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Role of foods of animal origin as dietary sources of vitamin D

By

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Declaration

I can confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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May 2017

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Abstract

Mounting evidence suggests lower vitamin D status is associated with increased risk of cardiovascular disease (CVD) and type 2 diabetes (T2D), which are the leading causes of morbidity and mortality in the world. Findings from the Caerphilly Prospective Study (CAPS) (n=452 men), showed a higher dietary vitamin D intake was associated with lower fasted plasma triacylglycerol concentration, an independent risk marker for CVD, after over 20 years follow-up. Over the past decade hypovitaminosis D of the general population has become a concern throughout the world due in part to limitations in the endogenous vitamin D synthesis from ultraviolet radiation, which has increased the importance of dietary vitamin D intake. There are only a few foods naturally rich in vitamin D, such as oily fish and egg yolk, however the vitamin D content and form can vary and oily fish is only regularly consumed by a small section of the UK population. To address this, a retail study was conducted to investigate the vitamin D content and form (vitamin D_3 and 25-hydroxyvitamin D_3 (25(OH) D_3)) in eggs from different production systems (indoor, organic and free-range) and supermarkets (n=3) between July to November of 2012. Vitamin D_3 was significant higher in free range and organic, compared with indoor eggs, while 25(OH) D_3 was only higher in organic eggs. Total vitamin D content (vitamin D_3 and 25(OH) D_3) of each egg was approximately 2 µg, which would contribute 20% of vitamin D recommended dose of 10 µg/day. However, there is debate over the possible detrimental effect on human health of the relatively high cholesterol content of eggs. Further findings from CAPS demonstrated that higher egg consumption was not associated with incident of CVD, T2D or all-cause mortality, but a higher egg consumption (up to 1 egg per day) was associated with a higher risk of stroke and elevated fasting glucose in subjects with impaired glucose tolerance (fasting glucose ≥6.1 mmol/L) and/or T2D.

Due to the relatively low natural enrichment of vitamin D in foods, fortification has become a recognised strategy to increase dietary vitamin D intake. Milk is used successfully as a vehicle for vitamin D fortification in a few countries, but there remains some uncertainty about the effects of milk consumption on risk of CVD. Thus, an updated dose-response meta-analysis which included all of the published prospective cohort studies up to May 2016 was conducted. It was found that milk was not associated with CVD or all-cause mortality, and suggested a beneficial role of fermented dairy or cheese by lowering the risk of CVD and mortality. Vitamin D fortified milk and dairy are not available in many countries such as the UK. Furthermore, limited evidence suggests that supplementation of 25(OH) D_3 has a greater efficacy for improving vitamin D status, than vitamin D_3. Thus, a further study was designed with the aim to increase the vitamin D content of milk by a food chain approach by feeding vitamin D (vitamin D_3 or 25(OH) D_3) supplements to dairy cows. This study showed feeding dairy cows with 25(OH) D_3 either pre-calving or post-calving was more effective in raising plasma 25(OH) D_3 concentration than vitamin D_3 supplementation, but vitamin D concentration in the milk was not affected by treatments. The mean 25(OH) D_3 concentration of the enriched milk was 0.88 µg/L. Thus, fortification was favoured as a strategy for increasing dietary vitamin D intake. A randomised, controlled, cross-over and double-blinded 24-hour acute intervention study was conducted in 17 men with sub-optimal vitamin D status (mean plasma 25(OH) D concentration was 31.7 ± 3.4 nmol/L) to compare the effects of 20 µg 25(OH) D_3 with 20 µg vitamin D_3 fortified dairy drink and a control dairy drink on vitamin D status (plasma 25(OH) D_3) and CVD risk markers. Consumption of 25(OH) D_3 fortified dairy drink was found to be more effective and faster at raising plasma 25(OH) D_3 concentrations postprandially. In summary, vitamin D fortified foods are needed to address the high prevalence of low vitamin D status within population. Fortification using 25(OH) D_3 would appear to have advantages over vitamin D_3.

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Chapter 1 - Introduction

Background of vitamin D

Vitamin D is known to be essential for normal bone growth and quality, thus, the classic functions of vitamin D relate to calcium absorption, homeostasis and bone mineralisation with deficiency leading to childhood rickets and adult osteomalacia (1). More recently, there is mounting evidence to show that vitamin D is involved in many additional non-skeletal functions in the body and the role of vitamin D deficiency in increasing the risk of many common and serious diseases, including osteoporosis, cardiovascular disease, common cancers and diabetes (2). The estimated benefit of increased vitamin D status in reducing the economic burden of disease in terms of CVD in Western Europe could be €7480 million/year (3).

Vitamin D is a fat-soluble vitamin, humans usually obtain vitamin D naturally from sunlight. The physiologically active vitamin D form is 1, $25(OH)_2$ D which is synthesised after two hydroxylation reactions in the body, the first in the liver where vitamin D is transformed to 25-hydroxyvitamin D_3 (25(OH) D), the second occurs in the kidneys where 25(OH) D is converted to$1,25(OH)_2$ D (1; Figure 1.1). Plasma or serum 25(OH) D is used as an indicator of vitamin D status (4). Although there is no agreement on the specific threshold of vitamin D status on disease outcomes, a low serum or plasma concentration of 25(OH) D (<25 nmol/L) is regarded as increasing the risk of rickets (1). Estimates of vitamin D status indicate widespread inadequacy with low status most prevalent in the Middle East and South Asia (5). Even within Europe, Hypponen and Power (6) concluded that the prevalence of hypovitaminosis D in the general population was alarmingly high especially during winter and spring. In UK, 23% of adults are estimated to have plasma vitamin D below 25 nmol/L (7). There are several reasons which contribute to the low vitamin D status, such as increasingly indoor lifestyle, skin pigmentation, ageing and sunscreen use all of which reduce the cutaneous production of vitamin D (8). Therefore, dietary intake of vitamin D has become

more important than before (9) and in recognition of this in 2016 the UK Scientific Advisory Committee on Nutrition (SACN) revised the national population dietary recommendations from zero to 10 μg vitamin D daily for all adults. However, there are very few foods that are naturally enriched with vitamin D, such as egg yolk, oily fish (10) and strategies to improve dietary intake are essential.

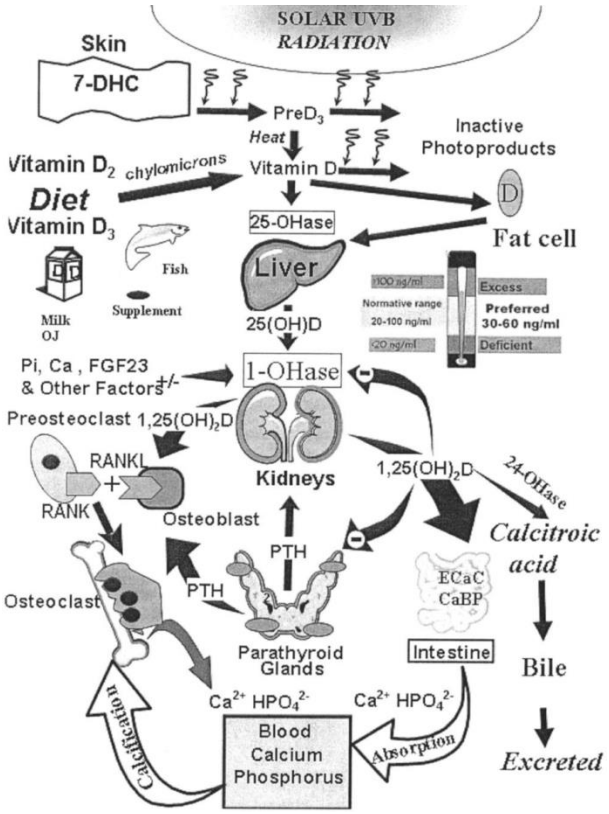

Figure 1.1 Schematic representation of the vitamin D synthesis (Holick and Chen, 2008).

Strategies of increase vitamin D intake from diet

In general, there are two ways to increase vitamin D intake from diet. An earlier study indicated it is feasible to enrich vitamin D in eggs by feeding a vitamin D supplement to poultry (11). In addition, a study (12) showed free-range farming is an efficient strategy to enrich vitamin D in eggs. However, a recent study showed that consumption of one egg per day is not associated with increased risk of CVD in the general population, but was associated with an increased risk of CVD in diabetic subjects (13). The other method is adding vitamin D into food as vitamin D fortified foods, which are available in a few countries, such as USA

and Canada (14). As milk is consumed by the vast majority of the population on a daily basis, milk is the predominant vehicle for vitamin D fortification in USA and Canada (14, 15). However, one recent publication with data from two large Swedish cohorts (16) reported that higher milk consumption was associated with a doubling of mortality risk including CVD mortality in the cohort of women. Since this paper was published in 2014, there has been mounting debate from different researchers regarding its seemingly contradictory results relative to other studies and meta-analyses (17, 18). Therefore, it is important to determine whether the chosen foods of milk or eggs have any long term detrimental effect on the populations' health before researching a strategy on enriched or fortified vitamin D in natural foods.

Aims and objective of the thesis

The overall objective of current thesis is to investigate the role of foods as dietary sources of vitamin D, particular eggs and dairy. The specific research question, hypothesis and objective pertinent to this thesis is summarised at the beginning of each chapter.

There are three sections in the current thesis:

Section 1: Introduction and Literature Review, including Study 1.

- Study 1. To critically review vitamin D intake from natural, enriched and fortified foods. Furthermore, to review the evidence from human intervention studies on the relative effects of 25(OH) D_3 and D_3 supplementation on vitamin D status.

Section 2: Role of eggs dietary sources of vitamin D, including Studies 2, 3 and 4.

- Study 2. To examine the effect of dietary vitamin D intake on CVD events and all-cause mortality in a prospective epidemiological study - evidence from the Caerphilly Cohort.

- Study 3. To examine the effect of egg consumption on CVD events and diabetes in an epidemiological study - evidence from the Caerphilly Cohort.
- Study 4. To examine the vitamin D content (vitamin D_3 and 25(OH) D_3) of retail eggs in the UK, and possible effect of production system (indoor vs outdoor), supermarket and purchase date.

Section 3: Role of dairy dietary sources of vitamin D, including studies 5, 6 and 7.

- Study 5. A comprehensive systematic review followed by a dose-response meta-analysis was conducted to examine linear and non-linear associations between milk and dairy products with CHD, CVD events and all-cause mortality using existing prospective cohort studies of adequate quality.
- Study 6. To investigate the effect of feeding cows different rates and forms of vitamin D on vitamin D forms and concentration in blood and milk.
- Study 7. To investigate the acute effect of a dairy drink fortified with either vitamin D_3 or 25(OH) D_3 on vitamin D status and predictors of CVD risk in humans.

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Chapter 2 - Literature review: Are 25(OH) D_3 enriched or fortified foods needed for increasing vitamin D status?

The present chapter aims to provide a review of the vitamin D intake from natural, enriched and fortified foods. Furthermore, to review the evidence from human intervention studies on the relative effects of 25(OH) D_3 and D_3 supplementation on vitamin D status.

JG conceived and wrote the manuscript, all authors critically reviewed and approved.

Are 25(OH) D_3 enriched or fortified foods needed for increasing vitamin D status? : A review

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Abbreviations: Cholecalciferol: vitamin D_3; Calcifediol: 25(OH) D_3; randomised controlled trial: RCT: Body mass index (BMI).

Abstract

Humans derive vitamin D from the diet or synthesise it using ultraviolet radiation on the skin. However, there are several limitations for humans to get sufficient vitamin D through sunlight. Thus, diet has become more important for contributing to vitamin D intake and status. Unfortunately, there are only a few types of foods (e.g. egg yolk, oily fish) naturally rich in vitamin D. Therefore, vitamin D enriched foods from supplementing the animals' diet with vitamin D or vitamin D fortification of foods have been studied as strategies to increase vitamin D intake. By reviewing vitamin D enrichment studies, it was clear that the cholecalciferol (vitamin D_3) and calcifediol (25(OH) D_3) contents of egg yolk, fish and milk did increase in response to vitamin D_3 supplementation of diets for hens, fish or cows. However, evidence from supplementation studies with laying hens showed 25(OH) D_3 supplementation to the diet only resulted in a pronounced increase of 25(OH) D_3 in the eggs. Therefore, the benefits of supplementing the animals' diet with vitamin D_3 or 25(OH) D_3 will depend on which form of vitamin D has more impact on raising human vitamin D status or health outcome. From the second part of the review of randomised controlled trials, it is apparent that a 25(OH) D_3 oral supplement can be absorbed faster and is also more efficient in raising serum 25(OH) D concentration compared with vitamin D_3 supplementation, although evidence showing the biological activity of 25(OH) D_3 varies between 3.13 to 7.14 times that of vitamin D_3 due to the different characteristics of the investigated subjects or study design. Furthermore, supplementation with 25(OH) D_3 may have more benefits on human health to the general population or clinical patients. Therefore, fortification by using 25(OH) D_3 would appear to have advantages over vitamin D_3. Further studies are needed to assess the effects of 25(OH) D_3 enriched or fortified foods in clinical trials to fill the research gaps.

Key words: Vitamin D deficiency, food enrichment, food fortification, vitamin D_3, 25(OH) D_3

Introduction

Vitamin D is usually synthesised in the skin when exposed to ultraviolet radiation, so it has been known as ‘sunshine vitamin’[1]. Traditionally, it has been thought that the primary role of vitamin D is related to calcium absorption and bone health. Children and adults with vitamin D deficiency have an increased risk of development of rickets or osteomalacia [2]. A resurgence of childhood rickets has recently highlighted the need for adequate vitamin D status in many parts of the world [3-5]. In addition, mounting evidence from epidemiology indicates that vitamin D status is inversely associated with risk of cardiovascular disease, cancers and diabetes [1, 6], although there is some uncertainty about what defines an adequate vitamin D status [7].

Several studies indicate that vitamin D deficiency is prevalent and is considered a serious issue throughout the world [8-10], even in sunnier climates such as Australia and New Zealand [11, 12]. There are several factors which have contributed to the low vitamin D status commonly seen today such as lifestyle changes (increased indoor lifestyle, sun screens use), latitude, human characteristics (e.g. skin pigmentation, ageing, clothing, low-fat diet trend) [13, 14]. Therefore, foods that contribute to vitamin D intake have become more important than before. However, there are only a few foods naturally enriched with vitamin D, such as oily fish and egg yolks [15].

In the first section of this review the possibility of the enrichment of vitamin D in foods of animal original through feeding supplements of cholecalciferol (vitamin D_3) and/or calcifediol (25(OH) D_3) to laying hens, fish and bovines is considered. The second section summaries information from human intervention studies which compare the relative effects of 25(OH) D_3 and vitamin D_3 in increasing serum 25(OH) D concentration.

Vitamin D absorption, synthesis, and metabolism

Generally the term vitamin D refers to vitamin D_2 and vitamin D_3. Vitamin D_2 is produced by fungi whilst vitamin D_3 is produced by humans or animals [16]. Humans usually synthesise vitamin D_3 in the skin [17] where 7-dehydrocholesterol in the epidermis is converted to previtamin D_3 when skin is exposed to sunlight. Then, previtamin D_3 undergoes a temperature-dependent isomerisation to vitamin D_3 over a period of about three days [6]. Whilst, vitamin D (vitamin D_2 or vitamin D_3) can also be obtained from the diet [17], as it is fat-soluble it is absorbed with long-chain triglycerides in the small intestine [18]. It is then incorporated into chylomicrons and transported in lymph to the blood and into the general circulation [19].

After entering the circulation, there were two hydroxylation reactions to convert vitamin D to the biologically active form [6]. The first hydroxylation reaction is in the liver where vitamin D is hydroxylated to 25(OH) D by the vitamin D-25-hydroxylase (25-OHase); The second hydroxylation reaction is in the kidney where 25(OH) D is converted to 1, $25(OH)_2$ D by the 25-hydroxyvitamin D-1α-hydroxylase (1-OHase) [6]. The 1, $25(OH)_2$ D metabolite is the biologically active form of vitamin D. The different side chain in the vitamin D_2 and vitamin D_3 molecules are maintained during the transformations, vitamin D_2 being converted to 25(OH) D_2 and then to $1,25(OH)_2$ D_2 whilst vitamin D_3 is converted to 25(OH) D_3 and then to 1,25(OH) D_3 [20].

Foods of animal origin as dietary sources of vitamin D

Within the few vitamin D rich foods the vitamin D content can differ considerably between food suppliers. One US retail study analysed the vitamin D content of eggs collected from 12 individual retail supermarkets across the country and reported a broad range of vitamin D_3 and 25(OH) D_3 concentrations 9.7-18 µg/kg and 4.3-13.2 µg/kg, respectively [21]. A recent UK study [22] showed vitamin D_3 and 25(OH) D_3 concentrations of eggs were significantly different depending on the egg production system. Egg yolks produced by birds kept in indoor

systems had much lower concentrations of vitamin D_3 than the egg yolks produced from outdoor systems, while 25(OH) D_3 concentrations of the eggs was only higher in organic eggs. Similarly, vitamin D contents of fish have been shown to vary due to the different production system. The study of Lu et al. [23] showed that vitamin D_3 content of wild salmon was three times higher than that of the farmed salmon. In addition, studies [24, 25] have shown 25(OH) D_3 content of several species of marine and freshwater fish to be less than 0.02 μg/100g. Therefore, foods generally regarded as rich sources of vitamin D may not be sustainable contributors of vitamin D intake to the general population, due to variability in vitamin D content which in turn may be influenced by production systems or different species.

Enrichment of animal origin foods as dietary sources of vitamin D

Vitamin D enriched eggs

In general there are two main ways to enrich the vitamin D content of eggs: increased sunlight exposure and vitamin D supplementation of the birds' diet. Because hens can synthesis vitamin D from natural sunlight, free-range egg production systems may be an inexpensive way to enrich vitamin D in their eggs. A study by Kuhn et al. [26] showed free-range to be an effective way to enrich vitamin D in eggs, (mean 14.3 μg/100 g dry matter) whereas the vitamin D content of the commercial free-range eggs had relatively low vitamin D contents (mean 3.8 μg/100 g dry matter).

Several studies [27-32] have shown that the vitamin D_3 content of the eggs can be enhanced by feeding vitamin D_3 supplements to the hens (Table 1). The results of all studies showed the egg yolk vitamin D_3 concentration was efficiently increased by vitamin D_3 supplementation of their diet. In addition, the study of Yao et al. [31] indicated a linear dose-response relationship existed between vitamin D_3 dietary supplementation and vitamin D_3 concentrations of the egg yolk. As 25(OH) D_3 is a metabolite of vitamin D_3, 25(OH) D_3

content in eggs can also be enhanced by supplementing the birds' diet with vitamin D_3 when vitamin D_3 supplement was fed to hens. However, the response in 25(OH) D_3 in the egg yolk is much less than that of vitamin D_3. Study of Browning et al. [32] indicated that a 2-fold increase in 25(OH) D_3 of egg yolk and a 4-fold increase in vitamin D_3 of egg yolk resulted from a 4-fold increase in the vitamin D_3 in the diet (2,500 to 10,000 IU/kg). Similarly, evidence from another study [27] showed that the increase of vitamin D_3 in egg yolk was about 7-fold as a result of feeding a diet with a vitamin D_3 content increased by 3.5 times (from 2496 to 8640 IU/kg), while the corresponding increase in 25(OH) D_3 content was only about 1.5-fold.

There are only a few studies examining the effect of feeding birds with diets supplemented with 25(OH) D_3. In the EU, 25(OH) D_3 has only recently been authorised for addition to poultry diets, and the maximum content of the vitamin D_3 and 25(OH) D_3 combination for laying hens is 80 µg/kg [33]. The study of Browning et al. [32] showed the addition of 25(OH) D_3 to the vitamin D_3 supplement resulted in elevation of the 25(OH) D_3 content of the egg yolk, but there was no significant increase in the vitamin D_3 content of the egg yolk. A further study investigated dietary supplementation with 25(OH) D_3 only [30], and showed that only 25(OH) D_3 in the egg yolk was increased but not vitamin D_3. These studies suggest that 25(OH) D_3 in the diet can be absorbed directly without transfer to vitamin D_3.

Vitamin D enriched fish

There are very few studies [34-37] on enriching the vitamin D content of fish (Table 2). Mattila et al. [36] has fed rainbow trout with different doses of vitamin D_3 supplements up to 21,560 IU/kg, but results showed no significant differences in the vitamin D_3 of the fish fillet. In contrast, the study of Horvli et al. [34] with Atlantic salmon showed a dose-response relationship between the vitamin D_3 in the fish meat and the vitamin D_3 in the diet up to 1,147,200 IU/kg. Similar high vitamin D_3 supplementation dose was reported in another two

studies [35, 37], which showed that the elevated vitamin D_3 content of the fish liver or whole fish had been achieved by the additional vitamin D_3 to the diet. However, the 25(OH) D_3 contents of the vitamin D_3 enriched fish were not measured in these studies, and no studies have examined the effects by feeding fish with 25(OH) D_3.

Vitamin D enriched milk

The summary of the studies investigating the vitamin D enrichment of milk by supplementing the bovine diet with vitamin D is presented in Table 3. An earlier study by Thompson et al. [38] provided large single doses of vitamin D_3 supplementation of 5×10^6 IU or 1×10^7 IU to bovine feed, and reported a corresponding increase in vitamin D_3 in the milk, which peaked between 3 and 7 days of supplementation. Furthermore, a few studies [39-42] have investigated the longer term effect of supplemental vitamin D_3 on the vitamin D content of the milk. The study of Hollis et al. [39] showed that a 10-fold enhancement of vitamin D_3 intake from 4,000 to 40,000 IU/d resulted in a 7.5-fold increased vitamin D_3 concentration of the milk and a 2-fold increase in 25(OH) D_3. The study of McDermott et al. [41] compared three different doses of vitamin D_3 with a control diet, and showed an increased level of vitamin D_3 and 25(OH) D_3 in the milk. However, the relationship of increasing extent of supplementation doses and vitamin D_3 or 25(OH) D_3 concentrations of the milk was not linear. Furthermore, the study of Weiss et al. [42] has investigated the effect of feeding 18,000 IU/day vitamin D_3 to pre-calving cows for 13 days, the vitamin D_3 and 25(OH) D_3 of the milk were ranged from 13.0-18.0 IU/L and 14.3-40.8 IU/L, respectively. In addition, the study has also included treatment of 240,000 IU and dietary cation-an-ion difference of -138 mEq/kg per day for 13 days, concentrations of 25(OH) D_3 in the milk was increased but the treatment effect disappeared after 28 days.

Evidence from human dietary intervention studies with vitamin D enriched animal-derived foods

Despite numerous animal-based vitamin D enrichment studies on eggs, fish and milk, there are few randomised controlled trials (RCTs) on the effect of consuming vitamin D enriched foods on the vitamin D status of the consumer. To our knowledge, only one recent study [43] has investigated the effect of vitamin D enriched eggs on vitamin D status compared with commercial eggs. Weekly consumption of seven vitamin D_3 enriched eggs or 25(OH) D_3 enriched eggs was compared with commercial eggs consumption ≤2 eggs/wk for 8-weeks during winter. The results showed that compared with subjects who consumed commercial eggs whose serum 25(OH) D decreased from baseline of 41.2 ± 14.1 nmol/L to 34.8 ± 11.4 nmol/L after 8-week intervention, serum 25(OH) D of the subjects who consumed vitamin D_3 enriched eggs or 25(OH) D_3 enriched egg were maintained at their starting values, the serum 25(OH) D of post-intervention of subjects who consumed vitamin D_3 or 25(OH) D_3 enriched eggs were 50.4 (SD=21.4) and 49.2 (SD=16.5) nmol/L, respectively. In addition, a study by Hayes et al. [38], showed that vitamin D_3 enriched eggs and 25(OH) D_3 enriched eggs did not significantly change serum 25(OH) D concentration, maybe because vitamin D intake from both treatments was low and eggs in both treatments had similar vitamin D concentrations (vitamin D_3 3.54±1.04 μg/egg; 25(OH) D_3 4.54±1.38 μg/egg). Although there are limited human dietary intervention studies on vitamin D enriched foods, the study of Mattila et al. [30] demonstrated that the effect of foods enriched with either vitamin D_3 or 25(OH) D_3 on human vitamin D status depends on their relative effectiveness in raising serum 25(OH) D concentration. However, previous study [44] indicated that there is no consensus on the relative biological activity of 25(OH) D_3 compared with vitamin D_3 in raising human serum or plasma 25(OH) D_3 concentrations. Furthermore, UK food composition tables [45] indicated there is no certainty on the relative potency of 25(OH) D_3 compared to vitamin D_3, although 5 was used currently for calculating the total vitamin D of foods. Therefore, we summarized the

evidence from randomized controlled studies (RCTs) which reported the changing of serum 25(OH) D concentrations by giving 25(OH) D_3 supplement to examine the effect of 25(OH) D_3 on raising serum 25(OH) D concentrations. In addition, we calculated the relative effectiveness of oral 25(OH) D_3 and vitamin D_3 if the two treatments were included within the same study.

Human intervention studies on the relative effects of 25(OH) D_3 and D_3 supplementation on vitamin D status.

Heterogeneity of intervention studies

Eleven RCTs which investigated the effects of 25(OH) D_3 treatment were identified [46-56] (Table 4). Nine studies administrated 25(OH) D_3 supplementation only, except 2 studies which provided a combination supplement of 25(OH) D_3 and calcium [46, 49]. Five of the 11 studies [47, 49-52] supplemented 25(OH) D_3 to generally healthy subjects whereas the other 6 studies supplemented 25(OH) D_3 to clinical patients. Most studies reported the serum 25(OH) D concentration at both beginning and end of the treatment, except one study [55] which only reported the serum 25(OH) D concentration at the end of treatment. In terms of characteristics of the investigated subjects, five studies included both men and women [46, 48, 51, 53, 55], while the other studies only included men or women. In addition, most studies reported the age and body mass index (BMI) of the subjects, except two studies [46, 48] that did not report the BMI range.

