy in the Site File.

**7.10: Site file**

On behalf of the Sponsor, Meddoc AS will supply the investigators with a Site File. The Site File should contain all documents relevant for the study. The investigator is responsible for keeping the Site File updated and secure that all required documents are present in the Site File. The Site File will be inspected during the monitor visits.

*Research protocol HV-Jarlsberg/III***VIII: Data Management****8.1. Case Record Forms (CRF)**

Prior to study start, a data entry instruction document will be made. In this study a copy of source data will be collected on a paper CRF. Source data consist of both printouts from the laboratory examinations and CRF data as baseline characteristics clinical examination collected by the investigator on paper and entered by site. In case of printing CRF data from InCRF instead of using paper CRF, it is important that also these are stored. The printed CRF data need investigator's signature and date.

**8.2. Study Database**

The validated data management system InCRF will be used for collecting the CRF data. The system selected is compliant with GCP guidelines and subject to 21 CFR (Code of federal regulations) FDA part 11 requirements. The final database will be stored in the Statistical Analysis system (SAS ver. 9.4 or later).

**8.3: Data handling**

A data entry person at site will enter the CRF data in InCRF and the Data Manager will perform the initial data validation. In case of missing data, logical errors or interpretation problems, a Query will be sent to the site via e-mail for clarification/correction in InCRF. In the meantime, a copy of the query will be sent to monitor for the routine monitoring tasks follow up. When all the needed participants have finalized the clinical part, and the investigator has electronically signed the CRFs, the DM will do the final verification checks and perform final database hard-lock when all errors are corrected.

Screening analysis for logical errors will be evenly performed on this database and errors will be corrected after new information is collected from the site. When all the detected errors are corrected, the main basic database will be locked. The database will be transformed to a labeled SAS database, which also will be locked for all possible changes or additions. In this copy, the responsible statistician can make derivations but no corrections of the data. If corrections are needed, the main basic study database has to be re-opened and corrected. The international procedure for such changes will be followed.

*Research protocol HV-Jarlsberg/III***IX: Discontinuation**

An HV may discontinue the study at any time if, in the view of the investigator, it is in the participant's best interests.

If an HV does not show up to an agreed visit, the investigator should try to motivate her to continue. However, if the HV has decided not to continue, the HV should be asked to attend a control visit as described for the end of the study.

**9.1: Discontinuation not related to the study question**

A patient who discontinues the study for administrative reasons or reasons documented not related to the cheese intake classifies as "Drop out" and will be replaced by a new volunteer. Drop-outs will be described specially in the statistical analysis and included in the Intention-To-Treat (ITT) analysis by using the procedure "Last observation carried forward" (LOCF).

**9.2: Discontinuation related to the study question**

HV discontinuing the study for reasons that are related to or might be related to the cheese intake will be classified as "Patient Withdrawal". These participants will not be replaced. They will be included in the Per-Protocol (PP) and the ITT analysis using the LOCF procedure.

*Research protocol HV-Jarlsberg/III***X: Ethical consideration****10.1: Consideration of steering committee**

The study will be carried out according to the Helsinki declaration with latest amendments, Good Clinical Practice (GCP) and International Ethical Guidelines for Health-related Research Involving Humans (CIOMS guidelines). The participants are HV and will only be included in this clinical trial after approval of the trial by the regional Ethical Committee (REK) and after the HVs have received oral and written information and signed informed consent.

The products to be used in this trial are cheese commercially available in Norway. To the best of our knowledge, no AE is reported except from person with intolerance of milk and milk product. It is known that vitamin K2 passing a certain daily dose may increase and strengthen the bone tightness and may therefore have a prophylactic effect on osteoporosis. This disease occurs frequently in older population, especially among women passing the menopausal age. Jarlsberg cheese is shown to be one of the Norwegian produced cheeses with the highest level of vitamin K2. The aims of this study are comparing the effect on the Osteocalcin level of Jarlsberg cheese with another cheese without content of vitamin K2. Additionally, to estimate the long-term optimal dose effect of Jarlsberg cheese related to the osteocalcin – and vitamin K2 level and to verify a daily maintenance dose of Jarlsberg cheese in healthy women. All participants invited to this clinical trial are entitled to make their decision based on the fullest amount of information available at that time. In order to make the choice, they will be given a written document expressed in a clear concise language of their native tongue to consider. The document will tell potential participants about the aim of the study. Additionally, that blood sampling will be performed between five and seven times in connection with clinical examinations during the two study parts.

*Summary:* All the included volunteers will receive the daily intake of cheese and clinical examinations free of charge and receive a modest compensation for participation. All participants will be given oral and written information and have to give their written consent to participate in the study. To the best of our knowledge, this study fulfils the entire international requirement to an ethical controlled clinical trial.

**10.2: Approval of the project**

This study will be performed in Norway and the study protocol together and other requested information will be sent for approval by Regional Ethical Committee (REK). Inclusion of participants will not be started before the approval is received.

