Original Research Article

Determination of maintenance Jarlsberg® cheese dose to keep the obtained serum osteocalcin level; a response surface pathway designed de-escalation dose study with individual starting values

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ABSTRACT

Background: Daily maximum effective dose (MED) of Jarlsberg® increased the serum osteocalcin (tOC) level, vitamin K2 and affected the lipid pattern positively. The aim of the study was to estimate and verify a daily maintenance dose.

Methods: 12 healthy female volunteers (HV) were included in a de-escalation study after a six week run-in period on the daily MED of 57 g Jarlsberg® cheese. A 3-level within-patient response surface pathway (RSP) design with individual starting values was developed. Another 12 HVs were included in a new study with a six week run-in period on MED followed with six weeks on the estimated maintenance dose. All HVs were premenopausal female between 20 and 52 years of age. The main variable in the studies was the tOC level.

Results: tOC, cOC and the vitamin K2 variants increases significantly (p<0.01) during the run-in period on daily MED of Jarlsberg® in both studies. The maintenance daily dose was estimated to 45 g (95% CI: 38-52 g/day) and used in the new study. The tOC level was reduced from 19.8 ng/ml (95% CI: 12.0-27.6) obtained in the run-in period to 18.5 ng/ml (95% CI: 11.7-25.3) during the maintenance part. This represents a reduction of 6.6%. The sum of vitamin K2 variants changed from 0.58 ng/ml on MED of Jarlsberg® to 0.59 ng/ml (95% CI: 0.37-0.82) during the maintenance period.

Conclusions: Daily MED of Jarlsberg® cheese increases tOC, cOC and the vitamin K2 level. The maintenance Jarlsberg® dose was estimated to 45 g/day and verified as sufficient.

Keywords: Dose de-escalation, Increased osteocalcin level, Jarlsberg® cheese, Osteocalcin ratio, RSP-design, Vitamin K2

INTRODUCTION

Bone loss remains a substantial problem among the elderly. It is well established that dietary calcium and vitamin D are beneficial for skeletal health. Research has documented that vitamin K has beneficial effects on the skeleton.1 Dairy products are good calcium sources and important for bone formation and cheese is an important source of vitamin K2. This indicates that cheese consumption may strengthen bones and reduce the risk of osteoporosis.

The effect of eating cheese rich in vitamin K2 have not been studied abundantly.
Activated osteocalcin has a key role in bone-formation and maintenance working with calcium and vitamin D collectively. Osteocalcin is one of the 17 body’s Gla proteins, 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.

Vitamin K2 or menaquinone (MK) is found in several variants. The short-chained MK-4 can be formed from vitamin K1 in humans and is found in animal products such as liver. The long chained vitamin K2 variants like MK-7, MK-8, MK-9 and MK-9 (4H) are of bacterial origin and mainly found in certain fermented foods. In the western diet, fermented dairy products like cheese are the main source of these vitamin K2 variants.

The long chained MKs have been found to have greater extra hepatic activity compared to K1 and MK-4, possibly due to more efficient uptake and much longer serum half-life. Prospective cohort studies have demonstrated health benefits that can be attributed to the intake of vitamin K2, but not K1. The main contributor to the vitamin K2 is cheese containing MKs with long side chains.

The dominating vitamin K2 variant is MK-9 in most cheeses, but the amount varies considerably. However, some cheeses also contain MK-9 (4H). Jarlsberg® cheese in particular is rich in this compound. 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 investigated in controlled clinical trials. Because of its high vitamin K2 content, Jarlsberg® cheese is well suited for such studies.

Recently, a dose-response study with healthy premenopausal Norwegian women was performed with a 3-level between patient RSP design. The study was performed on 19 women with daily intake of Jarlsberg® cheese during a period of 5 weeks. This recent study estimated the MED of Jarlsberg® cheese to 57 g/day and resulted in an increase of both the total serum osteocalcin level (tOC) and vitamin K2 levels. To the best of our knowledge, this is the first time medical or serum half life of osteocalcin was measured in humans.