Acute pharmacokinetic action of vitamin D_3 and 25(OH) D_3

An early study [57] gave meals with single doses of 25(OH) D_3 of 1.5, 5 or 10 μg/kg body-weight to generally healthy subjects and showed that the peak serum 25(OH) D_3 concentration was reached within 4-8 hours after ingestion. A later study by Jetter et al. [52] compared the

pharmacokinetic absorption of vitamin D_3 and 25(OH) D_3 by providing a single dose of 20 µg vitamin D_3 and 25(OH) D_3 to postmenopausal women. The time to reach maximum plasma 25(OH) D_3 concentration was 22 and 11 hours for vitamin D_3 and 25(OH) D_3, respectively. In addition, the peak concentration of plasma 25(OH) D_3 (43.9 nmol/L) of 25(OH) D_3 supplementation was numerically higher than vitamin D_3 supplementation (34.7 nmol/L), although there it was not significantly different. This study further compared the effect of a higher single dose (140 µg) of vitamin D_3 and 25(OH) D_3 with the time to reach peak plasma 25(OH) D_3 being 21 and 4.8 hours for vitamin D_3 and 25(OH) D_3 supplementation, respectively. In addition, the maximum plasma concentration of 25(OH) D_3 for 25(OH) D_3 treatment (44 nmol/L) was numerically higher than for vitamin D_3 treatment (35 nmol/L) but not significantly different. The results suggest that 25(OH) D_3 was absorbed more quickly than D_3 possibly because 25(OH) D_3 has higher solubility in aqueous media than vitamin D_3 due to its more polar chemical structure [58]. Furthermore, as this metabolite of vitamin D_3 is produced in the liver, the hepatic metabolism of vitamin D_3 to 25(OH) D_3 is circumvented and consequently the conversion from vitamin D_3 to 25(OH) D_3 would be negligible [59]. Patients with liver disease have an impaired ability to synthesis 25(OH) D_3 from vitamin D_3 [60]. The study of Sitrin et al. [61] verified that 25(OH) D_3 could be absorbed more efficiently than vitamin D_3 after oral supplementation in patients with chronic cholestatic liver disease. Therefore, supplementation with 25(OH) D_3 is not only more efficient at increasing vitamin D status in generally healthy people, but may also have a specific role in tackling lower vitamin D status in patients who are suffering from liver diseases.

Chronic effects and relative effectiveness of vitamin D_3 and 25(OH) D_3 treatments

Regarding the expected higher biological effect of 25(OH) D_3 in raising serum 25(OH) D level after a longer term administration, several studies have confirmed that oral consumption of 25(OH) D_3 is highly effective in raising serum 25(OH) D level (Table 3). However, the

majority of the evidence in support of a higher impact of 25(OH) D_3 supplementation compared with vitamin D_3 on serum 25(OH) D_3 level is available from only four studies [51, 52, 54, 56] where both 25(OH) D_3 and vitamin D_3 treatments were included in the same study. Although an earlier study of Barger-Lux et al. [47] has applied three different doses of vitamin D_3 (25, 250, 1250 µg) or 25(OH) D_3 (10, 20, 50 µg) in their daily trial to the subjects for 8 weeks and 4 weeks, respectively. However, the effects of 25(OH) D_3 and vitamin D_3 treatments are not directly comparable as the interventions were not at the same dose or treatment time. Thus, the study of Barger-Lux was excluded from the relative effectiveness analysis. In order to compare the relative effectiveness of 25(OH) D_3 and vitamin D_3 supplementation on raising serum 25(OH) D concentrations, we calculated a comparison factor for each µg of orally consumed 25(OH) D_3 or vitamin D_3 in 4 studies (Table 5). The comparison factor of 25(OH) D_3 and vitamin D_3 were calculated by using endpoint serum 25(OH) D minus baseline serum 25(OH) D, and then divided the dose of the supplementation. Then, calculation of the relative effectiveness of 25(OH) D_3 to vitamin D_3 by using comparison factor of vitamin D_3 divide by the comparison factor of 25(OH) D_3.

The highest relative effectiveness was found in the study by Catalano et al. [54]. Weekly treatment of 140 µg 25(OH) D_3 or 140 µg vitamin D_3 supplements were provided to osteopenic and dyslipidaemic postmenopausal women for 24 weeks. Supplementation of 25(OH) D_3 raised serum 25(OH) D from a baseline of 55.7 nmol/L to 125.6 nmol/L, while vitamin D_3 treatment increased serum 25(OH) D much less from baseline 50.8 nmol/L to 60.7 nmol/L. Thus, the conversion factor derived from this study is 7.14.

Vitamin D dietary recommendations are generally between 10 to 20 µg/day [10], thus, there are a few studies which have compared the effectiveness of 25(OH) D_3 and vitamin D_3 using doses of 20 µg in their treatments. Cashman et al. [51] provided daily supplements of 20 µg vitamin D_3 or 20 µg 25(OH) D_3 to adult men and women with a mean age of 57 years and with baseline serum 25(OH) D of 28.9 nmol/L during winter. After 10 weeks of

supplementation, the subjects' serum 25(OH) D rose to 134.6 nmol/L and 69.0 nmol/L for the 25(OH) D_3 and vitamin D_3 treatments, respectively. A conversion factor of 4.99 represented the relative effectiveness of each μg of 25(OH) D_3 relative to vitamin D_3 for raising serum 25(OH) D concentration. However, lower relative conversion factors were achieved in other studies using the same dose of 20 μg vitamin D_3 and 25(OH) D_3. Bischoff-Ferrari et al. [62] supplemented healthy postmenopausal women with 20 μg 25(OH) D_3 or vitamin D_3 for 16 weeks during the winter. They found that for the 25(OH) D_3 treatment, serum 25(OH) D increased to 173.4 nmol/L from a baseline of 30.7 nmol/L, whereas for the vitamin D_3 treatment serum 25(OH) D increased to 77.4 nmol/L from a baseline level of 35.4 nmol/L. The conversion factor of each μg of 25(OH) D_3 had 3.40 times compared with vitamin D_3 in raising serum 25(OH) D level. A similar low conversion factor was found in another study [56]. Post-menopausal osteoporotic women were given either 20 μg vitamin D_3 or 20 μg 25(OH) D_3 over 6 months or 12 months. The serum concentration of 25(OH) D for the 25(OH) D_3 treatment reached 161.0 nmol/L and 188.0 nmol/L from a baseline of 37.2 nmol/L after 6 months or 12 months administration respectively, while the comparable values for the vitamin D_3 treatment were an increase to 80.0 nmol/L and 86.2 nmol/L from a baseline of 40.5 nmol/L. So the conversion factor of 25(OH) D_3 relative to vitamin D_3 treatment at 6 months and 12 months were 3.13 or 3.29, respectively.

In summary, of the studies reviewed, the relative effectiveness of 25(OH) D_3 relative to vitamin D_3 for raising vitamin D status (Table 5), ranged from 3.13 to 7.14. Previous studies [13, 14] have demonstrated that the season may have influences on vitamin D status. There were two studies [47, 51] were conducted during the winter which may have minimised any confounding influence of cutaneous vitamin D synthesis from ultraviolet radiation. Other studies have longer intervention periods of six months or more, which could not have avoided cutaneous synthesis. Furthermore, baseline status may be another factor that influences the relative conversion factor. The study of Catalano et al. [54] had the highest conversion factor

of 7.14 in the current review, and the baseline concentration of 25(OH) D of the study participants was higher (>50 nmol/L) than the others. Therefore, the different relative effectiveness seen in different studies may be due to the different characteristics of the subjects or different study designs.

Overall, it seems that 25(OH) D_3 can more effectively increase serum 25(OH) D concentrations than vitamin D_3 and may also be absorbed faster reaching a serum 25(OH) D plateau earlier than vitamin D_3 supplementation. Furthermore, supplementation with 25(OH) D_3 may also have more benefits on human health compared with vitamin D_3. Bischoff-Ferrrari et al. [62] reported that 20 μg 25(OH) D_3 supplementation over four months led to a 5.7 mmHg decrease in systolic blood pressure and also improvements in several markers of innate immunity.

For patients with different diseases and receiving long term medication, three studies [63-65] showed that several drugs interfere with vitamin D and bone metabolism, which resulted in patients being more likely to have low vitamin D status. Thus, it is not only important to increase vitamin D status in the generally healthy population but also in patients with specific illnesses and receiving certain drugs. Therefore, the studies using 25(OH) D_3 treatments in patients were also summarised in Table 3 [46, 48, 53-56]. It was consistently reported that chronic 25(OH) D_3 supplementation effectively increased serum 25(OH) D concentrations. Ortego-Jurado et al. [55] showed a lower daily dose of 8.85 μg 25(OH) D_3 to be more effective than a 20 μg dose of vitamin D_3 for increasing vitamin D status in patients with autoimmune disease who were treated with a low dose of glucocorticoids throughout the year. Similarly, the study of Banon et al. [53] showed that a monthly dose of 400 μg 25(OH) D_3 was safe and effective at improving vitamin D status of HIV-infected patients throughout the year.

Furthermore, supplementation with 25(OH) D_3 may have additional benefits on patients' health. Previously, 25(OH) D_3 was recommended for patients with kidney disease since

25(OH) D_3 has a direct action on bone metabolism [66]. Hahn et al. [46] provided a daily 40 µg 25(OH) D_3 and 500 mg calcium supplement to patients who had glucocorticoid-induced osteopenia for 18 months. The treatment markedly increased vitamin D status from 39.2 nmol/L to 204.9 nmol/L. In addition, this study showed 25(OH) D_3 treatment can improve mineral and bone metabolism. Also, Jean et al. [48] offered haemodialysis patients who suffered with vitamin D deficiency a daily dose of 16 µg 25(OH) D_3 for 6 months; vitamin D status reached 126 nmol/L from 30 nmol/L, at the same time 25(OH) D_3 supplementation corrected the excess bone turnover. Similarly a study by Catalano et al. [54] not only found that weekly 25(OH) D_3 had a significantly higher effect on vitamin D status than vitamin D_3 of the same dose with additional benefits that 25(OH) D_3 improved plasma lipid levels (increase HDl-cholesterol (P=0.02) and decrease LDL-Cholesterol (P=0.02)) when added to an ongoing atorvastatin treatment.

General discussion

As an alternative to vitamin D enriched foods, vitamin D fortification of foods may also be an option for tackling vitamin D deficiency throughout the world. In general, fortification of foods refers to mandatory and voluntary fortification. The contribution of vitamin D fortified foods to vitamin D intake by the public varies considerably between countries as there are different food standard policies [10]. In practice, vitamin D_2 or vitamin D_3 is used for the fortification. Vitamin D_2 is produced from some fungi whilst vitamin D_3 is produced in the skin by animals and humans via ultraviolet radiation [67]. Previous meta-analysis of RCTs [68] showed that vitamin D_3 supplementation is more effective in raising vitamin D status than vitamin D_2. However, a further comprehensive systematic review and meta-analysis of 33 RCTs [69] showed the effect of vitamin D_3 supplement on serum 25(OH) D_3 response depends on supplemental dose, duration, age of subjects and baseline level, which has further indicated a higher serum 25(OH) D increasing was found when intervention study with a dose

20 μg/d or more, subjects whose age >80 years, administration period is at least 6 to 12 months or subjects had lower baseline 25(OH) D level. Therefore, better strategies are needed on raising vitamin D status in the public through the years.

Conclusion

There is no doubt that vitamin D insufficiency has become a world problem, especially in winter. There are a few natural foods rich in vitamin D. Thus, vitamin D enriched foods produced through a food chain approach such as feeding animals vitamin D supplements or vitamin D fortified foods are needed to guarantee an adequate dietary intake of vitamin D by the general population.

The present review summarised the limited number of RCTs investigating the effect of 25(OH) D_3 supplementation on serum 25 (OH) D concentration. We conclude that it is difficult to get consensus of the biological conversion factor of 25(OH) D_3 supplementation relative to vitamin D_3 for raising vitamin D status, due to different influencing factors such as different person characteristics (age, BMI), baseline vitamin D status and time of the year. However, it is unquestionable that 25(OH) D_3 supplementation is more efficient in raising serum 25(OH) D level and also appears to be absorbed faster by the human body than the same dose of vitamin D_3 supplementation. Secondly, by reviewing available evidence on vitamin D enriched eggs, fish or milk, it is a practical possibility to increase the vitamin D content of eggs, fish or milk by addition of vitamin D supplements into diet of poultry, fish or dairy cattle. However, there are a few RCTs investigating the impact of these vitamin D enriched foods on improving vitamin D status. Therefore, 25(OH) D_3 enriched or fortified foods should be explored more in the future, and additional RCTs should be conducted to investigate the effect of 25(OH) D_3 enriched or fortified foods on vitamin D status of the general population and patients with long-term health conditions.

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Table 1. Summary of enrichment studies investigating the impact of vitamin D supplemental poultry feeding on vitamin D content of egg yolk.

	Vitamin D supplement (IU/kg)			Vitamin D concentration of egg yolk (μg/100g)	
References	Vitamin D_3	$25(OH)D_3$	Feeding duration (weeks)	Vitamin D_3	$25(OH)D_3$
(Mattila et al, 1999) [27]	1,064	-	6	1.4	0.5
	2,496	-	6	3.5	0.9
	8,640	-	6	22.0	1.5
(Matila et al, 2003) [28]	11,200	-	4	30.0	1.9
(Mattila et al, 2004) [29]	2,500	-	4	3.8	-
	6,000	-	4	13.6	-
	15,000	-	4	33.7	-
(Browning et al, 2014)	2,500	-	9	6.5	1.6
[32]	5,000	-	9	10.5	2.1
	10,000	-	9	26.2	3.0
(Yao et al, 2013)	2,200	-	3	3.0	-
[31]	9,700	-	3	21.6	-
	17,200	-	3	41.0	-
	24,700	-	3	60.3	-
	102,200	-	3	870.4	-
(Browning et al, 2014)	2500	0	9	6.5	1.6
[32]	2500	1380	9	6.0	3.3
	2500	2760	9	4.9	4.5
	5000	0	9	10.5	2.1
	5000	1380	9	7.4	4.5
	5000	2760	9	8.1	5.8
	10,000	0	9	26.2	3.0
	10,000	1380	9	23.6	3.7
	10,000	2760	9	30.9	8.1
(Mattila et al, 2011)	-	2200	6	≤ 0.2	2.1
[30]	-	4880	6	≤ 0.2	4.3

Table 2. Summary of enrichment studies investigating the impact of vitamin D supplemental fish's feeding on vitamin D content of fish.

	Supplements to feeding		
References	Vitamin D_3 supplement (IU/kg)	Feeding duration (weeks)	Vitamin D_3 of fish (µg/100g)
(Horvli et al, 1998)	1600	11	1 (fillet)
[34]	88400	11	21 (fillet)
	1147200	11	210 (fillet)
(Vielma et al, 1998)	2500	12	1.3 (liver)
[35]	250000	12	73 (liver)
	2500000	12	6900 (liver)
(Mattila et al, 1999)	3560	16	5.7-15.4 (fish fillet)
[36]	6960	16	6.1-9.9 (fish fillet)
	21560	16	7.1-15.6 (fish fillet)
(Graff et al, 2002)	8000	9	≤25 (whole fish)[1]
[37]	200000	9	80 (whole fish)[1]
	2280000	9	650 (whole fish)[1]

[1]Estimated from graph

Table 3. Summary of enrichment studies investigating the impact of vitamin D supplements to bovine's feeding on vitamin D content of milk.

References	Supplements to feeding (IU/day)			Vitamin D concentration of milk (IU/L)		
	Vitamin D_3	$25(OH)D_3$	Feeding duration	Vitamin D_3	$25(OH)D_3$	$1,25(OH)_2D_3$
(Hollis et al, 1981)[1]	4000		NA	1.72	14.88	0.22
[39]	40000		NA	12.88	27.40	0.17
(Reeve et al, 1982)[1] [40]	15000		30 days	11.2	5.8	0.2
(Mcdermott et al, 1985)	0		14 weeks[2]	3	10	4
[41]	10,000		14 weeks	8	17	1
	50,000		14 weeks	6	30	5
	250,000		14 weeks	13	37	4
(Weiss et al, 2015)	18,000	-	13 days before calving	13.0-18.8	14.3-40.8	-
[42]	-	DCAD[3]+240,000	13 days before calving	-	24.3-147.6	-

[1]Feeding duration of pre-calving or post-calving are unknown.
[2] Including two weeks before calving and 12 weeks after calving.
[3] DCAD: dietary cation-anion difference of -138 mEq/kg.

Table 4. Summary of study details and serum 25(OH) D concentration in randomized controlled trials with 25(OH) D_3 supplementation in adults (order by year).

References	Subjects characteristics (trail time during the year, subjects (gender), age, BMI)	25(OH)D_3 supplementation group					Control group (if available)				
		Duration	25(OH)D_3 treatment	n	Baseline 25(OH)D (nmol/L)	Endpoint 25(OH)D (nmol/L)	Duration	Vitamin D treatment	n	Baseline 25(OH)D (nmol/L)	Endpoint 25(OH)D (nmol/L)
(Hahn et al, 1979) [46]	Whole year, patients (women and men) with glucocorticoid-induced osteopenia 46 years, BMI (NA)	18 months	40 µg 25(OH)D_3 /d + 500 mg calcium/d	9	39.2 (3.24)	204.9 (12.7)					
(Barger et al, 1998) [47]	January-April, men 28 years, 25.7 kg/m^2	4 weeks	10 µg 25(OH)D_3/d	7	67 (25)	107 (4.4)	8 weeks	25 µg vitaminD_3/d	13	67 (25)	95.6 (5.3)
		4 weeks	20 µg 25(OH)D_3/d	6	67 (25)	143.1 (6.2)	8 weeks	250 µg vitaminD_3/d	10	67 (25)	213.1 (12.0)
		4 weeks	50 µg 25(OH)D_3/d	4	67 (25)	273.4 (17.3)	8 weeks	1250 µg vitaminD_3/d	14	67 (25)	710 (42.7)
(Jean et al, 2008) [48]	March-September, haemodialysis patients (women and men) 67 years, BMI (NA)	6 months	16 ± 5 µg 25(OH)D_3/d	149	30 (19)	126 (48)					
(Cavalli et al, 2009) [49]	April to July, postmenopausal women 65-75 years, 24.5 kg/m^2	12 weeks	125 µg 25(OH)D_3/week + 500 mg calcicum/d	25	50.2 (12.0)	75.5 (13.9)					
		12 weeks	250 µg 25(OH)D_3/month + 500 mg calcium /d	28	50.9 (17.7)	69.5 (19.4)					
		12 weeks	500 µg 25(OH)D_3/month +500 mg calcium/d	27	52.4 (7.5)	77.4 (8.2)					
(Russo et al, 2011) [50]	January- April, women (7 premenopausal and 11 postmenopausal), 24-72 years, 23.5 kg/m^2	16 weeks	500 µg 25(OH)D_3/month	18	45.2 (31.2)	104.8 (no SD)1					
(Cashman et al, 2012) [51]	January-April, women and men, 57 years, 28.9 kg/m^2	10 weeks	20 µg 25(OH)D_3/d	12	38.2 (9.9)	134.6 (26.0)	10 weeks	20 µg vitaminD_3/d	13	49.7 (16.2)	69.0 (8.7)
			7 µg 25(OH)D_3/d	25	42.5 (8.9)	70.7 (9.9)					
(Jetter et al,2014)[2] [52]	January-July, postmenopausal women 50-70 years, 18-29 kg/m^2	16 weeks	20 µg 25(OH)D_3/d	5	30.65 (10.18)	173.40 (3.94)	16 weeks	20 µg vitaminD_3/d	5	35.39 (9.01)	77.35 (3.97)
		single dose	140 µg 25(OH)D_3	5	33.92 (14.68)	74.88 (no SD)	single dose	140 µg vitaminD_3	5	21.44 (2.45)	34.32 (no SD)
(Catalano et al, 2015) [54]	September-March, osteopenic and dyslipidemic postmenopausal women 59 years, 27.1 kg/m^2	24 weeks	140 µg 25(OH)D_3 once a week	29	55.7 (16.4)	125.6 (38.6)	24 weeks	140 µg vitaminD_3 once a week	28	50.8 (13.6)	60.7 (19.5)

References	Subjects characteristics (trail time during the year, subjects (gender), age, BMI)	$25(OH)D_3$ supplementation group					Control group (if available)				
		Duration	$25(OH)D_3$ treatment	n	Baseline 25(OH)D (nmol/L)	Endpoint 25(OH)D (nmol/L)	Duration	Vitamin D_3 treatment	n	Baseline 25(OH)D (nmol/L)	Endpoint 25(OH)D (nmol/L)
(Banon et al, 2015) [53]	Whole year, patients (women and men) had HIV-infected, 44 years, 14.8-43.9 kg/m^2	Summer	400 µg $25(OH)D_3$ once / month	123	37.19	85.6	Summer	No vitamin D supplementation	242	52.67	98.6
		Fall	400 µg $25(OH)D_3$ once / month	123	37.19	69.4	Fall	No vitamin D supplementation	242	52.67	84.1
		Winter	400 µg $25(OH)D_3$ once/ month	123	37.19	45.4	Winter	No vitamin D supplementation	242	52.67	54.9
		Spring	400 µg $25(OH)D_3$ once/ month	123	37.19	56.9	Spring	No vitamin D supplementation	242	52.67	78.4
(Otego-Jurado et al, 2015) [55]	Whole year, patients (women and men)had autoimmune diseases, undergoing glucocorticoids therapy, 56 years, 27.9 kg/m^2	Spring-summer	8.85 µg $25(OH)D_3$/d	49	NA	84.1	Spring-summer	20 µg vitaminD_3/d	86	NA	71.4
		Fall-winter	8.85 µg $25(OH)D_3$/d	49	NA	88.9	Fall-winter	20 µg vitaminD_3/d	86	NA	61.4
(Navarro-Valverde et al, 2016) [56]	Whole year, postmenopausal osteoporotic women, 67 years, 26.4 kg/m^2	6 months	20 µg $25(OH)D_3$ /day	10	37.2 (4.2)	161.0 (21.7)	6 months	20 µg vitaminD_3/day	10	40.5 (4.7)	80.0 (2)
		12 months	20 µg $25(OH)D_3$ /day	10	37.2 (4.2)	188.0 (24.0)	12 months	20 µg vitaminD_3/day	10	40.5 (4.7)	86.2 (23.7)
		6 months	266 µg $25(OH)D_3$ once/week	10	38.0 (3.7)	213.5 (80.0)					
		12 months	266 µg $25(OH)D_3$ once/ week	10	38.0 (3.7)	233.0 (81.2)					
		6 months	266 µg $25(OH)D_3$ once /2 weeks	10	39.5 (4)	164.5 (41.7)					
		12 months	266 µg $25(OH)D_3$ once /2 weeks	10	39.5 (4)	210.5 (22.2)					

NA: not available

[1]Estimated from graph

[2] Same study of (Jetter et al, 2014) and (Bischoff-Ferrari et al, 2012)

Table 5. Summary of randomized controlled trials with both 25(OH) D_3 and vitamin D_3 in adults to calculate the relative effectiveness of 25(OH)D_3 and vitamin D_3 supplementation in raising serum 25(OH)D level.

References	Treatment (dose, duration)	serum 25(OH)D raising (nmol/L) per 1 µg [1]	Relative effectiveness [2]
(Cashman et al, 2012) [51]	20 µg 25(OH)D_3/d × 10 weeks	4.82 [a]	4.99
	20 µg vitamin D_3/day × 10 weeks	0.97 [b]	
(Jetter et al,2014) [52]	20 µg 25(OH) D_3/d × 15 weeks	7.12 [a]	3.40
	20 µg vitamin D_3/d × 15 weeks	2.51 [b]	
(Catalano et al, 2015) [54]	140 µg 25(OH) D_3/week × 24 weeks	0.50 [a]	7.14
	140 µg vitamin D_3/week × 24 weeks	0.07 [b]	
(Navarro-Valverde et al, 2016) [56]	20 µg 25(OH)D_3 /d × 6 months	6.19 [a]	3.13
	20 µg vitamin D_3/d × 6 months	1.98 [b]	
	20 µg 25(OH)D_3/d × 12 months	7.54 [a]	3.29
	20 µg vitamin D_3/d × 12 months	2.29 [b]	

[1] Serum 25(OH) raising (nmol/L) per microgram supplementation = (endpoint serum 25(OH) D - baseline serum 25(OH) D)/dose)
[2] Relative effectiveness=a/b within same study

Chapter 3 - Vitamin D intake and risk of cardiovascular diseases and all-cause mortality: evidence from the Caerphilly Prospective Cohort Study.

The present chapter aims to examine the effect of dietary vitamin D intake on CVD events and all-cause mortality in epidemiological study- evidence from Caerphilly Cohort.

JG, JAL, DIG designed the study; JG conducted the research and wrote the manuscript. JRC, PCE and JEP contributed to the interpretation of the findings.

Vitamin D intake and risk of cardiovascular diseases and all-cause mortality: evidence from the Caerphilly Prospective Study

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[2] Address correspondences to Jing Guo, E-mail: jing.guo@pgr.reading.ac.uk.

[3] Supported by the Barham Benevolent Foundation.

[4]Abbreviations used: CVD, cardiovascular disease; MI, myocardial infarction; systolic blood pressure: SBP; diastolic blood pressure (DBP); CAPS, Caerphilly prospective cohort study.

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Abstract

Background: Prospective data on the associations between vitamin D dietary intake and risk of cardiovascular disease (CVD) and all-cause mortality are limited and inconclusive.

Objectives: To comprehensively investigate the associations between vitamin D dietary intake and CVD risk and all-cause mortality.