The database and storage will be in Norway and must be approved by the Data Register in Norway.

**10.3: Informed consent**

Before the start of the trial, the investigator will explain the confidentiality of participation in this research project, the objectives of the trial, the specific requirements for the participating volunteers, the trial design and the consequences of participation. Additionally, the



*Research protocol HV-Jarlsberg/III*

investigators have to obtain written informed consent from the participants before inclusion in the study.

**10.4: Protection of personal data**

The monitor may know the identity of the participants during verification of the source data. However, the monitor has unconditional professional secrecy.

All participant-related material leaving the GP-centers will be anonymous so that the volunteer only can be identified by date of birth, initials and HV's study identification number. The investigator is responsible for keeping a list with the full names, their citizens' number and corresponding study numbers according to the demands in GCP.

The participants will receive all the Jarlsberg cheese in the study or free. Additionally, they will get the clinical examination for free and receive a moderate economical compensation for the participation. In case they get extra transportation costs for the participation, this will be covered by the study.

*Research protocol HV-Jarlsberg/III***XI: Statistical model****11.1: Handling of discontinuation**

All volunteers fulfilling the inclusion and exclusion criteria, given informed consent to participate and started with at least one intake of the trial cheese, are classified as included in the study.

In the Intention-to-Treat (ITT) analysis, all these patients will be included and the procedure Last Observation Carried Forward (LOCF) will be used. The participants classified as Drop-out will be excluded from the Per-protocol (PP) analysis.

**11.2: Presentation of the result**

Assumed continuously distributed variables will be expressed by mean values with 95% confidence intervals [15]. As an index of dispersion Standard Deviation (SD) will be used. In case of skewed distribution, the variable will be tried logarithmic transformed and the analyses performed on the transformed data. The results will be retransformed for presentation.

Categorized and discrete distributed variables will be expressed in contingency tables [16]. Additionally, important prevalence will be expressed in percentage with 95% confidence intervals constructed by using the theory of Simple Binomial Sequences.

**11.4: Comparison of groups**

Comparison between and changes within groups will both be performed two-tailed and differences considered significant if the p-values are found less or equal to a significance level of 5%.

For investigation of assumption in distribution of continuous variable, Shapiro – Wilk test will be used [15]. In case of skewness, the variables will be tried logarithmic transformed and the analyses performed on the transformed data.

Comparison of groups with regard to continuously distributed variables will be performed by using Analysis of Variance (ANOVA) weighted for GP-site and the initial observation as covariates [17]. Changes within groups will be performed by using ANOVA model with repeated measurements [18]. Contingency Table Analysis will be used for both comparison between and changes within groups regarding categorized and discrete distributed variables [16].

**11.3: Sample size**

With a significant level of 5%, a power of 90% and a clinical relevant difference in total Osteocalcin increase of one-time SD between the two groups, at least 48 HV have to be included. By correcting for the number of participating GP and drop-outs during the first part of the study, 32 HV will be included in each group.

*Research protocol HV-Jarlsberg/III***XII: Operational matters****12.1: Investigator's agreement**

Before start, the primary investigator will confirm the agreements to participate in the trial by signing the Investigator's Agreement Form with the Sponsor.

**12.2: Instructions**

The project manager, supported by the clinical monitor, will instruct the investigator at the start-up visit and during the study.

**12.3: Amendments to the protocol**

Changes in the protocol can be required by REK, investigators, project manager or TINE. Changes must be given in written amendments and numbered in the original protocol. It is forbidden to add new parameters consisting of measurements on the patients in the study unless they are covered as amendments in the protocol or taken due to the health and safety of the participant.

**12.4: Protocol deviations**

Deviations from the protocol should be restricted as much as possible and will be fully recorded and justified. The project manager will be informed as soon as possible of all protocol deviations.

**12.5: Compliance monitoring**

The project manager and the investigator will ensure that the site is suitable for the trial and that the participants are well informed. They shall check protocol compliance, handling of the test articles and recording of data during the stages of the trial. A report is prepared of each visit and kept in the trial master file (TMF).

**12.6: Responsibilities**

The investigator will acknowledge the responsibilities and the agreement to participate in the trial by dating and signing the agreement form. The project manager will verify that adequate arrangements have been made for the observations, measurements and recording of the data.

**12.7: Confidentiality**

This clinical trial is a precondition for further studies. TINE will therefore use the obtained data and results for marketing the product. The main study database will be stored in the product database of the Sponsor.

The project manager and the investigator may demand and have the right to have the results published in an international medical journal. The draft of the manuscript has to be presented to the Sponsor for comments, discussion and final approval. TINE cannot stop the publication unless it is proved that publication of the results may damage the marketing of the product.

*Research protocol HV-Jarlsberg/III*

The project manager or the investigator cannot present the results in any meeting or congresses without approval by TINE. The data obtained in this study has to be handled confidentially.