The aim of this study was to verify the significant increase of tOC and coCO caused by the detected MED, to develop an RSP design with an individual starting dose, to estimate a maintenance Jarlsberg® cheese dose to keep the obtained tOC level after 6 weeks on MED and to demonstrate that this is a sufficient daily maintenance dose.

METHODS

The study population consisted of healthy Norwegian women (HV) 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 three preceding weeks or participating in another clinical trial the last six weeks were excluded.

The de-escalation study material consisted of 11 Caucasian and 1 Asian HV from the study population, recruited at Skjetten medical centre and primary medicine. The study was approved by the Norwegian regional ethical committee (REK) and performed from January 2020 to May 2020. Mean age of the study sample was 38.4 years ranging from 21.7 to 46.8 years with a mean body mass index (BMI) of 24.2 kg/m² range: 19.5-
29.8 kg/m². Nine of the HVs were married or in cohabitation, two were divorced and one was single. None were smokers and one was breastfeeding. One HV was using vitamin D supplements due to deficiency, one suffered from hypothyroidism and three were using contraceptives. All medical treatments were kept unchanged during the study.

The maintenance study material consisted of 11 Caucasian and 1 Asian HV from the study population, recruited from Sundet medical centre and primary medicine. The study was approved by the Norwegian regional ethical committee (REK) and performed from April 2020 to July 2020. The mean age of the study sample was 33.6 years ranging from 21.0 to 41.6 years with a mean BMI of 26.2 kg/m² range: 20.2-35.4 kg/m². Nine of the HVs were married or in cohabitation, two were divorced and one was single. None were breastfeeding or smokers. Three HVs were using vitamin D supplements due to deficiency, one suffered from hypothyroidism, two from allergy, one from asthma and one from fibromyalgia. One HV reported using contraceptives. All medical treatments were kept unchanged during the study.

Clinical procedure

The HVs in both the de-escalation and the maintenance study were initially included in a six week run-in period, in which they consumed 60 g or 6 slices Jarlsberg® cheese daily. Clinical investigations and blood sampling were performed initially and at the end of the six weeks run-in period. In addition to common laboratory variables, the blood samples were used for measurements of tOC and the vitamin K2 variants MK-7, MK-8, MK-9 and MK-9 (4H). The HVs were asked not to change their usual diet, except for replacing other cheeses with Jarlsberg® cheese.

The de-escalation study consisted of three design levels, each of three weeks duration. The starting dose used in the first design level was individually calculated based on the HV’s response in the 6 week run-in. Assumed a given HV obtained X% increase in the tOC level during the run-in period. The daily Jarlsberg® dose for the first design level in the de-escalating part was reduced with X%. The upper limit of the dose reduction was set to 67%. HVs obtaining an increase in the tOC level ≥67% during the run-in period received a starting Jarlsberg® dose of 20 g/day. Blood samples for the measurement of tOC and vitamin K2 variants were collected after three weeks at all three design levels.

The maintenance study consisted of one HV group in which the MED of Jarlsberg® was reduced to the estimated maintenance dose from the de-escalation study after six weeks. Blood samples for measurements of tOC and the vitamin K2 variants were collected initially, after six weeks of the daily optimized Jarlsberg® dose and after six weeks of the maintenance dose.

Study design

The dose de-escalation study was conducted as a one-dimensional, within-patient 3-level RSP designed trial with individual starting dose. The daily dose of Jarlsberg® cheese was used as intervention variable and the percent increase in tOC from the run-in period as the outcome variable. If the tOC level decreased >5% from the run-in period after 3 weeks during a given design level, the daily cheese dose was increased. In case the obtained change in the tOC level was within the interval ±5%, the dose was kept unchanged in the following design level. If the tOC level increased >5%, the Jarlsberg® dose was reduced for the HV in question in the following design level. The size of the dose increased or decreased depending on the degree of the change and was calculated in accordance with the RSP procedure (Table 1). The daily Jarlsberg® cheese window for this study was (20-60 g) or (2-6 slices). A tOC increase ≥25% classified for a large dose reduction, an increase <25% and >5% as small dose reduction and a change of ±5% as acceptable. On the other hand, a tOC reduction from the run-in period ≥25% resulted in a large dose increase and a reduction <25% and >5% in a small dose increase.