Methods: Vitamin D dietary intake was assessed in 452 healthy men who were free from CVD and type 2 diabetes at baseline (1979-1983 years) in the Caerphilly Prospective Cohort Study (CAPS). The associations of vitamin D dietary intake and CVD risk markers were examined cross-sectionally at baseline and longitudinally at the 5 year, 10 year and over 20 year follow-up examinations. Also, the predictive value of vitamin D dietary intake for cardiovascular events and all-cause mortality at 20 years was examined.

Results: After 20 years follow-up, 72 stroke cases, 142 myocardial infarctions (MI), 43 heart failures and 281 cases of all-cause mortality were identified. There was no significant association between vitamin D dietary intake and stroke, MI, heart failure or all-cause mortality. However, higher vitamin D dietary intake was associated with a decreased concentration of plasma triacylglycerol at baseline and 5-years examination. In addition, a modest positive association was found between vitamin D dietary intake and diastolic blood pressure (DBP) after 20-years follow-up.

Conclusions: The results of the current study suggest that a higher vitamin D dietary intake is associated with a lower plasma triacylglycerol level and a higher DBP. Further research is needed to confirm these findings.

Key words: vitamin D, cardiovascular disease, all-cause mortality, Caerphilly Prospective Study, blood pressure, triacylglycerol

Introduction

Cardiovascular disease (CVD) is one of the main causes of morbidity and mortality in the world. There is mounting evidence indicating an association between vitamin D deficiency and CVD (1-4). Recently, the UK Scientific Advisory Committee on Nutrition (SACN) (5) reported that in the UK 22-24% of individuals of 19-64 years, and 17-24% of those ≥65 years and above were vitamin D deficient (plasma 25(OH) D_3 <25 nmol/L). Humans obtain vitamin D generally from synthesis in the skin due to sunlight ultraviolet radiation and/or foods. However, a number of relatively recent lifestyle changes (e.g. increased working indoors, sunscreen use), personal characteristics (ageing, skin pigmentation) and geographic reasons (latitude), limit the ability to synthesise adequate vitamin D from sunlight (6). As a result vitamin D intake from foods has become more important than previously. This has led SACN to recommend a daily intake of 10 μg/day of vitamin D in all adults within the UK (5).

The association between vitamin D deficiency and increased risk of CVD (1-4) and all-cause mortality (7-9) has been investigated, but few prospective cohort studies have analysed the relationship between dietary vitamin D intake and CVD risk and all-cause mortality. In a 10-year cohort of 361 men and 394 women (10), dietary vitamin D intake was shown to have a protective association for stroke, but not myocardial infraction (MI). Another women's cohort study (11) reported no association between vitamin D dietary intake and all-cause mortality. As the evidence on the association of vitamin D dietary intake and CVD risk or all-cause mortality from prospective cohort studies is limited, we therefore investigated the associations between dietary vitamin D intake and CVD events, CVD risk markers and all-causer mortality using the Caerphilly Prospective Cohort Study (CAPS) which has over 20 years of follow-up.

Methods

Study population

CAPS was initially set up in 1979-1983 to investigate the CVD risk factors based on 2512 men (45-59 years), representing 89% of the subjects living in Caerphilly and the adjacent area (12)**,** which were followed up at 5-year intervals. At Phase 2 (1984-1988), 561 men were lost from Phase 1 (1979-1983) and an additional 447 men were recruited to follow-up. In Phase 1, a representative 30% subsample of subjects (665 men) was randomly selected at baseline to complete a 7-day weighed dietary intake record (13). Food items were coded according to McCance and Widdowson (14). These men were given weighing scales with instructions on how to complete the weighed dietary intake for seven consecutive days. From these records the dietary vitamin D intake was estimated based on food composition data given by McCance and Widdowson (14)**,** additionally, several manufacturers were contacted to obtain more information on new foodstuffs containing mixtures of ingredients (13,15). In order to ensure consistency of the subject group throughout the study, the 134 men from the weighed intake sub-group who dropped out after Phase 1 were excluded from this analysis. In addition, 17 subjects who previously had a heart attack and subjects (n=62) with missing confounding factor data were excluded. Therefore, a total of 452 subjects were available for the current analysis.

Cardiovascular disease events and all-cause mortality

Identification of stroke and vascular disease events was described elsewhere (16, 17). In brief, incidents of all-cause mortality were censored by central Registry NHS in the UK. Identification of fatal and non-fatal vascular disease events (ICD 121-5, 10^{th} revision) including MI, heart failure and stroke (IC 163-4) were according to established criteria (16, 17). Fasting blood samples were taken at baseline and every five years to measure a wide range of CVD risk markers (12). At baseline (Phase 1), at 5-years (Phase 2) and at 10-years

(Phase 3) examinations, plasma glucose, total-cholesterol and triacylglycerol were measured together with systolic blood pressure (SBP) and diastolic blood pressure (DBP). Insulin and HDL-cholesterol were only measured in Phase 1 and 2. In Phase 5, the haemodynamic variables of SBP, DBP, aortic pulse wave velocity (aPWV), augmentation index (AIx) and mean arterial pressure were measured, the details are described elsewhere (18,19). Pulse pressure was calculated by subtracting DBP from SBP. The Friedewald formula (20) was used to calculate LDL-cholesterol.

Statistics

Data were analysed using STATA (version 13.0; STATA Corporation, 2014). The subjects were divided into four groups according to dietary vitamin D intake. For the analysis of baseline, 5-years and 10-years examinations, logistic regression and general linear regression statistic models were used to investigate the relationships of vitamin D intake with categorical and continuous variables of CVD risk markers, respectively. In addition, logistic regression analyses were used to estimate the odds ratio of stroke, MI, heart failure and all-cause mortality for the longitudinal analysis. The multivariate-adjusted model for all of the analyses first included confounding factors of age (years); body mass index (BMI, kg/m^2); social class (manual worker; non-manual worker), smoking (current smoker, never-smoked, ex-smoker), leisure activity (with heavy work or exercise in leisure time, without heavy work or exercise in leisure time), alcohol (as ethanol, ml/week) and food energy intake (MJ/day). In addition, as vitamin D is closely functionally related with calcium, the second multivariate-adjusted model was further adjusted with calcium dietary intake. Results were considered statistically significant at P=0.05 or less.

Results

The characteristics of the 452 subjects at baseline are shown in **Table 1**. The average vitamin D intake was 21.0 (SD=19.3) μg per week. Subjects in the lowest quartiles of vitamin D intake were significantly more likely to be smokers (P=0.001) and had higher food energy intake (P<0.001). After controlling for total energy intake from foods, those with the highest vitamin D intake per week tended to have a higher intake of fat (P=0.002), cereal fibre (P<0.001), vegetable fibre (P<0.001), and calcium (P<0.001). There were no associations between dietary vitamin D intake and age, BMI, social class, leisure activity, alcohol consumption, protein intake and carbohydrate intake.

Associations of vitamin D intake with different CVD risk markers were investigated at baseline as a cross-sectional analysis (Phase 1). There was a significant positive association between vitamin D intake and HDL-cholesterol (adjusted model 2 P=0.002; adjusted model 3 P=0.003) (**Table 2**), with subjects consuming highest vitamin D intake (≥27.3 μg/week) having 0.13 mmol/L higher HDL-cholesterol levels compared with subjects consuming the lowest vitamin D intake (0.1-9.9 μg/week). In addition, negative associations were observed between vitamin D intake and total/HDL-cholesterol ratio (adjusted model 2 P=0.005; adjusted model 3 P=0.008) and triacylglycerol concentrations (adjusted model 2 P=0.003; adjusted model 3 P=0.013) with the subjects consuming ≥ 27.3 μg/week vitamin D having 0.7 mmol/L lower total/HDL-cholesterol ratio and 0.5 mmol/L lower plasma triacylglycerol compared with subjects consuming the lowest vitamin D intake (0.1-9.9 μg/week). In addition, a positive association was found between vitamin D intake and pulse pressure (adjusted model 2 P=0.026; adjusted model 3 P=0.040).

In the longitudinal analyses of vitamin D intake and CVD risk markers at the 5-year examination (Phase 2), higher vitamin D intake was significantly negatively associated with plasma triacylglycerol concentrations (adjusted model 2 P=0.003; adjusted model 3 P=0.010) (**Table 3**). The highest vitamin D intake group had 0.48 mmol/L lower plasma triacylglycerol

than the lowest vitamin D intake group. There were no significant associations between vitamin D intake and other CVD risk markers at the 5-year examination. In the longitudinal analyses at the 10-year examination (Phase 3), only a modest negative association (P=0.056) was found between higher vitamin D intake and plasma triacylglycerol in the un-adjusted model but not in the multivariable adjusted models (**Table 4**).

After over 20-years follow-up (Phase 5), a tendency for a lower pulse pressure was seen in those with the highest vitamin D intake, but this did not reach significance (**Table 5**). In the analysis of the associations of SBP and DBP with vitamin D intake, DBP showed a positive correlations with vitamin D intake in the multivariate adjusted models (P=0.041 model 2, P=0.029 model 3), but no significant associations were found between vitamin D intake and SBP.

There were no significant associations between dietary vitamin D intake and other CVD risk markers, i.e. fasting glucose/insulin (**Supplemental material**), mean arterial pressure, pulse wave velocity and augmentation index (Table 5). Also there were no significant associations of vitamin D dietary intake and cardiovascular events (stroke, MI, heart failure) or all-cause mortality after over the 20-years follow-up (**Table 6**).

Discussion

In this UK prospective cohort study of middle aged men with over 20 years follow-up, we found higher dietary vitamin D intake was associated with lower plasma triacylglycerol concentrations at baseline and the 5-year examination, but not 10-year examinations. In addition, baseline cross-sectional analysis indicated significant positive associations between vitamin D intake and HDL-cholesterol and pulse pressure, with a negative association between vitamin D intake and total/HDL-cholesterol ratio. After over 20 years follow up, a modest positive association was found between vitamin D dietary intake and DBP. In

contrast, no associations were found between vitamin D and other metabolite markers or disease outcomes of stroke, MI, heart failure and all-cause mortality.

To our knowledge, this is the first prospective study to investigate the associations between dietary vitamin D intake and blood lipid profiles in a generally healthy population using both cross-sectional and longitudinal analyses. The negative association between dietary vitamin D intake and plasma triacylglycerol at baseline and 5-years examination agrees with the results of a 6-month randomized controlled trial (21) in post-menopausal women with type 2 diabetes, which showed that a daily 100 μg dose of vitamin D_3 significantly decreased the concentration of serum triacylglycerol (by 1.9 mmol/L, P=0.021). However, it is not clear why the negative association between vitamin D dietary intake and plasma triacylglycerol was found at the 5-year, but not the 10-year of the longitudinal examination. One possible reason may due to dietary change during the follow-up. One study (22) showed that there has been a trend towards a lower fat diet in UK since the 1980s. As vitamin D is fat soluble vitamin (5), it is likely that vitamin D intake has also declined and indeed the current study showed dietary vitamin D intake to be positively associated with fat intake. So the lack of association between dietary vitamin D intake and 10-year triacylglycerol examination may due to dietary vitamin D intake having declined during the follow up. In addition, our study is the first to show a significant positive cross-sectional association between vitamin D intake and pulse pressure, but no association was seen in the longitudinal analysis. In the analysis of the association between vitamin D and SBP or DPB, the only significant finding was a positive association between vitamin D intake and DBP after the 20-year follow-up and which needs confirmation in further studies.

There are very few studies that have reported associations between dietary vitamin D intake and CVD risk or all-cause mortality. Our null finding of dietary vitamin D agree with an earlier prospective study of the Iowa Women's Healthy Study (WHS) in 1999 (11), which

also found no association between dietary vitamin D intake and ischaemic heart disease mortality over an 8-year follow-up period. However, vitamin D dietary intake (4.30±3.3 μg/day) was reported in another 10 year follow-up prospective study of 361 men and 394 women (10) and suggested a protective role of dietary vitamin D intake on stroke but not MI. The different conclusions of the above studies may be due to the different characteristics of the study participants. For example, the mean initial age of the subjects in the CAPS (mean age of 51.7 years) and WHS (mean age of 53.8 years) were similar, but higher (age range of 65-99 years) in the investigation of Marniemi et al. (10). Furthermore, our study agrees with a systematic review of 56 randomised controlled trails, which did not find a significant association between vitamin D supplementation and total mortality risk (23).

The recent report of the UK Scientific Advisory Committee on Nutrition (5) recommended a daily Reference Nutrient Intake (RNI) of 10 μg vitamin D for the general population aged 4 years and above, including pregnant and lactating women. In CAPS, only 11 out of 452 subjects achieved the current RNI dose. Therefore, the effect of dietary vitamin D may have been minimised by the low dietary vitamin D intake. However, a few recent studies have used higher doses of vitamin D in their intervention trials (24-26), which also showed no associations of vitamin D supplementation with markers of CVD risk.

The strength of the CAPS is the long (over 20 years) follow-up period. This novel study presents both cross-sectional and longitudinal relationships between dietary vitamin D intake and CVD events. The longitudinal analysis was conducted at 5, 10 and over 20 years which provide the opportunity to test the consistency of the influence of vitamin D intake on CVD events. There are however several limitations of this study. First, vitamin D dietary intake was only assessed at baseline, and was not repeated in the other phases to assess the extent of any diet change. Second, the results apply to men only, which may not represent the effect in women. Finally, unknown residual confounding factors may have influenced the outcomes

seen. In particular, the vitamin D status of the subjects was not measured initially and during the follow-up of CAPS and there were no assessments of sunshine exposure. In addition, because the small cohort size of the current study may not be representative all of UK men, further prospective studies with large subject numbers can provide more evidence on effect of the vitamin D dietary intake on CVD risk and/or all-cause mortality.

Conclusion

The current investigation from CAPS prospective cohort study provides further evidence for the potential benefits of vitamin D intake on circulating triacylglycerol concentrations at baseline and also the 5-years examination. After over 20 years follow-up, higher vitamin D dietary intake is associated with a higher DBP. Future studies are needed to verify the current findings, especially randomised controlled intervention trials on the effect of dietary vitamin D intake on CVD risk markers in subjects of low vitamin D status.

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Table 1. Baseline characteristics (n=452) of participants by category of vitamin D intake[1].

	Vitamin D intake from foods (μg/week)				
Characteristics	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend[2]
Subjects, n	114	112	114	112	
Age, y	52±4.5	52±4.1	51±4.4	52±4.6	0.738
BMI, kg/m^2	26.3±3.1	25.9±3.1	26.3±3.6	26.1±3.1	0.781
Leisure activity, %	43.9	52.7	50.0	57.1	0.077
Manual workers, %	68.4	49.1	63.2	70.5	0.327
Current smokers, %	64.9	58.0	50.9	44.6	0.001
Energy intake, MJ/d	6.46±1.74	7.25±1.60	7.34±1.55	7.55±1.51	<0.001
Fat, % of food energy	36.0±6.2	36.5±6.0	38.1±4.8	37.9±5.1	0.002
Protein, % of food energy*	13.9±2.7	13.5±2.3	13.8±2.4	14.0±2.4	0.247
Carbohydrate, % of food energy*	45.7±8.2	46.1±7.5	46.6±5.8	45.0±6.6	0.604
Alcohol intake, ml ethanol/wk[&]	37.0±62.7	32.2±38.7	21.7±27.7	29.0±35.0	0.114
Fibre (vegetable sources), g/d[&]	8.5±0.3	8.4±0.2	8.4±0.3	8.6±0.3	<0.001
Fibre (cereal sources), g/d[&]	7.3±1.4	7.9±1.1	8.1±1.2	9.1±1.2	<0.001
Calcium intake, mg/week[&]	5567±268	6343±200	6335±225	6613±224	<0.001

* Original data were transformed to natural logarithms for regression model.

[&] Data were adjusted with energy intake from foods.

[1] All values are mean ± SD.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables).

Table 2. Cross-sectional analysis between baseline (Phase 1) vitamin D intake and markers of CVD risk[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Total cholesterol					
Participants, n	114	112	114	112	
Mean, mmol/L	5.98±1.11	5.99±1.56	5.93±1.08	5.81±0.94	
Unadjusted Coef.	ref	0.001	-0.053	-0.174	0.254
Multivariable-adjusted Coef.[2]	ref	0.012	-0.025	-0.109	0.487
Multivariable-adjusted Coef.[3]	ref	0.029	-0.007	-0.083	0.588
HDL-cholesterol					
Participants, n	112	110	113	111	
Mean, mmol/L	1.29±0.37	1.32±0.34	1.40±0.40	1.42±0.41	
Unadjusted Coef.	ref	0.025	0.104	0.122	0.006
Multivariable-adjusted Coef.[2]	ref	0.023	0.129	0.134	0.002
Multivariable-adjusted Coef.[3]	ref	0.020	0.126	0.131	0.003
Total/HDL-cholesterol*					
Participants, n	112	110	113	111	
Mean, mmol/L	5.13±2.40	4.83±1.92	4.53±1.35	4.43±1.42	
Unadjusted Coef.	ref	-0.045	-0.094	-0.120	0.004
Multivariable-adjusted Coef.[2]	ref	-0.039	-0.102	-0.112	0.005
Multivariable-adjusted Coef.[3]	ref	-0.035	-0.098	-0.107	0.008
LDL-cholesterol					
Participants, n	112	108	103	109	
Mean, mmol/L	4.30±1.12	4.28±1.51	4.21±1.08	4.09±0.98	
Unadjusted Coef.	ref	-0.017	-0.087	-0.202	0.182
Multivariable-adjusted Coef.[2]	ref	-0.018	-0.089	-0.159	0.298
Multivariable-adjusted Coef.[3]	ref	-0.007	-0.077	-0.142	0.352
Triacylglycerol*					

Participants, n	112	108	113	110	
Mean, mmol/L	2.06±1.20	1.81±1.21	1.62±0.91	1.56±0.98	
Unadjusted Coef.	ref	-0.130	-0.206	-0.262	<0.001
Multivariable-adjusted Coef.[1]	ref	-0.098	-0.163	-0.214	0.003
Multivariable-adjusted Coef.[2]	ref	-0.075	-0.138	-0.180	0.013
Systolic blood pressure					
Participants, n	114	112	114	112	
Mean, mmHg	140.5±20.9	139.8±18.1	141.6±20.1	140.6±17.0	
Unadjusted Coef.	ref	-0.678	1.114	0.045	0.810
Multivariable-adjusted Coef.[1]	ref	0.934	2.994	1.123	0.491
Multivariable-adjusted Coef.[2]	ref	1.066	3.136	1.321	0.454
Diastolic blood pressure					
Participants, n	114	112	114	112	
Mean, mmHg	89.4±12.0	90.1±11.7	88.7±12.4	87.4±11.9	
Unadjusted Coef.	ref	0.615	-0.754	-2.064	0.137
Multivariable-adjusted Coef.[1]	ref	1.237	-0.471	-1.935	0.146
Multivariable-adjusted Coef.[2]	ref	1.567	-0.113	-1.438	0.247
Pulse Pressure					
Participants, n	114	112	114	112	
Mean, mmHg	51.1±14.5	49.8±13.6	53.0±13.2	53.2±13.8	
Unadjusted Coef.	ref	-1.293	1.868	2.109	0.104
Multivariable-adjusted Coef.[1]	ref	-0.303	3.465	3.058	0.026
Multivariable-adjusted Coef.[2]	ref	-0.501	3.250	2.760	0.040

* Original data were transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Table 3. Longitudinal analysis between baseline (Phase 1) vitamin D intake and markers of CVD risk after 5 years of follow-up[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Total cholesterol					
Participants, n	109	110	111	109	
Mean, mmol/L	5.67±0.96	5.62±1.08	5.68±0.97	5.60±0.84	
Unadjusted Coef.	ref	-0.045	0.018	-0.068	0.733
Multivariable-adjusted Coef.[2]	ref	-0.018	0.042	-0.025	0.976
Multivariable-adjusted Coef.[3]	ref	0.027	0.086	0.038	0.687
HDL-cholesterol					
Participants, n	109	110	111	109	
Mean, mmol/L	0.98±0.26	1.03±0.23	1.06±0.23	1.00±0.26	
Unadjusted Coef.	ref	0.046	0.078	0.018	0.419
Multivariable-adjusted Coef.[2]	ref	0.034	0.080	0.010	0.464
Multivariable-adjusted Coef.[3]	ref	0.034	0.080	0.011	0.466
Total/HDL-cholesterol*					
Participants, n	109	110	111	109	
Mean, mmol/L	6.23±2.24	5.77±1.75	5.66±1.75	5.98±1.73	
Unadjusted Coef.	ref	-0.070	-0.084	-0.030	0.415
Multivariable-adjusted Coef.[2]	ref	-0.051	-0.081	-0.015	0.560
Multivariable-adjusted Coef.[3]	ref	-0.044	-0.073	-0.004	0.751
LDL-cholesterol					
Participants, n	109	110	111	109	
Mean, mmol/L	4.23±0.90	4.17±0.99	4.28±0.96	4.24±0.81	
Unadjusted Coef.	ref	-0.062	0.047	0.010	0.722
Multivariable-adjusted Coef.[2]	ref	-0.051	-0.081	-0.015	0.560
Multivariable-adjusted Coef.[3]	ref	-0.044	-0.073	-0.004	0.751

Triacylglycerol*					
Participants, n	109	110	111	109	
Mean, mmol/L	2.27±1.66	2.13±1.41	1.74±0.77	1.79±1.05	
Unadjusted Coef.	ref	-0.078	-0.194	-0.197	0.001
Multivariable-adjusted Coef.[2]	ref	-0.049	-0.172	-0.173	0.003
Multivariable-adjusted Coef.[3]	ref	-0.032	-0.154	-0.148	0.010
Systolic blood pressure					
Participants, n	113	109	113	111	
Mean, mmHg	148.9±26.8	146.8±0.3	144.9±22.6	145.5±21.2	
Unadjusted Coef.	ref	-2.032	-3.973	-3.417	0.206
Multivariable-adjusted Coef.[2]	ref	-0.233	-2.185	-2.083	0.403
Multivariable-adjusted Coef.[3]	ref	-0.201	-2.150	-2.034	0.419
Diastolic blood pressure					
Participants, n	113	109	113	111	
Mean, mmHg	86.2±12.3	84.3±10.7	84.1±10.9	83.0±11.9	
Unadjusted Coef.	ref	-1.873	-2.133	-3.194	0.042
Multivariable-adjusted Coef.[2]	ref	-1.475	-1.865	-2.793	0.080
Multivariable-adjusted Coef.[3]	ref	-1.340	-1.718	-2.587	0.112
Pulse Pressure					
Participants, n	113	109	113	111	
Mean, mmHg	62.7±20.0	62.5±16.1	60.8±18.2	62.4±16.0	
Unadjusted Coef.	ref	-0.159	-1.840	-0.222	0.750
Multivariable-adjusted Coef.[2]	ref	1.242	-0.320	0.711	0.938
Multivariable-adjusted Coef.[3]	ref	1.139	-0.432	0.553	0.995

* Original data were transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Table 4. Longitudinal analysis between baseline (Phase 1) vitamin D intake and markers of CVD risk after 10 years of follow-up[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Triacylglycerol *					
Participants, n	85	98	99	99	
Mean, mmol/L	2.13±1.42	2.08±1.52	1.80±0.90	1.78±0.90	
Unadjusted Coef.	ref	-0.048	-0.125	-0.132	0.056
Multivariable-adjusted Coef.[2]	ref	-0.018	-0.102	-0.101	0.112
Multivariable-adjusted Coef.[3]	ref	-0.004	-0.086	-0.077	0.197
Systolic blood pressure					
Participants, n	88	104	98	101	
Mean, mmHg	145.2±23.7	143.5±21.3	145.4±22.1	144.9±22.2	
Unadjusted Coef.	ref	-1.720	0.216	-0.271	0.890
Multivariable-adjusted Coef.[2]	ref	-0.371	1.907	1.368	0.527
Multivariable-adjusted Coef.[3]	ref	0.142	2.416	2.191	0.389
Diastolic blood pressure					
Participants, n	88	104	98	101	
Mean, mmHg	82.2±12.2	80.6±10.6	83.0±11.6	81.1±11.9	
Unadjusted Coef.	ref	-1.613	0.809	-1.032	0.922
Multivariable-adjusted Coef.[2]	ref	-1.281	0.899	-0.262	0.770
Multivariable-adjusted Coef.[3]	ref	0.877	1.300	0.385	0.518
Pulse Pressure					
Participants, n	88	104	98	101	
Mean, mmHg	63.0±18.2	62.9±17.5	62.4±16.3	63.8±17.1	
Unadjusted Coef.	ref	-0.108	-0.593	0.761	0.808
Multivariable-adjusted Coef.[2]	ref	0.910	1.008	1.630	0.529
Multivariable-adjusted Coef.[3]	ref	1.019	1.117	1.805	0.495

*original data were transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Table 5. Longitudinal analysis between baseline (Phase 1) vitamin D intake and markers of CVD risk after over 20 years of follow-up[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Mean Arterial Pressure,					
Participants, n	43	43	47	41	
Mean, mmHg	96.35±10.19	96.07±14.07	99.63±13.09	95.64±13.70	
Unadjusted Coef.	ref	-0.284	3.279	-0.702	0.824
Multivariable-adjusted Coef.[2]	ref	0.326	3.919	1.000	0.417
Multivariable-adjusted Coef.[3]	ref	0.985	4.556	1.937	0.275
Pulse wave velocity					
Participants, n	43	45	47	39	
Mean, m/s	11.89±2.61	11.40±2.82	11.77±2.71	11.48±2.78	
Unadjusted Coef.	ref	-0.495	-0.123	-0.411	0.663
Multivariable-adjusted Coef.[2]	ref	-0.413	0.052	-0.098	0.889
Multivariable-adjusted Coef.[3]	ref	-0.339	0.124	0.009	0.756
Augmentation index					
Participants, n	43	46	47	41	
Mean	27.35±8.49	25.30±10.68	27.04±8.81	24.70±9.26	
Unadjusted Coef.	ref	-2.044	-0.306	-2.654	0.355
Multivariable-adjusted Coef.[2]	ref	-2.147	-0.370	-4.051	0.097
Multivariable-adjusted Coef.[3]	ref	-1.389	0.363	-2.971	0.267
Systolic blood pressure					
Participants, n	43	46	47	41	
Mean, mmHg	143.3±16.2	141.9±18.6	143.7±20.9	139.8±20.3	
Unadjusted Coef.	ref	-1.368	0.379	-3.522	0.519
Multivariable-adjusted Coef.[2]	ref	-0.055	1.225	-1.464	0.864
Multivariable-adjusted Coef.[3]	ref	0.971	2.218	-0.003	0.894
Diastolic blood pressure					

Participants, n	43	46	47	41	
Mean, mmHg	72.3±9.2	72.2±11.5	76.1±10.4	74.3±12.0	
Unadjusted Coef.	ref	-0.128	3.847	1.966	0.170
Multivariable-adjusted Coef.[2]	ref	0.141	4.378	3.574	0.041
Multivariable-adjusted Coef.[3]	ref	0.456	4.683	4.023	0.029
Pulse pressure*					
Participants, n	43	46	47	41	
Mean, mmHg	71.0±15.8	69.8±14.4	67.5±17.1	65.5±17.9	
Unadjusted Coef.	ref	-0.013	-0.055	-0.087	0.064
Multivariable-adjusted Coef.[2]	ref	0.004	-0.047	-0.080	0.075
Multivariable-adjusted Coef.[3]	ref	0.014	-0.037	-0.066	0.130

* Original data were transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake, heart rate, vaso-active medication.