**12.8: Investigator and Sponsor withdrawals**

The investigator can finalize his or her participation in the study if TINE does not fulfill its duties according to the protocol or the Sponsor-investigator agreement.

TINE has the right to terminate the study at any time. The investigators will be paid according to the agreements in the Sponsor-investigator agreement. A written explanation will be sent to the investigators and REK according to present rules.

*Research protocol HV-Jarlsberg/III***XIII: References. G**

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## BMJ NUTRITION PREVENTION & HEALTH

Externally peer reviewed? Yes

Evidence type: Randomised controlled trial

Subjects: People

### Small daily portion of Jarlsberg cheese may help to stave off bone thinning

*Not linked to higher harmful cholesterol either; effects specific to this type of cheese*

A small (57 g) daily portion of Jarlsberg cheese may help to stave off bone thinning (osteopenia/osteoporosis) without boosting harmful low density cholesterol, suggest the results of a small comparative clinical trial, published in the open access journal **BMJ Nutrition Prevention & Health**.

The effects seem to be specific to this type of cheese, the findings indicate.

Jarlsberg is a mild and semi-soft, nutty flavoured cheese made from cow's milk, with regular holes. It originates from Jarlsberg in eastern Norway.

Previous research indicates that it may help boost levels of osteocalcin, a hormone that is associated with strong bones and teeth, but it's not clear if this effect is specific to Jarlsberg or any type of cheese.

In a bid to find out, the researchers studied 66 healthy women (average age 33; average BMI of 24) who were randomly allocated to adding either a daily 57 g portion of Jarlsberg (41) or 50 g of Camembert cheese (25) to their diet for 6 weeks.

At the end of this period, the group eating Camembert was switched to Jarlsberg for another six weeks.

Jarlsberg and Camembert have similar fat and protein contents, but unlike Camembert, Jarlsberg is rich in vitamin K2, also known as menaquinone (MK), of which there are several varieties.

The short-chained MK-4 is found in animal products such as liver. The long-chained MK-7, MK-8, MK-9 and MK-9(4H) originate from bacteria, and occur in certain fermented foods, such as cheese. Jarlsberg is particularly rich in both MK-9 and MK-9(4H).

Every six weeks blood samples were taken from all the participants to check for key proteins, osteocalcin, and a peptide (PINP) involved in bone turnover. Vitamin K2 and blood fat levels were also measured.

Blood sample analysis showed that the key biochemical markers of bone turnover, including osteocalcin, and vitamin K2 increased significantly after 6 weeks in the Jarlsberg group.



Among those in the Camembert group, levels of PINP remained unchanged while those of the other biochemical markers fell slightly. But they increased significantly after switching to Jarlsberg. PINP levels also increased.

Blood fats increased slightly in both groups after 6 weeks. But levels of total cholesterol and LDL (harmful) cholesterol fell significantly in the Camembert group after they switched to Jarlsberg.

Glycated haemoglobin (HbA1c)---the amount of glucose stuck in red blood cells---fell significantly (by 3%) in the Jarlsberg group, while it rose sharply (by 2%) in those eating Camembert. But after switching to Jarlsberg HbA1c fell significantly in this group too.

Calcium and magnesium fell significantly in the Jarlsberg group but remained unchanged in the Camembert group. After switching cheese, calcium levels dropped in this group too, possibly reflecting increased uptake of these key minerals in bone formation, say the researchers.

“Daily Jarlsberg cheese consumption has a positive effect on osteocalcin, other [markers of bone turnover], glycated haemoglobin and lipids,” write the researchers, concluding that the effects are specific to this cheese.

The bacteria (*Propionibacterium freudenreichii*) in Jarlsberg that produces MK-9-(4H) also produces a substance called DHNA, which, experimental studies suggest, might combat bone thinning and increase bone tissue formation, and possibly explain the increase in osteocalcin, they add.

They go on to suggest that Jarlsberg cheese might therefore help to prevent osteopenia—the stage before osteoporosis—as well as metabolic diseases, such as diabetes, although further research would be needed to confirm this, they emphasise.

“This study shows that while calcium and vitamin D are known to be extremely important for bone health, there are other key factors at play, such as vitamin K2, which is perhaps not as well known,” comments Professor Sumantra Ray, Executive Director, NNEdPro Global Centre for Nutrition and Health, which co-owns the journal.

The study also highlights an important research issue, he adds. “Different methods of preparation mean there are key differences in the nutrient composition of cheese which has often been regarded as a homogenous food item in dietary research to date. This needs to be addressed in future studies.”

But he cautions. “As this is a small study in young and healthy people designed to explore novel pathways linking diet and bone health, the results need to be interpreted with great caution as the study participants will not necessarily be representative of other groups. And it shouldn’t be taken as a recommendation to eat a particular type of cheese.”