The maintenance study was conducted as a single-armed study of 12 weeks duration with a dose reduction in accordance with the previously estimated maintenance dose after 6 weeks.

Vitamin K extraction and osteocalcin analysis

Vitamin K in cheese was extracted in heptane after fat hydrolysis. In order to extract vitamin K from plasma, included 0.75 ml plasma samples were mixed with 1.5 ml ethanol, then 1 ml heptane. The mixtures were vortexed for 30 s and centrifuged for 1 min at 1000 g. Samples of 0.5 ml from the upper phase were withdrawn, dried under vacuum and re-dissolved in 50 μl isopropanol prior to HPLC. Vitamin K was analysed by isocratic reverse phase chromatography on a Dionex ultimate 3000 HPLC system with fluorescence detection at 436 nm and emission at 50°C. Injected volumes were 3 μl. Standards vitamin K1, MK-7 and MK-9 were from Sigma and MK-9 from Toronto research chemicals. Standards MK-8 and MK-9 (4H) were extracted from anaerobically grown pure cultures of Escherichia coli and Propionibacterium freudenreichii, respectively.

cOC and ucOC were measured in plasma by immunoassays kits (Takara Bio, Ōtsu, Japan) by Vitas AS.
Clinical intervention

Jarlsberg® cheese is produced by TINE SA. Cheese from three production batches was used in this study. The participants received the cheese in 100 g packages containing cheese slices of 10 g. The average vitamin K content per 100 g of the cheese was 2.8 μg vitamin K1, 2.7 μg MK-4, 1.2 μg MK-7, 6.3 μg MK-8, 24.2 μg MK-9 and 30.0 μg MK-9 (4H).

Central variables

The two main variables in this study were the tOC and the osteocalcin ratio,

$$RO = \frac{cOC}{ucOC}$$

The supporting variables were sum of vitamin K2, the vitamin K2 variants MK-7, MK-8, MK-9 and MK-9 (4H). Additionally, the lipid variables, haematological and clinical chemistry were used as secondary variables.

Statistical analysis

The assumed continuously distributed variables were expressed by mean values with 95% confidence interval (CI). As an index of dispersion; standard deviations (SD) were given. Categorized variables were given in contingency tables. Changes in mean were tested by using a paired two tailed t test with a significant level of 5%. The sample space of the dose in the present studies may be expressed as,

$$\Omega_D = \{DL \leq \ldots \leq DU\},$$

where,

$$\mu$$ be the maintenance dose,

assume $$\mu$$ is contained in $$\Omega_D$$.

The increase from run-in assumed to be ordinal and the probability increases monotonically in a limited cheese dose interval. Isotonic regression was used for estimation of MED.

Approvals

The study was approved by the Norwegian south-east regional ethical committee, reference number REK-85226; EudraCT number: 2019-004576-19.

RESULTS

De-escalation study

During the six weeks run-in part of the daily Jarlsberg® MED of 60 g (6 slices), the means of tOC and cOC increased significantly (p<0.01). The ucOC was found unchanged (Table 2). The mean tOC increased from 19.1 ng/ml (95% CI: 12.5-25.7) at screening to 26.0 ng/ml (95% CI: 18.8-33.2 ng/ml) after 6 weeks. Additionally, the mean osteocalcin ratio RO increased significantly (p<0.01) with 53.2%.