[3] Additionally adjusted for calcium intake.

Table 6. Longitudinal analysis between baseline (Phase 1) vitamin D intake and cardiovascular disease risk after over 20 years of follow-up[1].

Longitudinal relationship between baseline (phase 1) vitamin D intake and stroke					
Vitamin D intake (μg/week)	No. of men	No. of event	OR* (non-adjust)	OR (adjusted Model 1)[2]	OR (adjusted Model 2)[3]
0.1-9.9	114	14	1	1	1
10-15.1	112	21	1.65 (0.79-3.43)	1.77 (0.84-3.76)	1.67 (0.79-3.57)
15.2-27.2	114	19	1.43 (0.68-3.01)	1.58 (074-3.40)	1.48 (0.68-3.20)
≥27.3	112	18	1.37 (0.64-2.90)	1.54 (0.71-3.37)	1.41 (0.64-3.13)
P for trend			0.541	0.361	0.508
Longitudinal relationship between baseline (phase 1) vitamin D intake and myocardial infarction					
Vitamin D intake (μg/week)	No. of men	No. of event	OR (non-adjust)	OR (adjusted Model 1)[2]	OR (adjusted Model 2)[3]
0.1-9.9	114	36	1	1	1
10-15.1	112	37	1.07 (0.61-1.87)	1.13 (0.63-2.02)	1.11 (0.62-2.00)
15.2-27.2	114	35	0.96 (0.55-1.68)	1.09 (0.61-1.95)	1.07 (0.59-1.93)
≥27.3	112	34	0.94 (0054-1.66)	1.14 (0.63-2.07)	1.12 (0.61-2.05)
P for trend			0.760	0.707	0.764
Longitudinal relationship between baseline (phase 1) vitamin D intake and heart failure					
Vitamin D intake (μg/week)	No. of men	No. of event	OR (non-adjust)	OR (adjusted Model 1)[2]	OR (adjusted Model 2)[3]
0.1-9.9	114	10	1	1	1
10-15.1	112	13	1.37 (0.57-3.26)	1.87 (0.74-4.72)	1.66 (0.65-4.24)
15.2-27.2	114	10	1.00 (0.40-2.50)	1.19 (0.45-3.14)	1.02 (0.38-2.73)
≥27.3	112	10	1.02 (0.41-2.55)	1.18 (0.44-3.15)	0.95 (0.35-2.59)
P for trend			0.851	0.989	0.661
Longitudinal relationship between baseline (phase 1) vitamin D intake and all-cause mortality					
Vitamin D intake (μg/week)	No. of men	No. of event	OR (non-adjust)	OR (adjusted Model 1)[2]	OR (adjusted Model 2)[3]
0.1-9.9	114	76	1	1	1
10-15.1	112	69	0.80 (0.47-1.38)	0.84 (0.46-1.52)	0.89 (0.49-1.64)
15.2-27.2	114	65	0.66 (0.39-1.14)	0.77 (0.43-1.39)	0.82 (0.46-1.49)
≥27.3	112	71	0.87 (0.50-1.50)	0.98 (0.53-1.81)	1.09 (0.59-2.03)
P for trend			0.474	0.890	0.858

* Odds ratio (OR) derived from logistic regression.

[1] All values are mean ± SD.

[2]Model 1: multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Supplemental Table 1. Cross-sectional analysis between baseline (Phase 1) vitamin D intake and plasma glucose and insulin[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Fasting glucose					
Participants, n	113	113	113	113	
Mean, mmol/L	4.90±1.02	5.07±1.81	4.86±0.66	4.93±1.17	
Unadjusted Coef.	ref	0.171	-0.038	0.024	0.794
Multivariable-adjusted Coef.[2]	ref	0.174	-0.022	0.068	0.989
Multivariable-adjusted Coef.[3]	ref	0.176	-0.019	0.071	0.989
Insulin*					
Participants, n	92	104	101	104	
Mean, mmol/L	1.08±2.24	1.09±3.10	1.07±4.32	0.76±0.88	
Unadjusted Coef.	ref	0.037	-0.128	-0.086	0.233
Multivariable-adjusted Coef.[2]	ref	0.043	-0.124	-0.042	0.406
Multivariable-adjusted Coef.[3]	ref	0.056	-0.109	-0.020	0.520

*original data is transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Supplemental Table 2. Longitudinal analysis between baseline (Phase 1) vitamin D intake and plasma glucose and insulin after 5 years of follow-up[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Fasting glucose					
Participants, n	109	110	111	109	
Mean, mmol/L	5.39±1.09	5.63±2.03	5.14±0.64	5.35±1.28	
Unadjusted Coef.	ref	0.243	-0.247	-0.036	0.302
Multivariable-adjusted Coef.[2]	ref	0.307	-0.231	-0.018	0.317
Multivariable-adjusted Coef.[3]	ref	0.322	-0.216	0.004	0.362
Insulin*					
Participants, n	49	43	52	47	
Mean, mmol/L	2.85±2.12	3.70±1.76	3.42±2.99	3.54±2.35	
Unadjusted Coef.	ref	0.501	0.313	0.361	0.050
Multivariable-adjusted Coef.[2]	ref	0.481	0.280	0.358	0.055
Multivariable-adjusted Coef.[3]	ref	0.463	0.225	0.314	0.126

*original data is transformed to natural logarithms for regression model.

[1] All values are mean ± SD.

[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.

[3] Additionally adjusted for calcium intake.

Supplemental Table 3. Longitudinal analysis between baseline (Phase 1) vitamin D intake and plasma glucose after 10 years of follow-up[1].

	Vitamin D intake from foods (µg/week)				
	0.1-9.9	10-15.1	15.2-27.2	≥27.3	*P* for trend
Fasting glucose					
Participants, n	86	96	99	99	
Mean, mmol/L	5.49±1.22	5.73±1.82	5.74±1.98	5.86±1.95	
Unadjusted Coef.	ref	0.238	0.245	0.372	0.182
Multivariable-adjusted Coef.[2]	ref	0.238	0.241	0.443	0.118
Multivariable-adjusted Coef.[3]	ref	0.246	0.250	0.458	0.112

[1] All values are mean ± SD; Insulin was not available at 10 years examination.
[2] Multivariable-adjusted model adjusted for age, BMI, social class (manual and non-manual workers), alcohol intake (non-drinker, drinker has been divided into 3 equal groups), smokers (non-smoker, current smoker, previous smoker), leisure activity (yes and no), food energy intake.
[3] Additionally adjusted for calcium intake.

Chapter 4 - Egg consumption and cardiovascular disease events, diabetes and all-cause mortality: evidence from Caerphilly Prospective Cohort Study (CAPS) and National Diet and Nutritional Survey (NDNS) (Abstract has been published at the 'Nutrition Society's 2015 summer meeting (Appendix-1)).

The present chapter aims to examine the effect of egg consumption on cardiovascular disease (CVD) events and diabetes in epidemiological study- evidence from Caerphilly Cohort.

JAL and DIG designed the research; JG and DAH conducted the research; JG and DAH analysed the data with guidance from JEP; JG, DAH, DIG and JAL wrote the paper; PCE and JRC contributed expertise on epidemiology and CVD respectively. DIG and JAL had primary responsibility for the final content of the manuscript. All authors read and approved the final manuscript. All authors critically reviewed and approved. Abstract has been published at the 'Nutrition Society's 2015 summer meeting (Appendices-1). Full paper of the revision version has been submitted to journal.

Association between egg consumption and cardiovascular disease events, diabetes and all-cause mortality

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[3] Supported by the Barham Benevolent Foundation.

[4] Abbreviations used: CVD: cardiovascular disease; MI: myocardial infarction; T2D: type 2 diabetes; IGT: impaired glucose tolerance; CAPS: Caerphilly prospective cohort study; NDNS: national diet and nutrition survey; FFQ: food-frequency questionnaire; RCT: randomized controlled trials; SBP: Systolic blood pressure; DBP: diastolic blood pressure; TGs: triacylglycerol; CRP: C-reactive protein; HbA1c: glycated haemoglobin; ICD: International Classification of Diseases; HR: hazard ratio.

[5] Running title: Egg consumption and diabetes and risk of CVD

[6] Author names for indexing: Guo, Hobbs, Cockcroft, Elwood, Pickering, Lovegrove and Givens

[7] Word count: 5564

[8] Conflict of Interest: the authors declare that they have no conflict of interest.

Abstract

Purpose The association between egg consumption and cardiovascular disease (CVD) or type 2 diabetes (T2D) remains controversial. We investigated the association between egg consumption and risk of CVD, T2D and mortality in the Caerphilly prospective cohort study (CAPS) and National Diet and Nutritional Survey (NDNS).

Methods CAPS included 2512 men aged 45 to 59 years (1979-1983). Dietary intake, disease incidence and mortality were updated at 5-year intervals. NDNS included 754 adults aged 19-64 years from 2008-2012.

Results Men free of CVD (*n*=1781) were followed up for a mean of 22.8 years, new incidence of stroke (*n*=248), MI (*n*=477), heart failure (*n*=201), mortality (*n*=1028) and type 2 diabetes (T2D) (*n*=120) was identified. Egg consumption was not associated with incident of MI, heart failure, mortality or T2D. In contrast, increased risk of stroke in subjects with T2D and/or impaired glucose tolerance (IGT, fasting plasma glucose ≥6.1 mmol/L), adjusted hazard ratios (95% CI) were 1.0 (reference), 1.09 (0.41, 2.88), 0.96 (0.37, 2.50), 1.39 (0.54, 3.56) and 2.87 (1.13, 7.27) for egg intake (n) of $0 \leq n \leq 1$, $1 < n \leq 2$, $2 < n \leq 3$, $3 < n < 5$, and $n \geq 5$ eggs/wk, respectively (*P* for trend=0.01). In addition, cross-sectional analyses of CAPS and NDNS revealed higher egg consumption was significantly associated with elevated fasting glucose in those with T2D and/or IGT (baseline *P*=0.02; 5-year-later examination *P*=0.04; NDNS *P*=0.01).

Conclusions Higher egg consumption was associated with higher blood glucose in men with T2D and/or IGT. The markedly increased incidence of stroke with higher egg consumption among T2D and/or IGT sub-group warrants further investigation.

Key words eggs, cardiovascular disease, type 2 diabetes, impaired glucose tolerance, all-cause mortality

Introduction

Cardiovascular diseases (CVD) are still the leading cause of morbidity and mortality, and the prevalence of type 2 diabetes mellitus (T2D) is also increasing globally. Diet plays an important role in prevention and management of both CVD [1] and T2D [2]. Eggs are a good source of a number of nutrients in the UK diet such as vitamin D, selenium, vitamin K and choline as well as high quality protein [3]. However, eggs also contain relatively large amounts of dietary cholesterol (350 mg/100g) [4], which has been associated with impaired glucose metabolism [5] and increased inflammation [6] in animal models and with elevated fasting glucose in humans [7]. Meta-analyses of intervention studies have shown that increased consumption of dietary cholesterol increases serum total, LDL and HDL cholesterol concentrations, as well as the ratio of total to HDL cholesterol [8, 9], although Gray and Griffin [10] conclude that these changes are small and are not clinically significant. However, findings from randomized controlled trials (RCT) investigating the effects of high egg consumption on blood lipid have not been consistent. For example, a recent randomized controlled trial of 140 overweight or obese subjects with prediabetes or T2D showed that high egg consumption (2 eggs/d for 6 d/wk) did not have an adverse effect on lipid profile of those with T2D [11]. Furthermore, Fuller et al [11] reported no effect of egg intake and glycemic control in this 3 month RCT in T2D, whereas an inverse association between egg intake and fasting plasma glucose was reported in a prospective cohort study [12].

Evidence from previous meta-analyses in relation to egg consumption and CVD mortality showed inconsistent results. Some studies [13, 14] have shown that consumption of up to one egg per day is not associated with increased risk of CVD in the general population, which is in contrast to a recent meta-analysis which reported up to one egg per day was associated with reduced risk of stroke [15]. Furthermore, inconsistent associations between egg intake and CVD in diabetic patients where observed. Shin et al [14] concluded egg consumption up to one egg per day was associated with an increased risk of CVD in diabetic patients, whereas

Rong et al (13) found egg intake up to one egg per day was associated with a reduced risk of hemorrhagic stroke in diabetic patients.

Therefore, our hypothesis was that a higher egg intake is unlikely to increase the risk of CVD events (myocardial infarction (MI), stroke, heart failure), T2D or all-cause mortality in the general population, but may have detrimental effects in those suffering from T2D. This hypothesis was tested by using evidence from the Caerphilly Prospective Cohort Study (CAPS) and years 1-4 of the UK National Diet and Nutrition Survey (NDNS).

Subjects and methods

Study population of Caerphilly Prospective Cohort Study

The Caerphilly Prospective Cohort Study (CAPS) was set up between 1979 and 1983 and was designed to investigate CVD risk factors, there was follow-up of the men and re-examined at 5-year intervals (Figure 1). Initially, 2512 men aged 45-59 years old living in Caerphilly, Wales, United Kingdom and the adjacent area were recruited onto the study [16]. However, 561 men were lost through attrition after Phase 1 (1979-1983) and an additional group of 447 men were recruited for replacement, giving a new total of 2398 men at Phase 2 (1984-1988). At 5 years later, a total of 2147 men revisited the clinical centre for the Phase 3 examination (1989-1993). The current study did not include data from Phase 1, as there were an inconsistent number of subjects between Phase 1 and Phase 2.

Dietary assessment

Diet was assessed at both Phase 2 and Phase 3 with the use of a semi-quantitative food-frequency questionnaire (FFQ). Participants were asked to report the number of eggs consumed on a weekly basis, with one unit of consumption equivalent to one egg. This FFQ was previously validated using a 7-day weighed diet diary in a representative sub-group of

665 men, representing 30 % of Phase 1 participants [17]. In order to present the best estimation of egg consumption, the mean egg intake at Phase 2 and Phase 3 was used for the longitudinal analysis, which also allowed estimation of the effect of the cumulative long-term diet. Subjects with pre-existing stroke (n=60), MI (n=98), and those with missing data or confounding factors (n=208) were excluded from for the longitudinal CVD analysis, which left a total of 1781 men. Subjects with pre-existing stroke, MI were also excluded for cross-sectional analyses at Phase 2 or Phase 3.

The mean egg intakes at Phase 2 and Phase 3 were calculated for 1781 subjects who reported egg consumption in both phases. As egg consumption was not a continuous variable, it was divided into categorical variables for the analysis.

Cardiovascular events, diabetes and all-cause mortality

The incident of T2D was self-reported from questionnaires in the Caerphilly cohort. Identification of vascular disease events and deaths by cause has been described elsewhere [18-20]. In brief, subjects were seen in Clinics centre, symptoms and illnesses suggestive of a stroke or heart attack were confirmed by the use of the London School of Hygiene chest pain questionnaire and the Oxford Stroke Questionnaire, subjects also had an electrocardiogram measurement during the visit. Appropriate searches of hospital and general practitioner databases were made to extract relevant clinical information. Vascular events (International Classification of Diseases (ICD) 121-5, 10^{th} revision) of fatal ischaemic heart disease and non-fatal MI and ischaemic stroke (ICD 163-4) were diagnosed by two independent expert clinicians and an epidemiologist using all available clinical evidence, including computed tomography, radiological and pathological information. Furthermore, the records of all men at the National Health Service Central Registry were flagged so that notification of death certificate was received directly, and cause of death was defined by ICD-9 Revision.

The aim of this analysis was to investigate the relationship between egg intake and CVD or mortality in the total population, as well as in a sub-group suffering from T2D. After removal of 16 subjects with T2D who had missing dietary or confounding factor data, 94 pre-existing T2D subjects remained for inclusion in the analysis. In order to have sufficient numbers for the statistical analysis, we combined the men with impaired glucose tolerance (IGT; *n*=319), defined by the WHO as fasting plasma glucose of 6.1 mmol/L or higher [21]. Subjects (*n*=73) who met the inclusion criteria for both T2D and IGT were counted once in the analysis, thus, T2D and/or IGT sub-group included 340 subjects which were included in the longitudinal analysis of the associations between egg consumption with CVD events or all-cause mortality.

Other measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP), plasma glucose, insulin, triacylglycerol (TGs), total/HDL-cholesterol, fibrinogen, homocysteine and C-reactive protein (CRP) were measured in fasting plasma or serum samples at Phase 2. LDL-cholesterol was calculated by using TGs, total cholesterol and HDL- cholesterol by the Friedewald formula [22]. Pulse pressure was calculated by subtracting DBP from SBP. However, only SBP, DPB, glucose, TGs, total cholesterol, fibrinogen were measured at Phase 3 in CAPS.

National Diet and Nutrition Survey

Data files from the National Diet and Nutrition Survey (NDNS) [23] years 1-4 of the rolling programme (2008-2009 to 2011-2012) were obtained from the UK Data Archive (www.data-archive.ac.uk). The data from 754 adults (males *n*=322 and females *n*=432) aged 19-64 years old were used to determine association between egg intake (g/day) and fasting blood glucose, glycated haemoglobin (HbA1c) and other biochemical measures of cardio-metabolic health,

including total-, HDL-, and LDL- cholesterol, the total-/HDL- cholesterol ratio, TGs, SBP, DBP, pulse pressure and CRP. Egg consumption was divided into tertiles for the analysis. Participants with a previous history of stroke (*n*=1), heart attack or angina (*n*=6) were excluded from the analysis. In addition, associations between egg consumption and metabolic markers were examined in the T2D and/or IGT sub-group, which included men with T2D (*n*=14) or IGT (fasting plasma glucose ≥ 6.1 mmol/L, *n*=56), subjects (*n*=11) who met inclusion criteria for both T2D and IGT groups were only counted once. Thus, the total number in the T2D and/or IGT sub-group was 59 subjects. The egg food group included whole eggs and dishes such as omelettes and scrambled eggs. Composite dishes such as egg fried rice and quiches were removed from the total egg consumption to fit with the analysis conducted on the CAPS.

Statistical analysis

All data analysis was conducted using STATA (version 13.0; STATA Corporation, 2014) and a 2-sided $P<0.05$ was considered statistically significant. In the longitudinal analysis of CAPS, Cox proportional hazard models were used to calculate non-adjusted and multivariate adjusted hazard ratios (HR) by comparing the time until onset of disease or mortality in cases in higher intake categories of egg consumption with that in the lowest egg group as the reference group. The survival time of the Cox proportional hazard models was the date of disease diagnosis or the last follow-up visit date. The first multivariate model controlled for a number of confounding factors in CAPS. These included the covariates age (years), body mass index (weight (kg)/height (m^2)), energy intake (kcal/day), alcohol consumption (as ethanol, ml/week), smoking (never-smoked, ex-smoked, current smoked), energy expenditure (kJ/day), social class (manual worker; non-manual worker), family history of MI (yes or no) and T2D (yes or no). The second multivariate model also controlled for sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption

(<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d). The possibility of an interaction between egg consumption and subgroup of T2D and/or IGT with respect to any of the outcomes was investigated by an analysis including an interaction term in the regression model.

In secondary analyses, we examined the association between egg consumption and metabolic markers in CAPs and NDNS. For cross-sectional analysis of CAPs, the association of egg consumption with a range of metabolic markers have been examined at Phase 2. In the sensitivity analyses of cross-sectional analysis, the associations of egg consumption with metabolic markers have also been evaluated at Phase 3 in order to test the consistency of the findings. Trends associated with increasing egg consumption were investigated using linear regression for the continuous variables and logistic regression for categorical variables. For cross-sectional analysis of the NDNS, we used confounding factors of age (years), gender (men or women), energy intake (kcal/day), alcohol consumption (as ethanol, g/day), T2D (yes or no) and smoking habit (smokers or non-smokers). General linear regression was used for the continuous variables and the Pearson chi-square test for the categorical variables. Original data were transformed to natural logarithms if required.

Results

CAPS: Baseline characteristics according to egg consumption

The mean egg intake of 1781 subjects was 2.9 (SD=2.1) eggs per week. Among participants, 14.4 % subjects consumed 5 eggs or more per week. The baseline characteristics of the subjects from the CAPS are shown in Table 1. The men in the highest quantiles of egg consumption were significantly more likely to be manual workers, smokers, consume more alcohol, have higher energy intake, higher energy expenditure and higher BMI. They also had a lower incident of family MI history. After controlling for energy intake from foods, the men with the highest egg consumption had a significantly higher intake of total fat, saturated fat

and sugar intake, but lower cereal or vegetable fibre, carbohydrate intakes, red meat and fruit intake.

CAPS: Egg consumption and CVD events, all-cause mortality and diabetes in longitudinal investigation

During the mean follow up of 22.8 years, incident cases of stroke (n=248), MI (n=477), heart failure (n=201) and all-cause mortality (n=1028) were reported in the subjects initially free from CVD events (Table 2). In multivariate Cox regression model, egg consumption was not associated with incident of MI, heart failure, or all-cause mortality. However, a significant trend of higher risk of stroke with increasing egg intake (adjusted model *P*=0.04) was observed, with HR of 1.60 (95% CI: 1.00, 2.57) for the highest (≥5 eggs/wk) vs lowest (0≤ eggs/wk ≤1) quantile of egg consumption.

In stratified analyses, the prevalent of T2D and/or IGT did not influence the association between egg consumption and MI, heart failure, or all-cause mortality (data not shown). When the subjects of T2D and/or IGT were removed from the analysis of stroke, there was no significant increase in risk of stroke across increasing quantiles of egg consumption (Table 3). However, when this analysis was performed on the T2D and/or IGT sub-group, a significant trend for increased risk of stroke (adjusted model *P*=0.01) with increasing egg consumption was identified, HR for incident stroke was 2.87 (95% CI: 1.13, 7.27) in the highest vs lowest quantile of egg consumption (*P* for interaction between egg consumption and T2D and/or IGT = 0.09 in non-adjust model, 0.08 in adjusted Model 1 and 0.07 in adjusted Model 2).

During the follow-up, a total of 120 new T2D cases were diagnosed for subjects free from CVD and T2D events. There was no association between egg consumption and incident T2D using either un-adjusted or multiple adjusted model (Table 4).

CAPS and NDNS: Associations between egg consumption and cardio-metabolic risk factors: cross-sectional analysis.

In Phase 2 of the CAPS, no associations were found between egg consumption and fasting plasma glucose concentration in the subjects free from CVD (Supplemental Table 1). However, when the analysis was repeated in a subgroup (*n*=268) of participants with T2D and/or IGT, there was a significant positive association between egg consumption and fasting glucose concentration (Supplemental Table 2). In this subgroup analysis subjects consuming ≥5 eggs/wk had 1.31 mmol/L higher fasting glucose compared with subjects consuming 1 eggs/wk or less. There were no significant associations between egg consumption and other biomarkers of CVD risk in the population as a whole (Supplemental Table 1) and subgroup analysis (Supplemental Table 2).

In Phase 3 of the CAPS there was a significant positive association between egg consumption and fasting glucose concentration (Supplemental Table 3). When the subjects with T2D and/or IGT were removed from the analysis, there was no association between egg consumption and fasting glucose concentration across increasing quantile of egg consumption. However, when the analysis was repeated in a subgroup of T2D and/or IGT (*n*=334) there was a significant positive association between egg consumption and fasting glucose (Supplemental Table 4). In this subgroup analysis subjects consuming ≥5 eggs per week had 0.72 mmol/L higher fasting glucose levels compared with subjects consuming ≤1 eggs/wk.

Cross-sectional analysis in NDNS [23] showed egg consumption was positively associated with fasting glucose and HbA1c concentrations (Supplemental Table 5). When the subjects with T2D and/or IGT were removed from the analysis, there was no significant increase in fasting glucose, but there was a significant positive trend for increased egg consumption and HbA1c concentration (*P*=0.02). In the T2D and/or IGT subgroup, egg consumption was significant associated with elevated fasting glucose and HbA1c concentrations (Supplemental

Table 6). There were no significant associations between egg consumption and other markers of cardiovascular risk (Supplemental Table 5).

Discussion

No overall associations between weekly egg consumption up to 5 eggs/week and risk of MI, heart failure, all-cause mortality and T2D were observed after a mean follow-up of 22.8 years, but there was a significant positive trend in stroke risk across the quantiles of egg intake. Further sub-group investigation showed that the significant trend disappeared after the men with T2D and/or IGT were removed. By investigation of the association of egg intake and risk of stroke in the sub-group of T2D and/or IGT only, significant positive associations was found. Secondary analyses in both CAPS and NDNS showed increased fasting glucose with higher egg intake in the sub-group of T2D and/or IGT. In addition, results of cross-sectional analyses of NDNS showed higher egg intake was associated with higher HbA1c in the general healthy population across tertiles of egg intake.

These results are consistent with the previous meta-analyses of prospective studies [13, 14], which showed no association between egg consumption and CVD events in the general population. Very few studies have reported a positive association between egg intake and CVD risk. Nevertheless, data from Physicians' Health Study [24] indicated the HR of heart failure was 1.33 (95% CI: 1.04, 1.70) with egg consumption ≥ 7 per week compared with that of <1 per week over 20 years of follow-up for physicians free of previous MI. In addition, in another analysis of the same cohort there was no association between egg intake and MI and stroke, but there was a significant positive association with all-cause mortality [25]. The difference in the observed association of egg consumption and heart failure or all-cause mortality between the current study and that by Djousse et al [24, 25] may be due to differences in the characteristics of the investigated subjects. Physicians were included in the study of Djousse et al [24, 25], whereas 64% of the men in the current study were manual

workers. An earlier British study in 1997 reported a 2.68 times increased risk of ischaemic heart disease deaths in 10,802 subjects reporting higher egg intake (>6/week) compared with lower egg consumption (<1 egg/week) after 13.3 years follow-up [26]. In our study we were not able to conduct a similar analysis, as the data for mortality resulting from different categories of heart disease were not available.

Our finding of no association between egg intake and risk of stroke in generally healthy men is consistent with previous studies [27-29]. However in our sub-group analysis of 340 men with T2D and/or IGT, we found a significant positive association between egg intake and the risk of stroke. To the best of our knowledge, our study is the first to show this association in subjects with T2D and/or IGT and needs confirmation in further studies.

In our secondary cross-sectional analysis of CAPS and NDNS, a positive association between egg intake and blood glucose was observed in the T2D and/or IGT subgroup. This may indicate that higher egg intake had a detrimental effect on the glucose metabolism in subjects with T2D and/or IGT, although this needs confirmation in randomized controlled dietary intervention trials. An earlier study also showed that higher egg consumption was associated with elevated fasting glucose in 394 middle-aged healthy men [7]. This is also supported by another cross-sectional analysis [30] that observed significant positive relationships between egg consumption and fasting glucose, insulin or insulin resistance, although the difference was very small. However, neither of these studies investigated the association in sub-groups with T2D and/or IGT [7, 30].

The concept of eggs as a cholesterol rich food, which may increase LDL-cholesterol and risk of heart disease, has been recognised for a long time. However, in our analysis, we found no association between egg intake and blood cholesterol concentrations, in agreement with Gray and Griffin [10] who concluded that the effect of dietary cholesterol on LDL-cholesterol was negligible compared with the effect of dietary saturated fatty acids. In contrast to our findings, a study in a Finnish population showed a reduction in fasting plasma glucose in the

highest egg consumption quartile (>45 g/d) at baseline and after a 4-year follow up in 2312 men [12]. This significant association at baseline only appeared when dietary cholesterol intake was included as a covariate. However, controlling for dietary cholesterol may lead to bias as this component of eggs could be responsible for the observed effects of eggs on plasma glucose [31].

Lastly, this is the first study to show a strong positive association between higher egg intake (>29 g/d) and elevated HbA1c concentration in a population without CVD or known T2D. Higher HbA1c is regarded as an important marker for pre-diagnosed T2D and CVD [32, 33]. There was no evidence in CAPS of any association between egg consumption and the development of T2D. The numbers of self-reported T2D cases are relatively small and were not validated by clinical diagnosis. However in a larger study, Djousse and colleagues [34] showed a significant positive association between egg consumption (≥7 weekly) and risk of T2D in two large prospective cohort studies of men (*n*=20,303) and women (*n*=36,295) with 1921 and 2112 cases of T2D incident after a follow up period of 20.0 years for men and 11.7 years for women, respectively. One possible explanation for the non-significant finding in CAPS is the relatively small subject group and low number of T2D (*n*=120), which may have limited the statistical power of the study.

The potential mechanism by which eggs could increase fasting plasma glucose and ischaemic stroke in the T2D and/or IGT subjects is unknown. Findings from a 3 month randomized controlled study showed that there was no negative effect of higher egg consumption (>12 eggs/week) on blood lipid profile compared with low egg consumption (<2 eggs/week) in overweight or obese subjects diagnosed with diabetes or prediabetes [11]. However, in that study the authors controlled for diabetes or prediabetes drug use which may have masked the effect of egg consumption. In the baseline analysis of CAPS subject characteristics, higher egg consumption was associated with a relatively unhealthy lifestyle including higher alcohol consumption, higher energy intake, more smokers, which may

indicate that the positive relationship between egg intake and blood glucose or ischaemic stroke in those with compromised glycaemic control, is due to residual confounding factors linked to the dietary pattern. Therefore, future research is needed to assess if there is a causality effect of egg consumption on blood glucose in prediabetes or diabetes subjects.

The major strength of the CAPS study is that it has a follow-up of over 20 years, which is one of the longest UK prospective cohort studies providing new evidence on the relationship between dietary factors and CVD events. Furthermore, in order to prevent chance findings, the cross-sectional analyses were repeated in two phases of the CAPS (5-year interval). However, egg consumption was only recorded as the weekly egg intake and did not account for eggs consumed from composite dishes. Thus, egg intakes may have been underestimated or misclassified which may have affected the observed associations. In addition, cooking methods and information on how eggs were consumed were not recorded, and this may have had effects on health outcomes [35]. In terms of the metabolic markers available in CAPS, insulin was only measured in a small proportion of subjects and was not measured in Phase 3, thus insulin resistance could not be estimated.

In conclusion, our study did not show any evidence for adverse effects of egg intake on the risk of CVD, T2D and all-cause mortality in healthy men. However, a detrimental association of modest egg intake on fasting glucose and risk of ischaemic stroke in T2D and/or IGT subjects was observed. The adverse cross-sectional association of egg consumption on HbA1C in a generally healthy population needs to be confirmed in future studies. Furthermore, cautious interpretation of these results is recommended, due to the limited sample size and number of disease and deaths. In addition, Nicklas et al [36] pointed out the statistical methods and residual confounding factors may have influenced the health outcomes. Therefore, large prospective cohort studies and RCTs are required to verify these findings.

Conflict of Interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Table 1 Baseline characteristics of participants from the Caerphilly Prospective Cohort Study according to egg consumption[1]

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend[2]
Subjects, n	274	464	469	318	256	
Age, y	61.5 ± 4.6	61.9 ± 4.3	61.7 ± 4.5	61.7 ± 4.5	61.6 ± 4.4	0.93
BMI, kg/m	26.5 ± 3.5	26.5 ± 3.4	26.7 ± 3.7	26.9 ± 4.0	27.1 ± 4.2	0.03
Energy expenditure, kJ/d	1440 ± 1557	1421 ±1426	1551 ±1651	1659 ± 1618	1850 ± 2042	0.001
Manual workers, %	53.3	59.7	63.5	70.8	80.1	<0.001
Family history of MI, %	43.2	38.6	38.4	30.4	36.7	0.02
History of hypertension, %	28.8	26.9	29.2	31.1	29.7	0.40
History of diabetes, %	3.3	1.7	0.9	5.3	3.9	0.07
Current smokers, %	22.6	26.9	36.2	43.4	48.0	<0.001
Energy intake, kJ/d	7449 ± 1890	8074 ± 1873	8547 ± 2051	8988 ± 2132	9821 ± 2578	<0.001
Fat, % of energy	33.9	35.1	35.8	36.5	37.1	<0.001
Saturated fat, % of energy	14.6	15.0	15.5	15.9	16.1	<0.001
Protein, % of energy	14.7	14.3	14.3	14.2	14.5	0.19
Carbohydrates, % of energy	49.3	48.3	47.6	47.1	45.9	<0.001
Fibre (vegetable sources)[3], g/d	11.4 ± 0.5	10.9 ± 0.5	11.1 ± 0.6	11.2 ± 0.6	11.3 ± 0.7	0.02
Fibre (cereal sources)[3], g/d	10.8 ± 1.2	10.3 ± 1.1	10.0 ± 1.3	9.3 ± 1.3	9.2 ± 1.6	<0.001
Sugar[3], g/d	76.8 ± 24.1	82.2 ± 23.9	87.3 ± 26.1	93.6 ± 27.2	104.3 ± 32.9	<0.001
Fruit[3], number/wk	9.3 ± 0.5	8.3 ± 0.5	7.9 ± 0.6	7.4 ± 0.6	7.7 ± 0.7	<0.001
Vegetable[3], times/wk	10.7 ± 4.7	10.0 ± 4.6	10.5 ± 4.7	10.6 ± 4.8	10.3 ± 4.8	0.99
Red meat[3], times/wk	17.4 ± 0.9	14.9 ± 0.9	14.8 ± 1.0	14.3 ± 1.1	14.8 ± 1.3	<0.001
Alcohol intake, ml/wk	15.6 ± 21.5	16.2 ± 20.3	17.8 ± 22.5	17.3 ± 19.7	20.5 ± 22.8	0.010

[1] All values are mean ± SD.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables).

[3] Energy-adjusted values.

Table 2 Longitudinal study of incidence of stroke, myocardial infarction, heart failure and all-cause mortality according to egg consumption of all subjects.

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Total subjects, n	274	464	469	318	256	
Stroke						
No. of events	33	57	57	48	53	
HR (non-adjust)	1	1.01 (0.66, 1.56)	1.01 (0.66, 1.55)	1.27 (0.82, 1.98)	1.82 (1.18, 2.80)	0.002
HR (adjusted Model 1)[1]	1	0.99 (0.65, 1.53)	0.97 (0.63, 1.50)	1.14 (0.72, 1.81)	1.58 (1.00, 2.52)	0.03
HR (adjusted Model 2)[2]	1	1.01 (0.65, 1.56)	1.00 (0.64, 1.55)	1.15 (0.72, 1.84)	1.60 (1.00, 2.57)	0.04
Myocardial infarction						
No. of events	73	117	137	86	64	
HR (non-adjust)	1	0.94 (0.70, 1.26)	1.11 (0.83, 1.47)	1.00 (0.73, 1.37)	0.94 (0.67, 1.31)	0.98
HR (adjusted Model 1)	1	0.96 (0.71, 1.29)	1.10 (0.83, 1.48)	0.99 (0.71, 1.37)	0.90 (0.63, 1.29)	0.75
HR (adjusted Model 2)	1	0.97 (0.72, 1.31)	1.14 (0.85, 1.52)	1.01 (0.72, 1.40)	0.91 (0.64, 1.31)	0.80
Heart failure						
No. of events	29	33	63	44	32	
HR (non-adjust)	1	0.66 (0.40, 1.09)	1.29 (0.83, 2.00)	1.33 (0.83, 2.13)	1.20 (0.72, 1.98)	0.03
HR (adjusted Model 1)	1	0.64 (0.39, 1.06)	1.17 (0.74, 1.83)	1.10 (0.67, 1.79)	0.89 (0.52, 1.52)	0.46
HR (adjusted Model 2)	1	0.65 (0.39, 1.08)	1.19 (0.76, 1.88)	1.09 (0.66, 1.81)	0.89 (0.51, 1.53)	0.49
All-cause mortality						
No. of events	135	249	293	187	164	
HR (non-adjust)	1	1.13 (0.92, 1.40)	1.35 (1.10, 1.66)	1.25 (1.00, 1.56)	1.44 (1.14, 1.80)	0.001
HR (adjusted Model 1)	1	1.08 (0.87, 1.33)	1.21 (0.98, 1.49)	1.03 (0.82, 1.30)	1.11 (0.87, 1.42)	0.58
HR (adjusted Model 2)	1	1.08 (0.87, 1.34)	1.20 (0.98, 1.49)	1.02 (0.81, 1.29)	1.08 (0.84, 1.38)	0.80

[1] Values are hazard ratios (95 % CIs) derived by Cox proportional hazards regression models adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no), diabetes mellitus (yes or no).

[2] Adjusted as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

Table 3 Longitudinal analysis of incident of stroke according to egg consumption in subjects with and without type 2 diabetes and/or impaired glucose tolerance from the Caerphilly Prospective Cohort study.

	Egg consumption (n, eggs/wk)					
	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Subjects without T2D and/or IGT[1]						
Subjects, n	221	397	378	248	197	
No. of events	25	47	45	34	31	
HR (non-adjust)	1	1.05 (0.64, 1.70)	1.05 (0.65, 1.72)	1.23 (0.73, 2.05)	1.45 (0.85, 2.45)	0.12
HR (adjusted Model 1)[2]	1	1.01 (0.62, 1.66)	0.97 (0.59, 1.60)	1.14 (0.67, 1.95)	1.28 (0.73, 2.24)	0.33
HR (adjusted Model 2)[3]	1	1.05 (0.64, 1.71)	1.01 (0.61, 1.66)	1.17 (0.68, 2.02)	1.32 (0.75, 2.34)	0.29
Subjects with T2D and/or IGT						
Subjects, n	53	67	91	70	59	
No. of events	8	10	12	14	22	
HR (non-adjust)	1	0.96 (0.38, 2.44)	0.86 (0.35, 2.10)	1.35 (0.57, 3.23)	2.71 (1.21, 6.09)	0.003
HR (adjusted Model 1)	1	1.10 (0.42, 2.86)	1.02 (0.40, 2.62)	1.35 (0.53, 3.43)	2.83 (1.15, 6.96)	0.01
HR (adjusted Model 2)	1	1.09 (0.41, 2.88)	0.96 (0.37, 2.50)	1.39 (0.54, 3.56)	2.87 (1.13, 7.27)	0.01

[1] Impaired glucose tolerance, i.e. fasting glucose ≥ 6.1 mmol/L.

[2] Values are hazard ratios (95 % CIs) derived by Cox proportional hazards regression models adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no).

[3] Adjusted as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

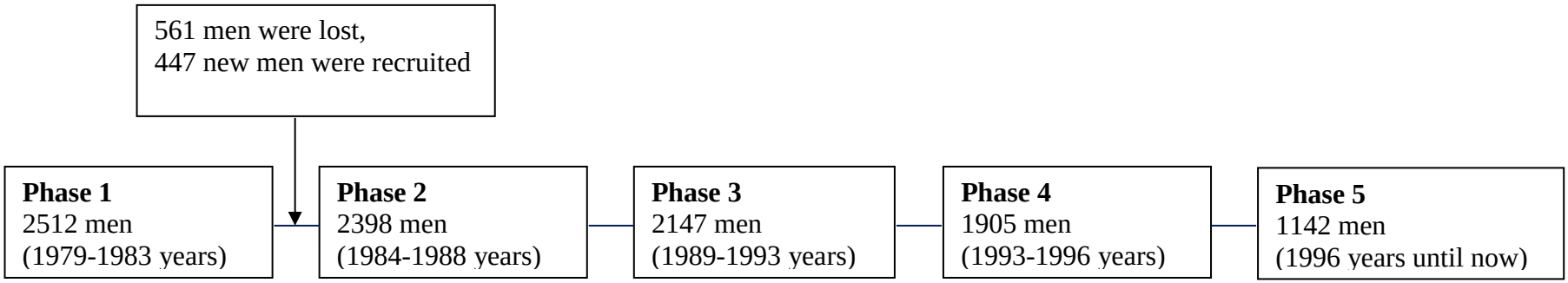
Table 4 Longitudinal study of incidence of type 2 diabetes according to egg consumption.

	Egg consumption (n, eggs/wk)					
	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Subjects, n	259	447	453	290	238	
No. of events	17	31	35	21	16	
HR (non-adjust)	1	1.17 (0.65, 2.12)	1.24 (0.69, 2.21)	1.20 (0.63, 2.27)	1.22 (0.61, 2.41)	0.59
HR (adjusted Model 1)[1]	1	1.08 (0.59, 1.97)	1.05 (0.57, 1.92)	1.25 (0.64, 2.44)	1.23 (0.60, 2.53)	0.48
HR (adjusted Model 2)[2]	1	1.05 (0.57, 1.93)	1.02 (0.55, 1.88)	1.24 (0.63, 2.45)	1.31 (0.63, 2.73)	0.39

[1] Values are hazard ratios (95 % CIs) derived by Cox proportional hazards regression models adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no).

[2] Adjusted as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

Figure 1 Timeline of the Caerphilly Prospective Cohort Study.



Supplemental Table 1 Cross-sectional (Phase 2) analysis of metabolic markers across egg consumption categories of all subjects in Caerphilly Prospective Cohort Study[1]

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Glucose[4]						
Participants, n	413	562	415	347	421	
Mean, mmol/L	5.25 (0.84)	5.43 (1.43)	5.36 (1.26)	5.41 (1.24)	5.49 (1.56)	
Coef. (Std. Err.) non-adjust	Reference	0.02 (0.00, 0.05)	0.01 (-0.01, 0.04)	0.02 (-0.00, 0.05)	0.03 (0000, 0.05)	0.05
Coef. (Std. Err.) adjusted Model 1[2]	Reference	0.02 (-0.00, 0.04)	0.01 (-0.01, 0.03)	0.02 (-0.00, 0.04)	0.01 (-0.01, 0.03)	0.44
Coef. (Std. Err.) adjusted Model 2[3]	Reference	0.02 (-0.00, 0.04)	0.01 (-0.02, 0.03)	0.01 (-0.01, 0.04)	0.01 (-0.02, 0.03)	0.70
Insulin[4]						
Participants, n	200	272	196	169	205	
Mean, mmol/L	5.28 (22.19)	3.74 (3.75)	3.64 (4.21)	3.17 (2.32)	3.43 (2.86)	
Coef. (Std. Err.) non-adjust	Reference	0.00 (-0.13, 0.13)	-0.01 (-0.15, 0.13)	-0.09 (-0.24, 0.06)	-0.06 (-0.20, 0.08)	0.20
Coef. (Std. Err.) adjusted Model 1	Reference	-0.01 (-0.14, 0.12)	-0.03 (-0.17, 0.11)	-0.13 (-0.28, 0.02)	-0.09 (-0.24, 0.07)	0.10
Coef. (Std. Err.) adjusted Model 2	Reference	-0.02 (-0.15, 0.11)	-0.03 (-0.17, 0.11)	-0.13 (-0.28, 0.03)	-0.08 (-0.23, 0.08)	0.15
Total cholesterol						
Participants, n	409	564	414	348	424	
Mean, mmol/L	5.64 (0.99)	5.56 (0.99)	5.62 (1.05)	5.64 (1.02)	5.68 (1.00)	
Coef. (Std. Err.) non-adjust	Reference	-0.08 (-0.21, 0.05)	-0.02 (-0.16, 0.12)	-0.00 (-0.15, 0.14)	0.04 (-0.10, 0.18)	0.26
Coef. (Std. Err.) adjusted Model 1	Reference	-0.09 (-0.22, 0.04)	-0.01 (-0.15, 0.13)	-0.00 (-0.15, 0.15)	0.05 (-0.09, 0.20)	0.18
Coef. (Std. Err.) adjusted Model 2	Reference	-0.09 (-0.22, 0.04)	-0.03 (-0.17, 0.11)	-0.02 (-0.17, 0.13)	0.03 (-0.11, 0.18)	0.35
HDL-cholesterol,						
Participants, n	409	564	414	348	424	
Mean, mmol/L	1.03 (0.25)	1.01 (0.24)	1.03 (0.24)	1.02 (0.25)	1.04 (0.27)	
Coef. (Std. Err.) non-adjust	Reference	-0.02 (-0.06, 0.01)	0.00 (-0.03, 0.03)	-0.01 (-0.04, 0.03)	0.01 (-0.03, 0.04)	0.36

Coef. (Std. Err.) adjusted Model 1	Reference	-0.02 (-0.05, 0.01)	0.01 (-0.03, 0.04)	0.01 (-0.02, 0.05)	0.02 (-0.01, 0.06)	0.05
Coef. (Std. Err.) adjusted Model 2	Reference	-0.02 (-0.05, 0.01)	0.00 (-0.03, 0.04)	0.01 (-0.03, 0.04)	0.02 (-0.02, 0.05)	0.08
LDL-cholesterol[4]						
Participants, n	409	564	414	348	424	
Mean, mmol/L	4.23 (0.94)	4.15 (0.90)	4.22 (0.96)	4.21 (0.96)	4.25 (0.92)	
Coef. (Std. Err.) non-adjust	Reference	-0.08 (-0.20, 0.03)	-0.01 (-0.14, 0.12)	-0.02 (-0.16, 0.11)	0.02 (-0.11, 0.14)	0.43
Coef. (Std. Err.) adjusted Model 1	Reference	-0.09 (-0.21, 0.02)	-0.00 (-0.13, 0.12)	-0.03 (-0.16, 0.11)	0.04 (-0.10, 0.17)	0.26
Coef. (Std. Err.) adjusted Model 2	Reference	-0.09 (-0.21, 0.03)	-0.02 (-0.14, 0.11)	-0.04 (-0.17, 0.10)	0.03 (-0.11, 0.16)	0.40
Triglycerides[4]						
Participants, n	409	564	414	348	424	
Mean, mmol/L	1.89 (1.06)	2.04 (1.80)	1.86 (1.11)	2.04 (1.57)	1.98 (1.29)	
Coef. (Std. Err.) non-adjust	Reference	0.03 (-0.03, 0.10)	-0.02 (-0.09, 0.05)	0.04 (-0.04, 0.11)	0.03 (-0.04, 0.10)	0.55
Coef. (Std. Err.) adjusted Model 1	Reference	0.01 (-0.05, 0.08)	-0.03 (-0.10, 0.03)	-0.01 (-0.08, 0.07)	-0.03 (-0.10, 0.04)	0.30
Coef. (Std. Err.) adjusted Model 2	Reference	0.01 (-0.05, 0.07)	-0.04 (-0.11, 0.03)	-0.01 (-0.09,0.06)	-0.04 (-0.11, 0.03)	0.18
Fibrinogen[4]						
Participants, n	252	340	280	236	253	
Mean, g/L	3.03 (0.78)	3.09 (0.85)	3.07 (0.85)	3.09 (0.89)	3.14 (0.83)	
Coef. (Std. Err.) non-adjust	Reference	0.02 (-0.02, 0.06)	0.01 (-0.03, 0.05)	0.01 (-0.03, 0.06)	0.03 (-0.01, 0.08)	0.23
Coef. (Std. Err.) adjusted Model 1	Reference	0.00 (-0.04, 0.04)	-0.00 (-0.04, 0.04)	-0.01 (-0.06, 0.03)	0.02 (-0.02, 0.07)	0.55
Coef. (Std. Err.) adjusted Model 2	Reference	0.01 (-0.03, 0.04)	-0.00 (-0.06, 0.03)	-0.01 (-0.06, 0.03)	0.02 (-0.03, 0.06)	0.77
Homocysteine						
Participants, n	412	560	408	346	424	
Mean, mmol/L	12.65 (4.91)	12.28 (5.07)	12.59 (4.77)	11.91 (4.81)	12.37 (5.52)	
Coef. (Std. Err.) non-adjust	Reference	-0.36 (-1.00, 0.28)	-0.05 (-0.74, 0.64)	-0.73 (-1.45, -0.01)	-0.28 (-0.96, 0.40)	0.30
Coef. (Std. Err.) adjusted Model 1	Reference	-0.37 (-1.01, 0.27)	-0.12 (-0.80, 0.57)	-0.74 (-1.47, -0.02)	-0.35 (-1.07, 0.36)	0.24
Coef. (Std. Err.) adjusted Model 2	Reference	-0.41 (-1.04, 0.22)	-0.21 (-1.55, 0.47)	-0.83 (-1.55, -0.10)	-0.61 (-1.33, 0.11)	0.06
C-reactive protein[4]						

Participants, n	280	395	285	242	288	
Mean, mg/L	2.42 (2.78)	3.29 (5.30)	2.94 (4.91)	2.96 (4.00)	3.22 (4.35)	
Coef. (Std. Err.) non-adjust	Reference	0.18 (0.02, 0.33)	0.10 (-0.07, 0.27)	0.16 (-0.02, 0.34)	0.17 (0.00, 0.34)	0.13
Coef. (Std. Err.) adjusted Model 1	Reference	0.10 (-0.05, 0.25)	0.06 (-0.10, 0.22)	0.03 (-0.14, 0.20)	0.04 (-0.13, 0.21)	0.98
Coef. (Std. Err.) adjusted Model 2	Reference	0.0 (-0.05, 0.24)	0.04 (-0.12, 0.20)	0.02 (-0.15, 0.19)	-0.01 (-0.18, 0.16)	0.54
Systolic blood pressure[4]						
Participants, n	427	577	427	356	433	
Mean, mmHg	147.0 (22.1)	146.5 (22.2)	144.8 (23.4)	148.1 (23.5)	146.1 (22.6)	
Coef. (Std. Err.) non-adjust	Reference	-0.00 (-0.02, 0.02)	-0.02 (-0.04, 0.00)	0.01 (-0.01, 0.03)	-0.01 (-0.03, 0.01)	0.85
Coef. (Std. Err.) adjusted Model 1	Reference	-0.01 (-0.03, 0.01)	-0.02 (-0.04, -0.00)	-0.00 (-0.02, 0.02)	-0.02 (-0.04, 0.00)	0.19
Coef. (Std. Err.) adjusted Model 2	Reference	-0.01 (-0.03, 0.01)	-0.02 (-0.04, -0.00)	-0.00 (-0.02, 0.02)	-0.02 (-0.04, -0.00)	0.14
Diastolic blood pressure						
Participants, n	426	577	427	356	433	
Mean, mmHg	84.7 (11.3)	85.3 (11.7)	83.9 (12.0)	85.5 (12.4)	85.0 (13.3)	
Coef. (Std. Err.) non-adjust	Reference	0.59 (-0.93, 2.11)	-0.75 (-2.38, 0.87)	0.83 (-0.88, 2.53)	0.30 (-1.33, 1.92)	0.75
Coef. (Std. Err.) adjusted Model 1	Reference	0.44 (-1.04, 1.93)	-0.80 (-2.39, 0.79)	0.49 (-1.21, 2.19)	-0.09 (-1.77, 1.60)	0.83
Coef. (Std. Err.) adjusted Model 2	Reference	0.48 (-1.00, 1.97)	-0.77 (-2.37, 0.82)	0.54 (-1.16, 2.25)	-0.34 (-2.03, 1.35)	0.64

[1] All values are mean ± SD.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables), adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no), diabetes mellitus (yes or no).