The mean Jarlsberg® starting dose in the first design level of the de-escalation part was 35 g/day (95% CI: 29-42 g/day) or 3.5 slices/day, varying from 20 g (2 slices) to 50 g (5 slices). In accordance with the RSP-procedure, the daily dose was increased from the first to the second design level for seven HV, remained unchanged in 2 and reduced in three HVs (Figure 1). The mean daily dose in the second design level was 38 g or 3.8 slices. From the second to the third design level, the daily dose was recommended to be increased in seven HVs, unchanged in three and reduced in two HVs. This would have given a mean dose of 40 g or 4 slices in the third design level. However, the dose compliance was reduced to 92% and only two HVs followed the recommendation. The actual mean dose was reduced to 36 g or 3.6 slices. Based on the results obtained in the third design level, the daily Jarlsberg® dose was recommended to be increased for 10 HVs, unchanged for one and reduced for one HV. The mean tOC decreased from 26.0 ng/ml after 6 weeks on optimal daily Jarlsberg® dose to 22.1 ng/ml (95% CI: 15.7-28.4 ng/ml) during the first design level (Figure 2A). The tOC level increased again to 24.6 ng/ml (95% CI: 17.2-32.0) in the second design level but reduced to 16.1 ng/ml (95% CI: 10.1-22.1 ng/ml) in the third design level. The maintenance daily dose of Jarlsberg® cheese was estimated to 45 g (95% CI: 38-52 g/day) or 4.5 slices/day. Serum of the long-chained vitamin K2 variants MK-7, MK-8, MK-9 and MK-9 (4H) increased significantly (p<0.01) during the 6 week run-in period with daily intake of optimized Jarlsberg® intake (Table 2). The mean sum of serum vitamin K2 increased from 0.31 ng/ml (95% CI: 0.14-0.47 ng/ml) at screening to 0.64 ng/ml (95% CI: 0.44-0.84 ng/ml) after 6 weeks (Figure 3A). The level of MK-7 continued to increase in the dose de-escalation part, whereas MK-8, MK-9, MK-9 (4H) and the sum of vitamin K2 decreased during the first design level but increased again in the second and third design level. The mean sum vitamin K2 decreased from 0.64 ng/ml after 6 weeks on optimal daily Jarlsberg® dose to 0.54 ng/ml (95% CI: 0.38-0.69) during the first design level (Figure 3A). During the second and third design level, the sum of vitamin K2 increases again to 0.72 ng/ml with 95% confidence intervals from 0.52 to 0.93 ng/ml and 0.55-0.89 ng/ml, respectively.

Maintenance study

During the six weeks run-in part on the optimal daily Jarlsberg® dose, the mean of both tOC and cOC increased significantly (p<0.01) whereas the mean of ucOC was nearly unchanged (Table 3). The mean sum tOC increased from 12.3 ng/ml (95% CI: 6.0-18.6) at screening to 19.8 ng/ml (95% CI: 12.0-27.6) after 6 weeks (Figure 2B). Additionally, the mean osteocalcin ratio RO increased significantly (p<0.01) by 94.6%.
Figure 1: Individual changes in the daily number of Jarlsberg slices during the three design levels in the dose de-escalation period of 3x3=12 weeks. The number of participants within each slice-box are given in number and illustrated with darkness of the shading. The straight lines show the individual change in the daily Jarlsberg dose. The lack in dose compliance was only recorded on the third design level and illustrated in the last column of the figure.

Figure 2: The development in sum osteocalcin level in n=12 volunteers during 6 weeks on optimized daily Jarlsberg® dose and 3x 3 weeks on reduced doses in the de-escalation study part (2A). Figure 2B shows the development in sum osteocalcin level in additionally n=12 volunteers during 6 weeks on optimized daily Jarlsberg® dose and during 6 weeks on the estimated maintenance dose. All the results are expressed by mean values with 95% confidence intervals.
Figure 3: The development in sum vitamin K2 level in n=12 volunteers during 6 weeks on optimized daily Jarlsberg® dose and 3x 3 weeks on reduced doses in the de-escalation study part (2A). Figure 2B shows the development in sum vitamin K2 level in additionally n=12 volunteers during 6 weeks on optimized daily Jarlsberg® dose and during 6 weeks on the estimated maintenance dose. All the results are expressed by mean values with 95% confidence intervals.