[3]Adjusted as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

[4] Original data were transformed to natural logarithms for regression model.

Supplemental Table 2 Cross-sectional (Phase 2) analysis of metabolic markers across egg consumption in subjects type 2 diabetes and/or impaired glucose tolerance from the Caerphilly Prospective Cohort study[1]

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Glucose[4]						
Participants, n	42	72	46	46	62	
Fasting glucose, mmol/L	6.89 (1.38)	7.88 (2.83)	7.73 (2.60)	7.45 (2.37)	8.20 (2.62)	
Coef.± Std. Err. (non-adjust)	Reference	0.10 (0.00, 0.21)	0.09 (-0.02, 0.20)	0.06 (-0.05, 0.20)	0.15 (0.05, 0.26)	0.04
Coef ± Std. Err. (adjusted Model 1)[2]	Reference	0.12 (0.02, 0.22)	0.11 (0.00, 0.23)	0.07 (-0.04, 0.18)	0.19 (0.08, 0.30)	0.008
Coef ± Std. Err. (adjusted Model 2)[3]	Reference	0.14 (0.03, 0.24)	0.11 (-0.00, 0.22)	0.05 (-0.06, 0.17)	0.16 (0.05, 0.27)	0.02
Insulin[4]						
Participants, n	22	39	21	21	28	
Mean, mmol/L	3.78 (2.60)	3.79 (3.49)	3.60 (1.65)	2.95 (1.54)	3.06 (1.97)	
Coef.± Std. Err. (non-adjust)	Reference	0.02 (-0.37, 0.41)	0.10 (-0.35, 0.54)	-0.16 (-0.35, 0.54)	-0.08 (-0.49, 0.34)	0.46
Coef ± Std. Err. (adjusted Model 1)	Reference	0.05 (-0.37, 0.47)	0.10(-0.36, 0.57)	-0.19 (-0.67, 0.29)	-0.15 (-0.60, 0.30)	0.29
Coef ± Std. Err. (adjusted Model 2)	Reference	0.04 (-0.43, 0.52)	0.07 (-0.47, 0.60)	-0.31 (-0.86, 0.25)	-0.21 (-0.70, 0.29)	0.17
Total cholesterol						
Participants, n	42	72	45	45	62	
Mean, mmol/L	5.43 (0.75)	5.69 (1.37)	5.37 (0.98)	5.61 (1.03)	5.54 (1.15)	
Coef.± Std. Err. (non-adjust)	Reference	0.26 (-0.17, 0.69)	-0.06 (-0.53, 0.42)	0.18 (-0.29, 0.66)	0.11 (-0.33, 0.55)	0.94
Coef ± Std. Err. (adjusted Model 1)	Reference	0.23 (-0.19, 0.65)	0.00 (-0.46, 0.46)	0.27 (-0.20, 0.74)	0.19 (-0.25, 0.64)	0.50
Coef ± Std. Err. (adjusted Model 2)	Reference	0.22 (-0.21, 0.66)	0.02 (-0.46, 0.50)	0.25 (-0.24, 0.74)	0.15 (-0.31, 0.60)	0.65
HDL-cholesterol,						
Participants, n	42	72	45	45	62	
Mean, mmol/L	1.05 (0.24)	0.91 (0.23)	0.95 (0.24)	1.00 (0.26)	0.99 (0.26)	
Coef.± Std. Err. (non-adjust)	Reference	-0.15 (-0.24, -0.05)	-0.10 (-0.21, 0.00)	-0.05 (-0.16, 0.05)	-0.07 (-0.16, 0.03)	0.82

Coef ± Std. Err. (adjusted Model 1)	Reference	-0.18 (-0.27, -0.08)	-0.11 (-0.21, -0.01)	-0.03 (-0.13, 0.07)	-0.04 (-0.14, 0.06)	0.24
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.18 (-0.27, -0.09)	-0.12 (-0.22, -0.12)	-0.04 (-0.14, 0.07)	-0.05 (-0.14, 0.05)	0.33
LDL-cholesterol[4]						
Participants, n	42	72	45	45	62	
Mean, mmol/L	4.00 (0.73)	4.18 (1.10)	3.95 (0.85)	4.18 (1.01)	1.08 (1.00)	
Coef.± Std. Err. (non-adjust)	Reference	0.18 (-0.19, 0.55)	-0.04 (-0.45, 0.37)	0.18 (-0.23, 0.59)	0.09 (-0.29, 0.47)	0.83
Coef ± Std. Err. (adjusted Model 1)	Reference	0.18 (-0.18, 0.54)	0.01 (-0.38, 0.41)	0.28 (-0.13, 0.69)	0.19 (-0.19, 0.57)	0.34
Coef ± Std. Err. (adjusted Model 2)	Reference	0.17 (-0.21, 0.54)	0.17 (-0.20, 0.54)	0.05 (-0.36, 0.46)	0.16 (-0.23, 0.55)	0.39
Triglycerides[4]						
Participants, n	42	72	45	45	62	
Mean, mmol/L	1.89 (0.86)	3.04 (3.91)	2.34 (1.61)	2.16 (1.20)	2.35 (2.05)	
Coef.± Std. Err. (non-adjust)	Reference	0.30 (0.08, 0.52)	0.14 (-0.10, 0.38)	0.07 (-0.17, 0.32)	0.10 (-0.13, 0.33)	0.57
Coef ± Std. Err. (adjusted Model 1)	Reference	0.31 (0.10, 0.52)	0.17 (-0.06, 0.40)	0.07 (-0.16, 0.31)	0.06 (-0.16, 0.28)	0.37
Coef ± Std. Err. (adjusted Model 2)	Reference	0.32 (0.11, 0.53)	0.16 (-0.07, 0.39)	0.02 (-0.21, 0.26)	0.05 (-0.17, 0.27)	0.23
Fibrinogen[4]						
Participants, n	31	44	28	31	29	
Mean, g/L	3.12 (0.96)	3.18 (0.98)	2.89 (0.80)	2.89 (0.71)	3.47 (1.02)	
Coef.± Std. Err. (non-adjust)	Reference	0.01 (-0.11, 0.14)	-0.08 (-0.22, 0.06)	-0.06 (-0.20, 0.07)	0.11 (-0.03, 0.25)	0.45
Coef ± Std. Err. (adjusted Model 1)	Reference	0.03 (-0.09, 0.15)	-0.07 (-0.21, 0.06)	-0.07 (-0.20, 0.06)	0.12 (-0.02, 0.03)	0.52
Coef ± Std. Err. (adjusted Model 2)	Reference	0.03 (-0.11, 0.16)	-0.06 (-0.21, 0.08)	-0.07 (-0.21, 0.07)	0.12 (-0.03, 0.26)	0.51
Homocysteine[4]						
Participants, n	43	71	44	46	62	
Mean, mmol/L	12.43 (4.45)	11.40 (3.45)	12.40 (6.57)	10.94 (3.10)	11.63 (6.48)	
Coef.± Std. Err. (non-adjust)	Reference	-1.03 (-2.93, 0.87)	-0.02 (-2.13, 2.09)	-1.49 (-3.57, 0.60)	-0.79 (-2.74, 1.16)	0.47
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.83 (-2.72, 1.06)	-0.42 (-2.52, 1.69)	-1.78 (-3.90, 0.33)	-1.07 (-3.08, 0.94)	0.23
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.74 (-2.68, 1.20)	-0.08 (-2.24, 2.08)	-1.47 (-3.66, 0.72)	-1.04 (-3.07, 1.00)	0.26
C-reactive protein[4]						

Participants, n	28	53	32	27	42	
Mean, mg/L	2.69 (2.64)	3.04 (4.34)	2.63 (1.70)	1.98 (1.58)	4.94 (5.90)	
Coef.± Std. Err. (non-adjust)	Reference	-0.05 (-0.48, 0.38)	0.05 (-0.43, 0.53)	-0.25 (-0.75, 0.53)	0.31 (-0.14, 0.76)	0.22
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.03 (-0.46, 0.40)	0.03 (-0.44, 0.50)	-0.37 (-0.87, 0.13)	0.24 (-0.22, 0.69)	0.54
Coef ± Std. Err. (adjusted Model 2)	Reference	0.05 (-0.40, 0.50)	0.07 (-0.42, 0.56)	-0.34 (-0.87, 0.18)	0.24 (-0.24, 0.71)	0.67
Systolic blood pressure[4]						
Participants, n	45	70	45	47	61	
Mean, mmHg	156.4 (25.3)	154.9 (20.2)	153.1 (26.7)	156.5 (25.4)	152.8 (25.3)	
Coef.± Std. Err. (non-adjust)	Reference	-0.01 (-0.06, 0.05)	-0.02 (-0.096, 0.04)	0.00 (-0.06, 0.07)	-0.02 (-0.08, 0.04)	0.53
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.01 (-0.07, 0.04)	-0.02 (-0.09, 0.04)	0.01 (-0.06, 0.07)	-0.04 (-0.10, 0.02)	0.36
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.01 (-0.07, 0.05)	-0.01 (-0.07, 0.06)	0.02 (-0.05, 0.09)	-0.04 (-0.10, 0.03)	0.47
Diastolic blood pressure						
Participants, n	45	70	45	47	61	
Mean, mmHg	87.7 (10.4)	87.4 (11.4)	87.6 (14.7)	86.1 (14.5)	86.7 (13.8)	
Coef.± Std. Err. (non-adjust)	Reference	-0.32 (-5.20, 4.56)	-0.09 (-5.48, 5.30)	-1.54 (-6.87, 3.79)	-0.97 (-5.99, 4.05)	0.59
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.87 (-5.66, 3.92)	-0.56 (-5.86, 4.75)	-0.99 (-6.33, 4.35)	-2.78 (-7.89, 2.34)	0.31
Coef ± Std. Err. (adjusted Model 2)	Reference	0.08 (-4.87, 5.03)	1.09 (-4.42, 6.59)	0.36 (-5.20, 5.93)	-2.05 (-7.26, 3.17)	0.43

[1] All values are mean ± SD; Impaired glucose tolerance, i.e. fasting glucose ≥ 6.1 mmol/L.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables), adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no).

[3]Adjusted as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

[4] Original data were transformed to natural logarithms for regression model.

Supplemental Table 3 Cross-sectional (Phase 3) analysis of metabolic markers across egg consumption categories of all subjects in Caerphilly Prospective Cohort Study[1]

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	*P*-trend
Glucose[4]						
Participants, n	450	505	301	230	229	
Fasting glucose, mmol/L	5.62 (1.48)	5.59 (1.45)	5.75 (1.77)	5.82 (1.98)	6.09 (2.17)	
Coef.± Std. Err. (non-adjust)	Reference	-0.00 (-0.03, 0.02)	0.01 (-0.02, 0.05)	0.02 (-0.01, 0.05)	0.06 (-0.01, 0.05)	<0.001
Coef ± Std. Err. (adjusted Model 1)[2]	Reference	-0.01 (-0.03, 0.02)	0.01 (-0.02, 0.04)	0.01 (-0.03, 0.04)	0.04 (0.01, 0.08)	0.03
Coef ± Std. Err. (adjusted Model 2)[3]	Reference	-0.01 (-0.03, 0.02)	0.01 (-0.02, 0.04)	0.01 (-0.03, 0.04)	0.04 (0.00, 0.07)	0.04
Total cholesterol						
Participants, n	453	503	303	267	192	
Mean, mmol/L	6.30 (1.24)	6.17 (1.17)	6.25 (1.17)	6.31 (1.04)	6.26 (1.14)	
Coef.± Std. Err. (non-adjust)	Reference	-0.13 (-0.27, 0.02)	-0.05 (-0.22, 0.11)	0.01 (-0.17, 0.19)	-0.04 (-0.22, 0.14)	0.85
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.18 (-0.35, -0.02)	-0.08 (-0.28, 0.11)	0.05 (-0.16, 0.26)	-0.07 (-0.29, 0.15)	0.72
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.20 (-0.37, 0.03)	-0.09 (-0.29, 0.10)	0.04 (-0.17, 0.25)	-0.09 (-0.31, 0.13)	0.84
Triglycerides[4]						
Participants, n	453	503	303	229	230	
Mean, mmol/L	1.98 (1.23)	1.84 (1.08)	1.86 (1.08)	1.90 (1.15)	2.02 (1.43)	
Coef.± Std. Err. (non-adjust)	Reference	-0.07 (-0.13, -0.00)	-0.05 (-0.13, 0.02)	-0.05 (-0.13, 0.04)	-0.02 (-0.10, 0.07)	0.71
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.09 (-0.17, -0.02)	-0.09 (-0.17, -0.00)	-0.11 (-0.20, -0.02)	-0.09 (-0.19, 0.01)	0.05
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.10 (-0.17, -0.03)	-0.09 (-0.18, -0.01)	-0.12 (-0.21, -0.03)	-0.10 (-0.19, -0.00)	0.05
Fibrinogen[4]						
Participants, n	444	498	302	227	227	
Mean, g/L	4.17 (0.95)	4.24 (0.80)	4.24 (0.80)	4.21 (0.92)	4.13 (0.83)	
Coef.± Std. Err. (non-adjust)	Reference	0.03 (0.00, 0.05)	0.05 (0.02, 0.08)	0.04 (0.01, 0.07)	0.02 (-0.01, 0.06)	0.03

Coef ± Std. Err. (adjusted Model 1)	Reference	0.01 (-0.02, 0.04)	0.04 (0.00, 0.07)	0.03 (-0.01, 0.06)	0.02 (-0.02, 0.06)	0.18
Coef ± Std. Err. (adjusted Model 2)	Reference	0.01 (-0.02, 0.04)	0.04 (0.00, 0.07)	0.03 (-0.01, 0.06)	0.02 (-0.02, 0.06)	0.17
Systolic blood pressure						
Participants, n	466	520	313	238	236	
Mean, mmHg	144.2 (21.9)	142.5 (20.8)	144.4 (23.2)	146.7 (20.4)	147.0 (23.8)	
Coef.± Std. Err. (non-adjust)	Reference	-1.73 (-4.48, 1.01)	0.19 (-2.95, 3.33)	2.47 (-0.95, 5.90)	2.85 (-0.59, 6.28)	0.02
Coef ± Std. Err. (adjusted Model 1)	Reference	2.12 (-5.21, 0.97)	0.01 (-3.59, 3.60)	1.34 (-2.51, 5.19)	2.47 (-1.59, 6.53)	0.08
Coef ± Std. Err. (adjusted Model 2)	Reference	-1.99 (-5.11, 1.12)	0.10 (-3.52, 3.72)	1.46 (-2.41, 5.34)	2.44 (-1.65, 6.53)	0.08
Diastolic blood pressure						
Participants, n	466	520	313	238	236	
Mean, mmHg	81.1 (11.8)	81.5 (12.1)	80.7 (12.2)	81.38 (12.0)	82.5 (11.9)	
Coef.± Std. Err. (non-adjust)	Reference	0.43 (-1.07, 1.93)	-0.36 (-2.08, 1.36)	0.29 (-1.58, 2.17)	1.43 (-0.45, 3.31)	0.28
Coef ± Std. Err. (adjusted Model 1)	Reference	0.15 (-1.55, 1.84)	-0.46 (-2.43, 1.52)	-0.25 (-2.36, 1.86)	1.17 (-1.06, 3.40)	0.57
Coef ± Std. Err. (adjusted Model 2)	Reference	0.30 (-1.41, 2.01)	-0.38 (-2.37, 1.61)	-0.15 (-2.27, 1.98)	1.28 (-0.97, 3.53)	0.52

[1] All values are mean ± SD.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables), adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no), diabetes mellitus (yes or no).

[3] Adjustd as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

[4] Original data were transformed to natural logarithms for regression model.

Supplemental Table 4 Cross-sectional (Phase 3) analysis of metabolic markers across egg consumption in subjects type 2 diabetes and/or impaired glucose tolerance from the Caerphilly Prospective Cohort study[1]

	Egg consumption (n, eggs/wk)					
Characteristics	1 (0≤ n ≤1)	2 (1< n ≤2)	3 (2< n ≤3)	4 (3< n <5)	5 (n ≥5)	P-trend
Glucose4						
Participants, n	76	91	61	44	62	
Fasting glucose, mmol/L	7.74 (2.60)	7.54 (2.49)	8.20 (2.68)	8.51 (3.25)	8.46 (3.05)	
Coef.± Std. Err. (non-adjust)	Reference	-0.02 (-0.11, 0.06)	0.06 (-0.04, 0.15)	0.08 (-0.03, 0.19)	0.08 (-0.02, 0.17)	0.02
Coef ± Std. Err. (adjusted Model 1)[2]	Reference	0.01 (-0.09, 0.11)	0.09 (-0.02, 0.21)	0.13 (-0.00, 0.26)	0.11 (-0.01, 0.23)	0.02
Coef ± Std. Err. (adjusted Model 2)[3]	Reference	0.01 (-0.10, 0.12)	0.09 (-0.03, 0.21)	0.13 (-0.01, 0.27)	0.09 (-0.03, 0.22)	0.04
Total cholesterol						
Participants, n	76	90	61	44	62	
Mean, mmol/L	6.31 (1.13)	6.19 (1.08)	6.15 (1.19)	6.19 (1.19)	6.13 (1.00)	
Coef.± Std. Err. (non-adjust)	Reference	-0.12 (-0.46, 0.22)	-0.15 (-0.53, 0.22)	-0.12 (-0.53, 0.30)	-0.18 (-0.55, 0.20)	0.40
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.17 (-0.57, 0.24)	-0.14 (-0.59, 0.31)	0.17 (-0.34, 0.68)	-0.03 (-0.50, 0.44)	0.67
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.25 (-0.68, 0.18)	-0.19 (-0.67, 0.28)	0.10 (-0.44, 0.65)	-0.08 (-0.57, 0.41)	0.76
Triglycerides[4]						
Participants, n	76	90	61	44	62	
Mean, mmol/L	2.35 (1.53)	2.06 (1.42)	2.08 (0.97)	2.66 (1.71)	2.18 (1.45)	
Coef.± Std. Err. (non-adjust)	Reference	-0.12 (-0.29, 0.05)	-0.05 (-0.24, 0.13)	0.11 (-0.09, 0.32)	-0.09 (-0.28, 0.10)	0.92
Coef ± Std. Err. (adjusted Model 1)	Reference	-0.08 (-0.28, 0.11)	-0.00 (-0.22, 0.22)	0.10 (-0.14, 0.35)	-0.07 (-0.29, 0.16)	0.90
Coef ± Std. Err. (adjusted Model 2)	Reference	-0.09 (-0.30, 0.12)	-0.03 (-0.26, 0.20)	0.11 (-0.15, 0.38)	-0.07 (-0.31, 0.16)	0.92
Fibrinogen[4]						
Participants, n	74	90	61	44	61	
Mean, g/L	4.08 (0.78)	4.20 (1.16)	4.23 (0.78)	4.38 (0.91)	4.26 (0.93)	
Coef.± Std. Err. (non-adjust)	Reference	0.01 (-0.05, 0.08)	0.04 (-0.03, 0.11)	0.07 (-0.01, 0.15)	0.04 (-0.03, 0.11)	0.12

Coef ± Std. Err. (adjusted Model 1)	Reference	0.02 (-0.06, 0.09)	0.04 (-0.05, 0.13)	0.05 (-0.06, 0.15)	0.04 (-0.05, 0.14)	0.30
Coef ± Std. Err. (adjusted Model 2)	Reference	0.01 (-0.08, 0.10)	0.04 (-0.06, 0.13)	0.04 (-0.07, 0.15)	0.05 (-0.05, 0.14)	0.28
Systolic blood pressure						
Participants, n	75	92	61	46	62	
Mean, mmHg	118.3 (22.3)	142.9 (20.1)	149.3 (23.1)	150.3 (20.7)	151.2 (22.9)	
Coef.± Std. Err. (non-adjust)	Reference	-5.43 (-12.09, 1.23)	0.99 (-6.39, 8.37)	1.98 (-6.04, 9.99)	2.90 (-4.45, 10.25)	0.11
Coef ± Std. Err. (adjusted Model 1)	Reference	-3.31 (-10.91, 4.29)	0.74 (-7.84, 9.32)	5.69 (-3.74, 15.13)	5.32 (-3.62, 14.25)	0.05
Coef ± Std. Err. (adjusted Model 2)	Reference	-5.35 (-13.30, 2.59)	-1.23 (-10.09, 7.62)	3.81 (-6.15, 13.76)	4.57 (-4.57, 13.72)	0.07
Diastolic blood pressure						
Participants, n	75	92	61	46	62	
Mean, mmHg	82.2 (12.1)	82.2 (10.3)	83.8 (11.6)	82.2 (12.1)	84.2 (10.9)	
Coef.± Std. Err. (non-adjust)	Reference	-0.00 (-3.47, 3.46)	1.57 (-2.27, 5.41)	-0.03 (-4.20, 4.14)	2.01 (-1.81, 5.83)	0.32
Coef ± Std. Err. (adjusted Model 1)	Reference	0.59 (-3.37, 4.56)	0.99 (-3.48, 5.46)	1.83 (-3.09, 6.75)	3.18 (-1.48, 7.84)	0.15
Coef ± Std. Err. (adjusted Model 2)	Reference	0.22 (-4.01, 4.45)	0.65 (-4.07, 5.36)	1.57 (-3.73, 6.87)	3.27 (-1.60, 8.14)	0.15

[1] All values are mean ± SD; Impaired glucose tolerance, i.e. fasting glucose ≥ 6.1 mmol/L.

[2] *P*-trend was assessed by linear regression (continuous variables) or by logistic regression (categorical variables), adjusted for age (continuous), BMI (continuous), energy intake (continuous), alcohol consumption (quartiles), smoking (never, past or current), energy expenditure (quartiles), social class (manual or non-manual), family history of myocardial infarction (yes or no).

[3] Adjustd as model 1 plus sugar intake (<50, 50-100, >100 g/d), fruit consumption (<7, 8-14, 15-21, or >21 times/wk), red meat consumption (<7, 8-14, 15-21, or >21 times/wk) and fibre (cereal and vegetable sources) (<10, 10-20, or >20 g/d).

[4] Original data were transformed to natural logarithms for regression model.

Supplemental Table 5 Cross-sectional analysis metabolic markers of adult males (19-64 y) across tertiles of egg consumption from the National Diet and Nutrition Survey (2009/10-2011/12)[1]

	Egg consumption (n)			
Characteristics	1 (n = 0 g/d)	2 (0< n ≤ 29 g/d)	3 (> 29 g/d)	*P*-trend
Fasting glucose				
Participants, n	356	204	187	
Mean, mmol/L	5.08 ± 0.80	5.17 ± 0.98	5.35 ± 1.70	
Coef.± Std. Err. (non-adjust)	Reference	0.10 (-0.10, 0.29)	0.27 (0.07, 0.47)	0.01
Coef ± Std. Err. (adjusted)[2]	Reference	0.12 (-0.04, 0.28)	0.21 (0.05, 0.39)	0.01
HbA1c				
Subjects, n	338	188	183	
HbA1c, mmol/L	5.45 ± 0.43	5.48 ± 0.47	5.65 ± 0.77	
Coef.± Std. Err. (non-adjust)	Reference	0.04 (-0.06, 0.13)	0.20 (0.10, 0.30)	<0.001
Coef ± Std. Err. (adjusted)	Reference	0.06 (-0.02, 0.14)	0.16 (0.08, 0.24)	<0.001
Total cholesterol				
Participants, n	345	194	185	
Mean, mmol/L	5.21 ± 1.06	5.37 ± 1.10	5.35 ± 1.15	
Coef.± Std. Err. (non-adjust)	Reference	0.15 (-0.04, 0.35)	0.14 (-0.05. 0.34)	0.12
Coef ± Std. Err. (adjusted)	Reference	0.09 (-0.10, 0.27)	0.05 (-0.14, 0.23)	0.53
Triglycerides				
Participants, n	345	194	185	
Mean, mmol/L	1.43 ± 1.25	1.30 ± 0.86	1.35 ± 0.83	
Coef.± Std. Err. (non-adjust)	Reference	-0.05 (-0.15, 0.05)	-0.01 (-0.11, 0.09)	0.71
Coef ± Std. Err. (adjusted)	Reference	-0.03 (-0.12, 0.66)	-0.06 (-0.16, 0.04)	0.22
HDL-cholesterol[3]				
Participants, n	345	194	185	
Mean, mmol/L	1.48 ± 0.43	1.56 ± 0.44	1.51 ± 0.45	
Coef.± Std. Err. (non-adjust)	Reference	0.06 (0.01, 0.11)	0.02 (-0.03, 0.07)	0.27
Coef ± Std. Err. (adjusted)	Reference	0.03 (-0.02, 0.07)	0.03 (-0.02, 0.08)	0.18
LDL-cholesterol				

Participants, n	334	190	183	
Mean, mmol/L	3.16 ± 0.92	3.27 ± 0.95	3.28 ± 1.02	
Coef.± Std. Err. (non-adjust)	Reference	0.11 (-0.06, 0.28)	0.13 (-0.05, 0.30)	0.12
Coef ± Std. Err. (adjusted)	Reference	0.07 (-0.09, 0.23)	0.04 (-0.13, 0.20)	0.57
Diastolic blood pressure				
Participants, n	354	202	187	
Mean, mmol/L	74.75 ± 11.71	74.93 ± 10.23	75.43 ± 10.26	
Coef.± Std. Err. (non-adjust)	Reference	0.17 (-1.73, 2.07)	0.68 (-1.27, 2.62)	0.51
Coef ± Std. Err. (adjusted)	Reference	0.12 (-1.72, 1.97)	0.21 (-1.68, 2.11)	0.82
Systolic blood pressure				
Participants, n	354	202	187	
Mean, mmol/L	124.43 ± 15.78	124.46 ± 14.87	127.16 ± 15.22	
Coef.± Std. Err. (non-adjust)	Reference	0.03 (-2.64, 2.69)	2.73 (-0.00, 5.46)	0.07
Coef ± Std. Err. (adjusted)	Reference	-0.01 (-2.42, 2.40)	0.81 (-1.67, 3.29)	0.56
Total/HDL ratio[3]				
Participants, n	345	194	185	
Mean, mmol/L	3.75 ± 1.36	3.61 ± 1.15	3.76 ± 1.25	
Coef.± Std. Err. (non-adjust)	Reference	-0.03 (-0.09, 0.03)	0.01 (-0.05, 0.06)	0.96
Coef ± Std. Err. (adjusted)	Reference	-0.01 (-0.06, 0.04)	-0.02 (-0.07, 0.03)	0.43
Pulse Pressure				
Participants, n	354	202	187	
Mean, mmol/L	71.30 ± 10.89	70.00 ± 9.77	70.51 ± 11.42	
Coef.± Std. Err. (non-adjust)	Reference	-1.30 (-3.16, 0.56)	-0.79 (-2.70, 1.11)	0.32
Coef ± Std. Err. (adjusted)	Reference	-0.76 (-2.60, 1.09)	-0.29 (-2.19, 1.60)	0.70
C-reactive protein				
Participants, n	345	194	185	
Mean, mmol/L	1.70 ± 1.01	1.66 ± 1.08	1.74 ± 1.06	0.50

[1] All values are mean ± SD.