Table 1: The dose-change procedure of daily intake of Jarlsberg® cheese during the de-escalation part.

| Cheese dose 1st design level | Response at design level 1 | Cheese dose 2nd design level | Response at design level 2 | Cheese dose 3rd design level |
|------------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|
| Osteocalcin level (%)        |                             | Osteocalcin level (%)        |                             |                             |
| ≥25 reduction                | m+m/k                       | ≥25 reduction                | m+m/k+m/k²                  |
| 5-25 reduction               | m+m/k²                      | 5-25 red                     | m+m/k+m/k³                  |
| 5-25 increase                | m+m/k²                      | ±5 change                    | m+m/k¹                     |
| ≥25 increase                 | m+m/k²                      | ≥25 increase                 | m+m/k²                     |
| X% reduction of the optimal Jarlsberg® dose of 60 g/day (6 slices); X% represents the increase in sum osteocalcin obtained during the 6 week run-in period |
| ±5 change                    | m                           | ±5 change                    | m                           |
| 5-25 increase                | m+m/k²                      | 5-25 increase                | m+m/k²                     |
| ≥25 increase                 | m+m/k²                      | ≥5 increase                  | m+m/k²                     |
| X% increase (4)              | m-m/k²                      | ≥25 reduction                | m-m/k²+m/k³                 |
| 5-25 red                     | m-m/k²                      | 5-25 red                     | m-m/k²+m/k³                 |
| ±5 change                    | m-m/k²                      | ±5 change                    | m-m/k²                     |
| 5-25 increase                | m-m/k²                      | ≥5 increase                  | m-m/k²                     |
| ≥25 increase                 | m-m/k²                      |                              |                             |
| ≥25 increase (5)             | m-m/k                       |                              |                             |
During the six weeks on optimal daily Jarlsberg® dose, the long-chained vitamin K2 variants MK-8, MK-9 and MK-9 (4H) increased significantly (p<0.01). No change was detected for MK-7 (Table 3). The mean sum of vitamin K2 variants increased significantly (p<0.01) from 0.35 ng/ml (95% CI: 0.28-0.41) at screening to 0.58 ng/ml (95% CI: 0.42-0.75) after 6 weeks (Figure 3B). The level of MK-7 increased slightly during the 6 weeks on the maintenance dose, whereas MK-8, MK-9, MK-9 (4H) were nearly unchanged (Table 3). The mean sum of the vitamin K2 variants changed from 0.58 ng/ml after 6 weeks on the optimal Jarlsberg® dose to 0.59 ng/ml (95% CI: 0.37-0.82) during the maintenance part (Figure 3B).
DISCUSSION

The six week run-in periods with daily MED of Jarlsberg® preceding the de-escalation and maintenance studies verified the increases in tOC levels, osteocalcin ratio and serum vitamin K2 as previously reported. The studies verified the increases in vitamin K status, measured as osteocalcin carboxylation, as well as serum vitamin K2. The obtained positive effects are not transient and can be retained by daily intake of Jarlsberg®. The enhanced carboxylation of osteocalcin can be ascribed to vitamin K2 in the cheese and the carboxylation of osteocalcin is important for bone strength. Osteocalcin is a Gla protein and is activated by carboxylation in a vitamin K dependent process. The extent of carboxylation of tOC is used an indicator of vitamin K status. However, the vitamin K status is also an indication of carboxylation of other Gla proteins like matrix Gla protein which when activated is an inhibitor of vascular calcification. The vitamin K status has been found to be negatively associated with cardiovascular disease and mortality in controlled clinical trials and longitudinal studies. Moreover, vitamin K2 is associated with positive effects on liver, kidney, neurological and inflammatory diseases as well as immune functions and obesity. Most of the vitamin K2 effects have been related to increase Gla protein carboxylation, but some are claimed to be independent on this process. This includes the stimulation of osteoblast activity including increased osteocalcin production and inhibition of bone resorption by osteoclasts. These effects have been seen in cell cultures and animal model systems, but to the best of our knowledge no other food has been reported to increase the serum osteocalcin level. This indicates that daily intake of MED of Jarlsberg® cheese could have this effect on bone. The positive health effects of osteocalcin stimulation do not affect the bone solely. Low tOC levels are also associated with several diseases such as risk of metabolic syndrome, type 2 diabetes and obesity.