[2] *P*-trend was assessed by linear regression (continuous variables) or by Pearson chi-square test (categorical variables), adjusted for age (continuous), food energy (continuous), alcohol consumption (tertiles), smoking (yes or no), sex (men or women), and incident of diabetes (yes or no).

[3] Original data were transformed to natural logarithms for regression model.

Supplemental Table 6 Cross-sectional analysis of metabolic markers across egg consumption in subjects with subjects type 2 diabetes and/or impaired glucose tolerance from the National Diet and Nutrition Survey (2009/10-2011/12)[1]

	Egg consumption (n)			
	1	2	3	
Characteristics	(n = 0 g/d)	(0< n ≤ 29 g/d)	(> 29 g/d)	*P*-trend
Fasting glucose				
Participants, n	25	18	16	
Mean, mmol/L	7.01± 1.53	7.20 ± 2.11	8.89 ± 4.34	
Coef.± Std. Err. (non-adjust)	Reference	0.19 (-1.50, 1.88)	1.88 (0.14, 3.63)	0.04
Coef ± Std. Err. (adjusted)[2]	Reference	0.51 (-1.24, 2.25)	2.25 (0.55, 3.95)	0.01
HbA1c				
Subjects, n	24	17	16	
HbA1c, mmol/L	6.15 ± 0.65	6.37 ± 0.75	7.01 ± 1.92	
Coef.± Std. Err. (non-adjust)	Reference	0.22 (-0.52, 0.97)	0.87 (0.11, 1.62)	0.03
Coef ± Std. Err. (adjusted)	Reference	0.36 (-0.38, 1.11)	1.10 (0.37, 1.83)	0.004
Total cholesterol				
Participants, n	25	17	16	
Mean, mmol/L	5.24 ± 1.51	4.98 ± 0.94	5.16 ± 1.04	
Coef.± Std. Err. (non-adjust)	Reference	-0.26 (-1.04, 0.52)	-0.07 (-0.87, 0.72)	0.79
Coef ± Std. Err. (adjusted)	Reference	-0.37 (-1.24, 0.50)	-0.26 (-1.10, 0.59)	0.51
Triglycerides				
Participants, n	25	17	16	
Mean, mmol/L	3.15 ± 3.03	1.78 ± 1.21	2.13 ± 1.29	
Coef.± Std. Err. (non-adjust)	Reference	-0.43 (-0.86, 0.00)	-0.20 (-0.64, 0.24)	0.28
Coef ± Std. Err. (adjusted)	Reference	-0.28 (-0.75, 0.19)	-0.19 (-0.65, 0.26)	0.37
HDL-cholesterol[3]				
Participants, n	25	17	16	
Mean, mmol/L	4.96 ± 2.34	3.99 ± 1.41	4.29 ± 1.19	
Coef.± Std. Err. (non-adjust)	Reference	-0.18 (-0.43, 0.06)	-0.08 (-0.34, 0.17)	0.42
Coef ± Std. Err. (adjusted)	Reference	-0.10 (-0.36, 0.17)	-0.08 (-0.33, 0.18)	0.52
LDL-cholesterol				
Participants, n	19	16	15	

Mean, mmol/L	2.92 ± 1.29	2.89 ± 0.82	3.06 ± 0.88	
Coef.± Std. Err. (non-adjust)	Reference	-0.03 (-0.74, 0.69)	0.15 (-0.58, 0.87)	0.70
Coef ± Std. Err. (adjusted)	Reference	-0.25 (-1.06, 0.55)	-0.05 (-0.84, 0.75)	0.91
Diastolic blood pressure				
Participants, n	24	18	16	
Mean, mmol/L	78.92 ± 10.88	77.17 ± 8.03	75.75 ± 7.46	
Coef.± Std. Err. (non-adjust)	Reference	-1.75 (-7.50, 4.00)	-3.17 (-9.12, 2.78)	0.28
Coef ± Std. Err. (adjusted)	Reference	-0.08 (-6.14, 5.98)	-2.53 (-8.39, 3.32)	0.40
Systolic blood pressure				
Participants, n	24	18	16	
Mean, mmol/L	132.83 ± 16.24	129.22 ± 9.13	128.31 ± 8.33	
Coef.± Std. Err. (non-adjust)	Reference	-3.61 (-11.39, 4.17)	-4.52 (-12.57, 3.53)	0.24
Coef ± Std. Err. (adjusted)	Reference	-0.60 (-9.04, 7.84)	-3.53 (-11.69, 4.63)	0.39
Total/HDL ratio[3]				
Participants, n	25	17	16	
Mean, mmol/L	4.96 ± 2.34	3.99 ± 1.41	4.29 ± 1.19	
Coef.± Std. Err. (non-adjust)	Reference	-0.18 (-0.43, 0.06)	-0.08 (-0.34, 0.17)	0.42
Coef ± Std. Err. (adjusted)	Reference	-0.10 (-0.36, 0.17)	-0.08 (-0.33, 0.18)	0.52
Pulse Pressure				
Participants, n	24	18	16	
Mean, mmol/L	73.79 ± 10.45	73.22 ± 9.84	73.31 ± 11.39	
Coef.± Std. Err. (non-adjust)	Reference	-0.57 (-7.15, 6.01)	-0.48 (-7.29, 6.34)	0.88
Coef ± Std. Err. (adjusted)	Reference	1.30 (-6.05, 8.65)	0.66 (-6.44, 7.77)	0.84
C-reactive protein				
Participants, n	25	17	16	
Mean, mmol/L	2.16 ± 0.90	1.88 ± 0.93	1.56 ± 0.73	0.13

[1] All values are mean ± SD; Impaired glucose tolerance, i.e. fasting glucose ≥ 6.1 mmol/L.

[2] *P*-trend was assessed by linear regression (continuous variables) or by Pearson chi-square test (categorical variables), adjusted for age (continuous), food energy (continuous), alcohol consumption (tertiles), smoking (yes or no), sex (men or women).

[3] Original data were transformed to natural logarithms for regression model.

Chapter 5 - Effect of production system, supermarket and purchase date on the vitamin D content of eggs at retail (Published: Food Chemistry 2016; 221:1021-5)

The present chapter aims to examine the vitamin D content (vitamin D_3 and 25(OH) D_3) of retail eggs in the UK, and possible effect of production system (indoor vs outdoor), supermarket and purchase date.

DIG designed the study. JG conducted the research with help from students Sarah Barnsley and Sophie Franks who collected the samples. JG wrote the manuscript. All authors read and approved the final manuscript.

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Effect of production system, supermarket and purchase date on the vitamin D content of eggs at retail

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ABSTRACT

The vitamin D content of eggs from three retail outlets was measured over five months to examine the effects of production system (organic vs. free range vs. indoor), supermarket and purchase date on the concentration of vitamin D_3 and 25-hydroxyvitamin D_3. Results demonstrated a higher vitamin D_3 concentration in free range (57.2 ± 3.1 μg/kg) and organic (57.2 ± 3.2 μg/kg) compared with indoor (40.2 ± 3.1 μg/kg) ($P < 0.001$), which was perhaps related to increased vitamin D synthesis by birds having more access to sunlight, while 25-hydroxyvitamin D_3 concentration was higher ($P < 0.05$) only in organic eggs. The interaction ($P < 0.05$) between system and supermarket for both forms of vitamin D may relate to some incorrect labelling. Concentration of 25-hydroxyvitamin D_3 was higher ($P < 0.05$) in July and September than in August. The results indicate variations in vitamin D concentrations in eggs from different sources, thus highlighting the importance of accurate labelling.

1. Introduction

The two major sources of vitamin D for humans are *in vivo* synthesis by exposure to sunlight and dietary intake. Holick and Chen (2008) reported the links of vitamin D deficiency with increased risk of many common and serious diseases, such as cardiovascular disease, osteoporosis, common cancers and diabetes, in addition to its association with calcium homeostasis. Maintaining a serum 25-hydroxyvitamin D (25(OH) D) concentration of at least 75 nmol/L is regarded as being necessary for prevention of most vitamin D-related diseases (Vieth, 2011). There are many factors which limit *in vivo* synthesis of vitamin D via ultraviolet radiation, such as a more indoor lifestyle, latitude, skin pigmentation, ageing and sunscreen use (Holick, 1995). Thus, the prevalence of vitamin D deficiency in Europe has become a very concerning issue (Cashman et al., 2016). In the UK, a study showed that 87% of 7437 white British participants (92% Scotland residents) had plasma concentrations of 25(OH) D of below 75 nmol/L during winter and spring (Hypponen & Power, 2007). Therefore, the vitamin D intake from dietary sources has become more important in maintaining adequate vitamin D status. However, only certain foods (e.g. fish, meat, offal, eggs) are naturally rich in vitamin D (Schmid & Walther, 2013), and many of these are not consumed widely.

Eggs contain, not only vitamin D_3, but also significant quantities of 25-hydroxyvitamin D_3 (25(OH) D_3) (Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1993; Schmid & Walther, 2013), with the accumulation of vitamin D in the egg yolk rather than egg white (Fraser & Emtage, 1976). Studies have shown that the 25(OH) D_3 metabolite is five times more effective at raising plasma 25(OH) D_3 concentration in humans and has been reported to be absorbed at a faster rate when compared with an equivalent dose of vitamin D_3 (Cashman et al., 2012; Jetter et al., 2013).

Recently, the vitamin D concentration of whole eggs was given as 3.2 μg/100 g in the UK official food database (McCance & Widdowson, 2015). Eggs are available from different husbandry production systems, including indoor, free-range and organic in the UK retail outlets (Department for Environment & Rural Affairs, 2010). Evidence from a previous enhancement study demonstrated that vitamin D in eggs was increased from birds exposed to ultraviolet radiation (Kühn, Schutkowski, Kluge, Hirche, & Stangl, 2014). Thus, vitamin D concentrations of eggs may vary due to different production systems which give the birds varying lengths of sunlight exposure. However, there are limited data on the vitamin D content of retail eggs from the different UK production systems. As customers will expect more expensive eggs to be of better quality, it is important to inform the consumer about the effect of different production systems on the nutritional

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composition of eggs. One previous UK study suggested that the vitamin D_3 concentration of hens' eggs was significantly affected by housing system, with the vitamin D_3 content of egg yolk produced outdoors being significantly higher (44.1–69.2 nmol/L) than that of egg yolk produced indoors (17.3–18.7 nmol/L) (Hobbs-Chell, Stickland, & Wathes, 2010). However, egg yolk 25(OH) D_3 concentration was not reported, and the study was not concerned with retail eggs.

The main objective of the current study was to explore the effects of production system (as labelled), supermarket and time of the year on the concentrations of vitamin D_3 and 25(OH) D_3 in the egg yolk from UK hens' eggs at retail. Although variation in vitamin D_3 content of eggs collected from UK farms due to production system has been reported (Hobbs-Chell et al., 2010), the data are unlikely to reflect eggs currently in the UK market. Accordingly, the current study focussed on the effect, not only of labelled production system, but also on supermarket and seasonal variation of two forms of vitamin D, vitamin D_3 and 25(OH) D_3 in UK retail eggs. This study also updates information on the vitamin D_3 and 25(OH) D_3 contents of eggs sold in the UK, which may improve the estimation of the contribution of eggs to vitamin D intakes of the general population.

2. Materials and methods

2.1. Sample collection

Eggs were purchased from three supermarkets (Supermarket 1, Supermarket 2, and Supermarket 3) in the Reading, Berkshire area, once per month, from July to November in 2012. On each occasion, packs of six eggs per box from three production systems (indoor, organic and free range, as identified on the label) were purchased from each supermarket, so a total of 270 eggs was collected. Following collection, eggs were transported directly to the laboratory, the yolks and whites of each egg were separated manually. The yolk was homogenised and decanted into 10 ml tubes before storage at −80 °C prior to vitamin D analysis. In total, 259 egg yolks (129 egg yolks for vitamin D_3 analysis; 130 egg yolks for 25(OH) D_3 analysis) were stored frozen, prior to analysis, as the egg whites and egg yolks of 11 eggs failed to separate during the processing. Nutritional information on the label of the purchased egg boxes was recorded for each sample.

2.2. Vitamin D_3 and 25(OH) D_3 analyses

The vitamin D_3 and 25(OH) D_3 concentrations of egg yolk samples were analysed by DSM Nutritional Products Ltd., (Basel, Switzerland). Vitamin D_3 analysis was carried out according to the method of Schadt, Gössl, Seibel, and Aebischer (2012).

The concentration of 25(OH) D_3 in the egg yolk samples was quantified by the standard method of the DSM Nutritional Products Ltd. using a LC-MS system (Agilent 1946). In brief, the sample was combined with d_6-25(OH) D_3 as an internal standard and the mixture dispersed in water. The suspension was extracted with tert-butyl methyl ether (TMBE). An aliquot of the TMBE phase was purified by semi-preparative normal-phase HPLC with a YMC-Pack-Sil column. An appropriate fraction was collected and analysed after solvent exchange by reversed-phase HPLC equipped with Aquasil C18 column and a mass selective detector.

2.3. Data analysis

A General Linear Model ANOVA (Minitab version 16; Minitab Inc., State College, PA, USA) was used to investigate the effect of (a) month of purchase (July to November 2012), (b) production system (indoor, organic or free-range) and (c) supermarket (S1, S2 or S3) on vitamin D_3 and 25(OH) D_3 concentrations. Tukey's pairwise multiple comparison test was used for *post hoc* analysis. Effects were considered significant when $P < 0.05$.

Total vitamin D concentration was calculated by using concentrations of vitamin D_3 + (5 × 25(OH) D_3) (McCance & Widdowson, 2015).

3. Results

3.1. Effect of production system

Concentrations of both vitamin D_3 and 25(OH) D_3 in egg yolk differed ($P < 0.001$), depending on production system (Table 1). Egg yolk from free range and organic systems contained a 42% greater concentration of vitamin D_3 than did those from the indoor system (Table 1). In addition, organic egg yolks had a higher ($P = 0.001$) concentration of 25(OH) D_3 than had egg yolks from free range and indoor systems, although no differences were observed between caged and free range systems.

3.2. Effect of purchase month

There was no effect of month purchased on the concentration of vitamin D_3 in egg yolks (Table 1; Fig. 1a). However, there was a significant effect of the system by month interaction ($P = 0.001$; Table 1), meaning that the vitamin D_3 concentration changes across different months varied by production system (Fig. 1a). The greatest ($P < 0.05$) concentration of vitamin D_3 in egg yolks tended to be found during summer months for indoor and organic eggs but, for free range eggs, the highest ($P < 0.05$) concentration was observed during the autumn months (Fig. 1a).

Month of collection had an effect ($P < 0.001$) on egg yolk 25(OH) D_3 concentration; however, as with vitamin D_3, no clear trend over time was observed (Fig. 1b). Again, a production system by month interaction was observed ($P = 0.001$; Fig. 1b). The lowest ($P < 0.05$) concentration of 25(OH) D_3 across all production systems was measured during August (Fig. 1b), but highest ($P < 0.05$) concentrations were observed during different months for each production system. In addition, no interaction ($P > 0.05$) was observed between supermarket and month on both vitamin D concentrations of the eggs.

3.3. Effect of supermarket

An effect of supermarket ($P = 0.009$) was observed for vitamin D_3 (Table 1; Fig. 2a) but not for 25(OH) D_3 (Table 1; Fig. 2b). The interaction effects of production system with supermarket were significant for both vitamin D_3 ($P < 0.001$) and 25(OH) D_3 ($P = 0.033$) (Table 1). For Supermarket 1, free range eggs were higher ($P < 0.05$) in vitamin D_3 concentration than were both caged and organic eggs. In addition, there was no interaction ($P > 0.05$) between supermarket and month for vitamin D_3 and 25(OH) D_3 (Table 1).

4. Discussion

4.1. General

The main objective of this study was to identify any differences in egg yolk vitamin D_3 or 25(OH) D_3 concentrations between three different production systems (indoor, free range and organic). To our knowledge, this is the first comparison study of both vitamin D forms between indoor and outdoor eggs from different UK retail supermarkets among varied months of the year.

Table 1
Concentrations of vitamin D_3 and 25(OH) D_3 (μg/100 g) of egg yolk as influenced by production system, month and supermarket (least square means ± pooled SE).

	Production system				*P* –Value for					
Vitamin D	Indoor	Free range	Organic	SEM	System	Month	Supermarket	System × month	System × supermarket	Supermarket × month
Vitamin D_3 (n = 130)	4.0[b]	5.7[a]	5.7[a]	0.3	<0.001	NS[1]	0.009	0.001	<0.001	NS
25(OH)D_3 (n = 129)	1.3[b]	1.4[b]	1.6[a]	0.06	0.001	<0.001	NS	0.001	0.033	NS
Total vitamin D[2]	10.4	12.6	13.8							

[a,b,c]Mean values with different superscripts within a row are significantly different (P < 0.05).
[1] NS, not significant (P > 0.05).
[2] Calculated as the sum of vitamin D_3 and (5 × 25(OH) D_3) concentrations (McCance & Widdowson, 2015).

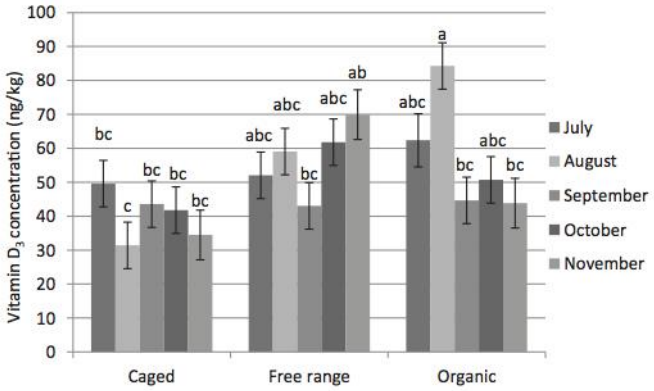

Fig. 1a. Effect of month on concentration (ng/kg) of vitamin D_3 in egg yolk from three production systems (least square means ± pooled SE). [a–d]Mean values with different letters are significantly different (*P* < 0.05) according to Tukey's pairwise multiple comparison test across all systems and months.

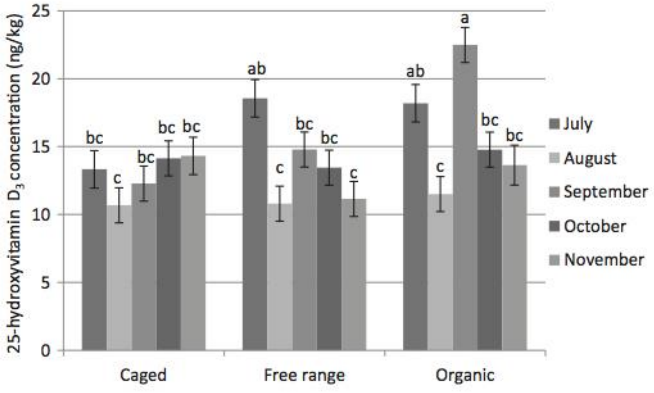

Fig. 1b. Effect of month on concentration (ng/kg) of 25-hydroxyvitamin D_3 in egg yolk from three production systems (least square means ± pooled SE). [a–d]Mean values with different letters are significantly different (*P* < 0.05) according to Tukey's pairwise multiple comparison test across all systems and months.

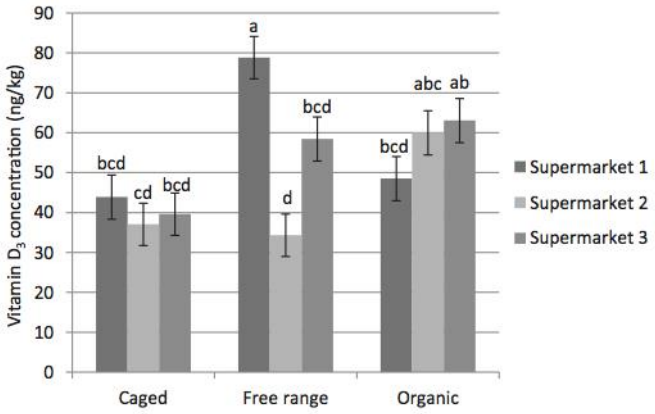

Fig. 2a. Effect of supermarket on concentration (ng/kg) of vitamin D_3 in egg yolk from three production systems (least square means ± pooled SE). [a–d]Mean values with different letters are significantly different (*P* < 0.05) according to Tukey's pairwise multiple comparison test across all systems and supermarkets.

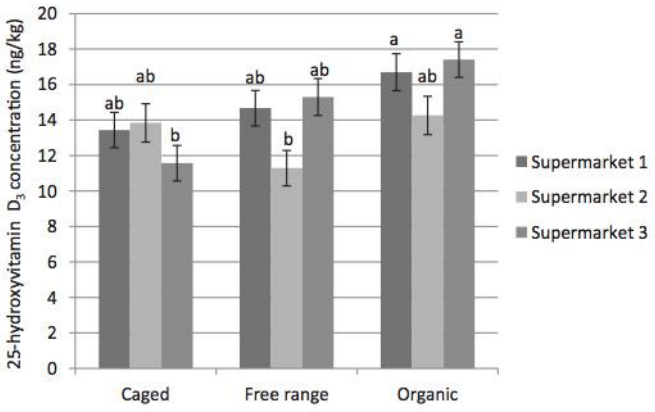

Fig. 2b. Effect of supermarket on concentration (ng/kg) of 25-hydroxyvitamin D_3 in egg yolk from three production systems (least square means ± pooled SE). [a–d]Mean values with different letters are significantly different (*P* < 0.05) according to Tukey's pairwise multiple comparison test across all systems and supermarkets.

4.2. Effect of production system

The vitamin D nutrition of birds is similar to that of humans (Bar, Sharvit, Noff, Edelstein, & Hurwitz, 1980); vitamin D is either synthesised *in vivo* by ultraviolet radiation from sunlight or consumed in the diet. In the UK, eggs produced by free range and indoor systems account for the majority of production systems (Department for Environment & Rural Affairs, 2013). Unlike the conventional indoor egg production system, free range and organic birds have more opportunity to be exposed to sunlight, as they can access pasture continuously during the day time with at least 4 square metres of range for one bird (RSPCA, 2014). As expected, the key finding of the current study is that both vitamin D_3 and 25(OH) D_3 were significantly different, according to production system. It is probable that the main reason for greater concentrations of vitamin D_3 and 25(OH) D_3 in eggs from free range and/or organic systems is higher sun exposure of the laying birds. Two previous studies also reported the effects of production systems on vitamin D concentration of eggs (Hobbs-Chell et al., 2010; Matt, Veromann, & Luik, 2009). Our results support previous UK data from Hobbs-Chell et al. (2010), who reported that eggs from free range and organic systems had higher vitamin D_3 concentrations than had those from a conventional indoor husbandry

system. However, an Estonian study (Matt et al., 2009) demonstrated that eggs from organic systems have lower vitamin D_3 content than have indoor eggs. The inconsistency in results of these earlier studies can probably be explained by the variation in production system management between different countries, such as the difference in the diet or pasture usage for the birds.

The variation of vitamin D_3 concentrations between production systems was of greater magnitude than that observed for 25(OH) D_3 concentrations in the current study. An enhancement study (Kühn et al., 2014) also reported that the concentration of 25 (OH) D_3 can be increased in response to sunshine exposure (free range vs indoor system) but the increase was less pronounced than that of vitamin D_3. In our study, no difference in the 25(OH) D_3 concentration of eggs was seen between the indoor and free-range eggs, but there was a significantly higher amount of the 25 (OH) D_3 in the organic eggs. The reason for this is unclear, as the vitamin D content of the diets from different production systems in the present study is not known.