The daily MED of Jarlsberg® cheese was found to be 57g. This is possibly unacceptably high in the long run for some people. A smaller dose reduction without damaging the developed effect on osteocalcin would be appreciated. The aim of the de-escalation study was to determine a maintenance daily dose of Jarlsberg® cheese to keep the tOC level obtained after six weeks on MED within ±5%.

Both baseline osteocalcin and osteocalcin increase after six weeks with daily MED of Jarlsberg® varied considerably among the HVs, hence a new version of the RSP-design was developed with individual starting doses. The HVs were used as their own references. The starting doses in the de-escalation study were calculated based on the individual increase in the tOC level obtained in the six week run-in period. The changes in dose between design levels were performed in accordance with the common RSP procedure. In this way the individual variation in osteocalcin increase during the run-in period was considered in estimation of the maintenance dose. The large variation in starting dose was substantially reduced during the first two design levels. To obtain good and reliable results in dose-response study, 100% dose compliance is required. This was obtained in the two first design levels, but substantially reduced in the third. In spite of this coincidence, the RSP-procedure was able to estimate a sufficient maintenance dose even the uncertainty was a bit larger than expected for such design. The compliance problem occurred during the last two weeks of the third design level and might be a weakness of the design. The HVs are used as their own controls which increases the statistical power, but also the study duration. Eighteen weeks is probably past the limit for HV in a clinical study.

This estimated maintenance dose was used on a new study sample of 12 HVs required from another study site in the same district. After a six week run-in period with a daily MED of Jarlsberg® cheese, all the HVs received the estimated maintenance dose. The mean tOC level during the six weeks on the maintenance dose was reduced by 6% from the end of the run-in period and thus verified as a sufficient maintenance dose.

The increase of the serum long chained vitamin K2 was remarkable. In the run-in periods, all the vitamin K2-variants increased significantly in both studies. The development in serum vitamin K2 showed a pattern in rise and falls identical to that of the tOC during maintenance study. This pattern was not so obvious in the de-escalation study due to the reduction in tOC on the last design level. It might be that the vitamin K2 level does not change as quickly as tOC. The parallelism in the development of sum vitamin K2 and tOC in the two studies except for the last design level in the de-escalation study, indicated a strong relation between the two variables. The lack of parallelism between tOC and sum of vitamin K2 in the third design level is likely related to the cheese dose compliance. Significant linear correlation between the two variables was found in both studies, but not as convincing as the parallelism indicated. Together with the changed pattern in the last design level of the de-escalation study indicating that more variables might be involved in the connection between tOC and vitamin K2. It might be that Jarlsberg® Cheese contains other factors besides vitamin K2 which stimulates the increase in the tOC level.

Daily intake of fat cheese might raise questions related to both weight gain and increased lipid parameters. However, recent studies have demonstrated that daily cheese intake, without concern to K2 and osteocalcin seems to reduce cardiac disease. Positive effects of daily Jarlsberg® cheese intake were detected on the lipid pattern and the blood pressure in the previous dose-response study. Similar findings were not verified in the present studies. Neither cholesterol nor blood pressure was significantly affected. The run-in-periods started
during the beginning stage of the corona pandemics in Norway. None of the participating HVs were infected during the project, but changes in both dietary pattern and physical activity in the study sample was reported. The coronavirus pandemic brought a lot of temporary changes in the study population and several of the participating HVs had to stay at home. A recent Italian study reported changes in lifestyle and eating habits because of the pandemic.26 Such factors may explain the inconsistencies related to blood lipids reported in the previous and the present studies. New studies in this field should be carried out under normal conditions.

CONCLUSION

Daily MED of Jarlsberg® cheese increases tOC, cOC and the vitamin K2 level. The maintenance Jarlsberg® dose was estimated to 45 g/day and verified as sufficient.

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