If levels of vitamin D_3 and/or 25(OH) D_3 in eggs from free range and organic systems were consistently higher than those from a conventional production system; this would provide the consumer of free range and organic eggs with an advantage in terms of vitamin D intake and potentially status. However, the significant interaction between the production system and supermarket for both vitamin D_3 and 25(OH) D_3 reflects inconsistencies in the ranking of both vitamin D forms by production systems between different supermarkets. This may indicate that the diets fed to birds at the farms supplying each supermarket were different or maybe some incorrect labelling exist, which would result in egg choice according to production system being less valuable.

The interaction between production system and collection month may suggest that the vitamin D_3 and 25(OH) D_3 concentrations in eggs produced indoors were more consistent than were the concentrations in eggs from free range and organic systems, possibly due to less variability in vitamin D synthesis from sunlight, since indoor birds only obtain vitamin D from their diet. For free range and organic birds, the potentially beneficial effect of exposure to sunshine may introduce unpredictable and changeable influences on vitamin D concentrations in eggs. There are several studies that have shown that vitamin D_3 and 25(OH) D_3 in eggs can be enhanced effectively by supplementing indoor birds with vitamin D_3- and 25(OH) D_3-enriched diets (Browning & Cowieson, 2014; Mattila, Lehikoinen, Kiiskinen, & Piironen, 1999; Yao, Wang, Persia, Horst, & Higgins, 2013). Therefore, for greater enrichment of total vitamin D in eggs, the combination of enhanced vitamin D in the hen's diet, together with exposure to sunlight, may present opportunities in the future. It may be noted, however, that, within the EU, there are upper limits imposed on the concentrations of vitamin D_3 (75 μg/kg diet; European Commission, 2004) and 25(OH) D_3 (80 μg/kg diet; European Commission, 2009) that may be added to the diet of laying hens. Moreover, the total dietary concentrations of vitamin D_3 and 25 (OH) D_3 in poultry must not exceed 80 μg/kg diet (European Commission, 2009). These regulations may reduce the opportunity for dietary enrichment.

4.3. Effect of purchase month and supermarket

In terms of the seasonal effect on vitamin D content of the eggs, an earlier study (Mattila, Vakonen, & Valaja, 2011) reported that egg yolk vitamin D_3 and 25(OH) D_3 contents were not significantly different between the spring and autumn. Our results for vitamin D_3 agree with Mattila et al. (2011) in that vitamin D_3 did not vary with month, but 25(OH) D_3 was affected by purchase month. Surprisingly, the lowest concentration of 25(OH) D_3 was observed during August for all production systems. With the limitations of a retail study (such as not knowing farm locations, the diet and vitamin D status of the producing birds and weather conditions at these locations during egg production), the reason for effect of purchase month on 25(OH) D_3 is unclear. It might be that there was less sunshine, or the ambient temperature was too high for the birds to be outside in the year the eggs were produced, or changing of the vitamin D content of the feed. Other factors, such as fearfulness or stress, can also influence the length of outdoor time of the birds (Mahboub, Müller, & Von Borell, 2004).

Variations observed between supermarkets for vitamin D_3 in the current study may be related to different conditions employed by egg producers supplying the supermarkets. A similar finding was reported in an US-based retail study (Exler, Phillips, Patterson, & Holden, 2013) where eggs were collected from twelve supermarkets, which found a wider range of vitamin D_3 (0.71–12.1 μg/100 g) or 25(OH) D_3 (0.43–1.32 μg/100 g) content of the hen's eggs. Due to the nature of retail studies, it is difficult to assess the reasons why supermarket affected vitamin D_3 but not 25(OH) D_3 concentrations in the egg yolks. One possible reason may be variation of vitamin D_3 concentration of the birds' diet. Previous egg enrichment studies (Browning & Cowieson, 2014; Mattila et al., 1999) have shown that supplementing the birds' diet with vitamin D_3 can result in higher vitamin D_3 and 25(OH) D_3 concentrations of the egg yolk, but the increased 25(OH) D_3 content is much less than that of vitamin D_3. This may be because 25(OH) D_3 is a metabolite of vitamin D_3. In addition, Mattila et al. (2011) showed that supplementing birds with a high dose of 25(OH) D_3 only increased 25(OH) D_3 in the egg yolk but not vitamin D_3.

4.4. Vitamin D intake from eggs

The results from the current study indicate that the mean concentrations of vitamin D_3 and 25(OH) D_3 for egg yolk are 5.14 μg/100 g and 1.42 μg/100 g, respectively. Assuming that the bioactivity of 25(OH) D_3 is five times that of the same dose of vitamin D_3 (McCance & Widdowson, 2015), the mean total effective vitamin D concentration ($D_3 + (5 \times 25(OH)\ D_3)$) of the egg yolk in this study would be 12.25 μg/100 g, which agrees well with those of the most recently published data (Benelam et al., 2012) which reported a mean egg yolk vitamin D concentration ($D_3 + (5 \times 25$ (OH) $D_3)$) of 12.8 μg/100 g. Furthermore, if the average egg yolk weight 16.31 g is taken into account, one egg yolk in the current study contains a total of 2 μg vitamin D.

For UK adults aged up to 65 years, a daily 10 μg of vitamin D has been recommended by SACN (SACN, 2015). Thus, one egg per day, from the current study, would contribute about 20% of the RNI of vitamin D. It must be noted that cooking may lead to loss of vitamin D in eggs (Jakobsen & Knuthsen, 2014; Mattila, Ronkainen, Lehikoinen, & Piironen, 1999); thus, the cooking temperature and method need to be considered to avoid reduction of active vitamin D intake, and this suggests an area for further research.

4.5. Strengths and limitations of the study

Whilst the current study has limitations in terms of the relatively small sample of eggs, the new data on vitamin D in retail eggs from differing production systems provide new information of value to the UK public. This study only collected samples between July and November, which does not represent all seasonal changes throughout the whole year. Also, since all of the eggs were purchased from retail outlets with no indication of producer location, these data may not be totally representative of the UK. Furthermore, observed variations in yolk concentration due to purchase months and/or supermarkets were difficult to explain, given that the producer details, bird diets, farming practices and weather conditions at time of production were not known.

Subsequent investigations should study variations in vitamin D content of eggs from different producers throughout the UK, taking account the effects of the birds' diet and sun exposure.

5. Conclusions

Results from the current study confirm that vitamin D_3 and 25 (OH) D_3 concentrations in egg yolk vary over time and between production systems. Eggs from outdoor production systems are likely to contain higher amounts of both vitamin D forms, but this may not be a consistent effect. Future work is needed on eggs collected from different areas of the UK throughout the whole year to provide more information on vitamin D content of retail eggs. In addition, further studies should focus on identifying the reasons behind these variations to enable a greater understanding of how variation in vitamin D content can be minimised, for the benefit of the consumer.

The current study indicates that the average effective vitamin D content of each egg is about 2 μg (excluding any effect of factors such as cooking), which would mean that one egg per day would contribute 20% of the UK RNI for vitamin D. However, in the absence of up to date information on the vitamin D content of other relevant foods, such as fish and meat, it is difficult to reliably estimate vitamin D intake from the diet of the general population in the UK. So future retail studies should investigate the vitamin D content of other vitamin D- containing foods to improve estimates of dietary vitamin D intake of the UK population.

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Chapter 6 - Milk and dairy consumption and risk of cardiovascular diseases and all-cause mortality: dose-response meta-analysis of prospective cohort studies

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The present chapter aims to to examine linear and non-linear dose-response associations between milk and dairy products with CHD, CVD events and all-cause mortality using existing prospective cohort studies of adequate quality.

JG, AA, DIG, JAL, and SSSM designed the research. JG performed the literature search, extracted data. JG, SSSM checked data. JG performed the analyses and drafted the paper. AA, DIG, JAL, SSSM critically reviewed and improved it. JG is guarantor.

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META-ANALYSIS

Milk and dairy consumption and risk of cardiovascular diseases and all-cause mortality: dose–response meta-analysis of prospective cohort studies

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Abstract With a growing number of prospective cohort studies, an updated dose–response meta-analysis of milk and dairy products with all-cause mortality, coronary heart disease (CHD) or cardiovascular disease (CVD) have been conducted. PubMed, Embase and Scopus were searched for articles published up to September 2016. Random-effect meta-analyses with summarised dose–response data were performed for total (high-fat/low-fat) dairy, milk, fermented dairy, cheese and yogurt. Non-linear associations were investigated using the spine models and heterogeneity by subgroup analyses. A total of 29 cohort studies were available for meta-analysis, with 938,465 participants and 93,158 mortality, 28,419 CHD and 25,416 CVD cases. No associations were found for total (high-fat/low-fat) dairy, and milk with the health outcomes of mortality, CHD or CVD. Inverse associations were found between total fermented dairy (included sour milk products, cheese or yogurt; per 20 g/day) with mortality (RR 0.98, 95% CI 0.97–0.99; $I^2 = 94.4\%$) and CVD risk (RR 0.98, 95% CI 0.97–0.99; $I^2 = 87.5\%$). Further analyses of individual fermented dairy of cheese and yogurt showed cheese to have a 2% lower risk of CVD (RR 0.98, 95% CI 0.95–1.00; $I^2 = 82.6\%$) per 10 g/day, but not yogurt. All of these marginally inverse associations of totally fermented dairy and cheese were attenuated in sensitivity analyses by removing one large Swedish study. This meta-analysis combining data from 29 prospective cohort studies demonstrated neutral associations between dairy products and cardiovascular and all-cause mortality. For future studies it is important to investigate in more detail how dairy products can be replaced by other foods.

Keywords Dairy · Milk · Fermented dairy · All-cause mortality · Cardiovascular disease · Dose–response meta-analysis

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Introduction

Cardiovascular disease (CVD) is the leading cause of mortality and disability worldwide [1]. Together with smoking, obesity and inactivity, diet is considered to be one of the most important prevention strategies for CVD

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[2]. Milk and dairy foods have been recommended in most dietary guidelines around the world, but the association of milk or dairy food consumption with CVD is still controversial [3, 4]. An earlier meta-analysis [5] which included 17 prospective cohort studies showed that milk intake was not associated with total mortality or CHD mortality, but there was a borderline significant inverse association with CVD mortality based on limited studies. There were not enough data to examine the effects of other dairy products or milk fat content. Since then, further prospective cohort studies have been published. For example, one recent Swedish publication with two large Swedish cohorts [6] reported that higher milk consumption was associated with a doubling of mortality risk including CVD mortality in the cohort of women. Since this paper was published in 2014, there has been mounting debate from different researchers regarding its seemingly contradictory results [7, 8]. This has caused new uncertainty about the effects of milk and dairy intake on human health. Recently, new meta-analyses of dairy consumption and risk of stroke [9], butter and risk of CVD, diabetes and mortality [10] have been published, showing predominantly neutral or marginally beneficial associations for all dairy products. Therefore, we conducted a comprehensive dose–response meta-analysis to examine linear and non-linear associations between milk and dairy products with all-cause mortality, CHD and CVD events using existing prospective cohort studies of adequate quality.

Methods

Literature search and study selection

This review was conducted based on guidelines of Meta-analysis of Observational Studies in Epidemiology [11]. Prospective cohort studies published up to Sep 2016 (without language restriction) were searched using PubMed, Embase, and Scopus database, the query syntax of searching is shown in the Supplemental Methods (see search strategy). After excluding duplicates and based on titles and abstracts, we excluded studies on animals, baseline age $\leq$18 years, or populations with prior CVD, diabetes, or any other chronic diseases. Eligible studies were selected by using predefined inclusion criteria of prospective cohort studies, healthy populations and original articles on the association of milk and dairy intake and all-cause mortality, CHD or CVD. In addition, supplementary hand searching of reference lists of previous reviews or meta-analyses was conducted. Of 59 eligible full articles, 29 articles [6, 12–39] met the inclusion criteria (see Fig. 1). Several authors or coworkers provided additional data for this meta-analysis [14, 16, 19, 23, 27, 28, 32, 34, 37, 40].

Data extraction and quality assessment

Data were extracted from published articles by using a structured extraction form, which included descriptive characteristics of the study, range of intake, median intake, number of participants, number of mortalities, CHD or CVD cases, person-years at risk, and relative risk (RR) with 95% CI for each unit of dairy intake. For studies that reported results from different multivariable-adjusted models, the model with the most confounding factors was extracted for the meta-analysis. If dairy intake was presented in servings or times per period of time [12–20, 22, 23, 34–36, 39], we converted the portion size into grams per day by using standard units of 244 g for milk (585 g for 1 pint of milk); 244 g for yoghurt and 40 g for cheese [41, 42]. One serving of total dairy, high-fat dairy and low-fat dairy was taken to be 200 g, similar to our previous meta-analysis [5]. When studies reported country specific conversion factors, these were used to calculate intake as g/day [26, 29, 30].

In some studies the mean intakes of dairy categories were not reported, in which case we calculated the mean value by using the lower and upper limit. For open-ended upper limits of intake, the same range as the lower category was applied. The categories of dairy types were defined in accordance with the definition in the original articles (Supplemental Table 2).

Two independent reviewers determined the quality of the 29 studies based on the Newcastle–Ottawa quality assessment scale (NOS, Supplemental Methods) [43]. By evaluation of selection, comparability and outcome, the rating system scores studies from 0 (highest degree of bias) to 9 (lowest degree of bias). Additionally we investigated the funding sources of all of the eligible studies. The four categories of funding were recorded as industry, partial funded by industry, research institution and unknown.

Statistical analysis

Meta-analyses of each dairy type were performed if the number of studies was three or more. Splined variables were generated by MKSPLINE in STATA version 13.0 to determine the most appropriate knot points of nonlinear associations from goodness-of-fit tests and Chi square statistics. Spine analysis and dose–response generalised least-square trend (GLST) meta-analysis were applied for the further analysis of linear or nonlinear associations. Incremental dose–response RRs were derived from the random-effects meta-regression trend estimation of summarised dose–response data. Ding's spaghetti plot was used to present the shapes of the association within individual studies, as described previously [44]. Forest plots were created to assess the linear dose–response slopes and

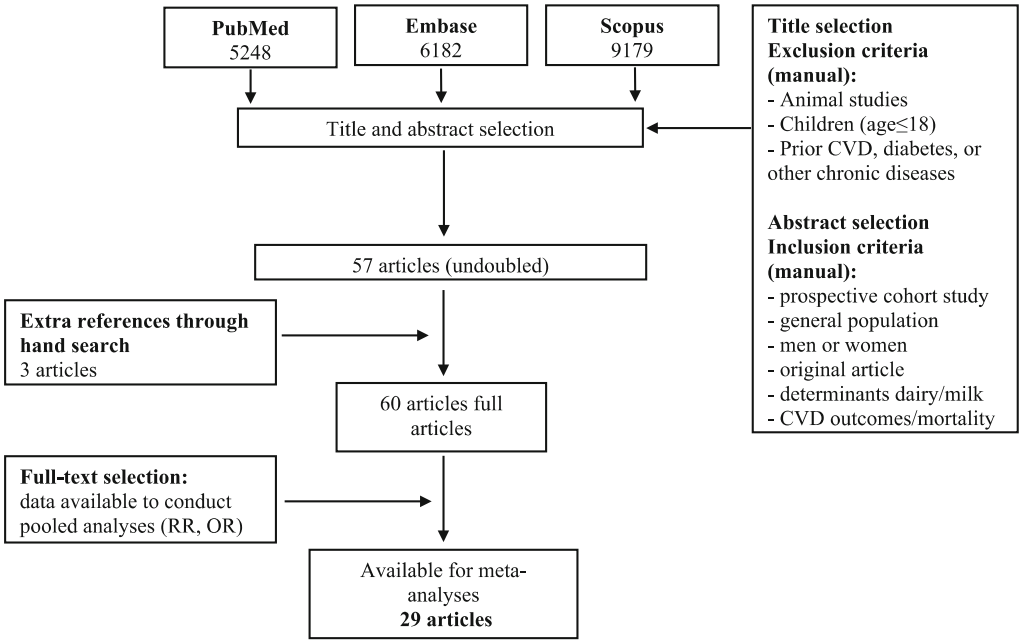

Fig. 1 Flowchart of meta-analysis on dairy consumption and incident CVD, CVD mortality and all-cause mortality

corresponding 95% CI across relative studies with increments of 200 g/day for total, high-fat, and low-fat dairy; 244 g/day for milk; 20 g/day for total fermented dairy (includes cheese, yogurt and soured milk products); 10 g/day for cheese; 50 g/day for yogurt. Sensitivity analysis was based on linear dose–response slopes by excluding one study population at a time.

To explore heterogeneity between studies, I-squared was calculated from Cochrane Q test [45]. In addition, sub-group analyses were performed providing that at least 6 study populations were available by age (≤50 years, >50 years), follow-up duration (≤10 years, >10 years), gender (men, women, both men and women), continent, confounding factors (whether analyses were or were not adjusted for the following 7 confounders age, sex, smoking, alcohol, body mass index (BMI), physical activity, food energy intake), BMI (≤25 kg/m^2, >25 kg/m^2) and Newcastle–Ottawa quality score < or ≥7. When number of the examined studies ≥10, potential publication bias was assessed by means of the Eggers test [46] and symmetry of the funnel plot. All of the statistical analyses were performed in STATA version 13.0 (StataCorp. College Station, Texas, USA). Two-sided *P* values <0.05 were considered as statistically significant.

Results

Overviews of key characteristics of the 29 prospective cohort studies are shown in Table 1. The included participants of each dairy exposure data on all-cause mortality, CHD or CVD are presented in Table 2. A total of 783,989 participants, 93,158 mortality cases, 28,419 CHD and 25,416 CVD were included in the analysis. There were 3 studies conducted in Asia (Japan and Taiwan) [28, 35, 39], 2 studies in Australia [24, 29], 7 in the United States [12, 14–16, 19, 22, 34] and the remaining 17 studies in Europe. A total of 6 studies presented sex-specific results, 3 studies were in men [18–20] and 3 in women [15, 16, 30]. There was one study [12] with missing data on age and 4 studies with missing BMI data [12, 21, 33, 36]. The estimated mean age was 57 years (range 34–80 years) and mean value of BMI was 25.4 kg/m^2 (range 22.3–27.1 kg/m^2). The duration of follow-up ranged from 5 to 25 years, with a mean follow-up of 13 years. Study characteristics of each dairy intake category by outcomes are shown in Table 2. Results of quality assessment are shown in the Supplemental Table 1, with 18 studies scoring ≥7. All of the studies were funded by a research institute except one study [13] without funding information, thus sub-group analysis was not conducted by funding source. There was no evidence of publication bias in the meta-analyses of milk or dairy consumption with different health outcomes (Supplemental Figs. 19–27).

Total, high-fat, and low-fat dairy

Total dairy intake (per 200 g/day) was not associated with the risk of all-cause mortality (Supplemental Figure 1; RR 0.99, 95% CI 0.96–1.03, 10 populations), CHD (Supplemental Figure 2; RR 0.99, 95% CI 0.96–1.02, 12 populations) or CVD (Supplemental Figure 3; RR 0.97, 95% CI 0.91–1.02). Considerable heterogeneity was observed in the meta-analyses of mortality (I^2 = 62.2%,

Table 1 Characteristics of 29 prospective cohort studies on dairy consumption and CHD, CVD risk or mortality

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Kahn et al. [12]	California Seventh-Day Adventists, USA	40	–	–	21	6180 deaths	27,530	Milk, Cheese	FFQ (unvalidated)	All-cause mortality; deaths were matched by computer tapes	Age, sex, smoking history, history of major chronic disease
Mann et al. [13]	Vegetarian, semi-vegetarians, and meat eaters; UK	38	34	22.3	13.3	392 deaths (64 fatal IHD)	10,802	Milk, Cheese	FFQ (unvalidated)	All-cause mortality, fatal IHD; National Health Service Central Register, causes of death was coded by investigator blinded	Age, sex, smoking, social class
Appleby et al. [14]	Oxford Vegetarian Study; UK	–	34	22.3	12	63 fatal CHD	10,800	Milk, Cheese	Simple FFQ (unvalidated)	Fatal IHD; National death certificate	Age, sex, smoking, socioeconomic status
Bostick et al. [15]	Postmenopausal women, Iowa; USA	0	61.5	26.8	8	387 fatal IHD	34,486	Total dairy, High-fat dairy	FFQ (validated)	Fata IHD; Registry and follow-up questionnaire	Age, total energy intake, body mass index, waist: ratio, history of diabetes mellitus, cigarette smoking status, postmenopausal oestrogen use, alcohol intake, education, marital status, physical activity, dietary vitamin E and saturated fat intake
Hu et al. [16]	Nurses' Health Study; USA	0	46.5	24.2	14	939 CHD	41,254	Total dairy, High-fat dairy, Low-fat dairy, Milk	FFQ (validated)	CHD (fatal and nonfatal); medical records reviewed by physicians blind to risk factors; deaths from registry, jps[ota; records or autopsy	Age, time period, BMI, smoking, menopausal status (including hormone replacement therapy), parental history of MI, vitamin E supplement, alcohol, history of hypertension, aspirin, physical activity, total energy intake
Fortes et al. [17]	Elderly residents from public home in Rome; Italy	32	80	25.6	5	53 deaths	162	Cheese	FFQ (validated)	All-cause mortality; Registry	Age, sex, education, BMI, smoking, cognitive function, chronic diseases

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Ness et al. [18]	Working men in west of Scotland; UK	100	48	25.3	25	2350 deaths (1212 fatal CVD, 892 fatal CHD)	5765	Milk	Questionnaire (check by interview)	All-cause mortality, fatal CVD, fatal CHD; National Healthy Service Central Registry	Age, smoking, BP, cholesterol, BMI, forced expiratory volume, social class, education, deprivation, siblings, car user, angina, ECG ischemia, bronchitis, alcohol
Al-Delaimy et al. [19]	Health Professionals Follow-up Study	100	53	25.4	12	14,468 IHD (fatal and non-fatal)	39,800	Total dairy, High-fat dairy, Low-fat dairy, Milk	FFQ (validated)	IHD (fatal and nonfatal); medical records reviewed, autopsy reports, death certificates	Age, time period, energy intake, history of diabetes, history of hypercholesterolemia and hypertension, family history of MI, smoking history, aspirin, BMI, alcohol intake, physical activity, vitamin E, trans fatty acids, PUFA:SFA ratio, total protein intake, fibre, folate, n-3 fatty acids, and a-linolenic acid
Elwood et al. [20]	Caerphilly cohort; UK	100	52	26.1	22	811 deaths, 628 CVD, 493 IHD	2512	Milk	FFQ (validated)	All-cause mortality, CVD (fatal and nonfatal), IHD (fatal and nonfatal); ECG examination, GP and hospital records	Age, total energy, smoking, social class, BMI, systolic BP, alcohol and fat, prior vascular disease
Knoops et al. [21]	HALE study (combination of SENECA and FINE studies)	66	75	–	10	1382 deaths	3117	Total dairy	Dietary history	All-cause mortality; general practitioners and/or hospital registers or vital status	Age, sex, alcohol, physical activity, smoking, number of years of education, BMI, chronic diseases, study centre
Paganini-Hill et al. [22]	Leisure World Cohort Study; USA	37	74	23.5	23	11,386 deaths	13,624	Milk	Questionnaire (unvalidated)	All-cause mortality; hospital discharge data, death indexes and ascertainment of death certificates	Age, sex, smoking, exercise, BMI, alcohol, hypertension, angina, MI, stroke, diabetes, rheumatoid arthritis, cancer

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Engberink et al. [40]	The Rottedam Study, Netherlands	38	66.9	26.2	11.2	1111 death (307 from CVD)	3971	Total dairy, High-fat dairy, Low-fat dairy, Cheese	FFQ (validated)	All-cause mortality, CVD mortality; medical record and digital record linkage	Age, sex, BMI, SBP, total cholesterol, family history of MI, use of oestrogen, smoking, educational level, alcohol consumption, total energy, saturated fat, intake of fruit, vegetables, meat, fish, coffee, and tea
Panagiotakos et al. [23]	ATTICA Study; Greece	50	53	27	5	30 CVD (fatal and non-fatal)	686	Total dairy, Cheese, Yogurt, Milk	FFQ (validated)	CVD (fatal and non-fatal); medical records	Age, sex, BMI, hypertension, diabetes, hypercholesterolemia, current smoking, physical activity
Bonthuis et al. [24]	Community-based sample, Australia	43	49.8	26.2	14.4	177 death (61 from CVD)	1529	Total dairy, High-fat dairy, Low-fat dairy, Milk, Yogurt, Full-fat cheese	FFQ (validated)	All-cause mortality, CVD mortality; National Death Index of Australia	Age, sex, total energy intake, body mass index, alcohol intake, school leaving age, physical activity level, pack years of smoking, dietary supplement use, b-carotene treatment during trial, presence of any medical condition, and dietary calcium intake.
Goldbohm et al. [25]	Netherland Cohort Study	48	61.6	24.4	10	16,136 death (2689 from IHD)	120,852	Total dairy, High-fat dairy, Low-fat dairy, High-fat fermented dairy, Low-fat fermented dairy, Cheese	150 item FFQ (validated)	All-cause mortality, IHD mortality; Dutch Central Bureau of Genealogy and the Dutch Central Bureau of Statistics	Age, education, cigarette, cigar, and pipe smoking, nonoccupational physical activity, occupational physical activity, BMI, multivitamin use, alcohol, energy, energy-adjusted mono- and polyunsaturated fat intakes, and vegetable and fruit consumption

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Sonestedt et al. [26]	The Malmo diet and cancer cohort, Sweden	38	57.3	25.2	12	2520 CVD	26,445	Total dairy, High-fat dairy, Low-fat dairy, Fermented dairy, Milk, Cheese	Dietary assessment method	CVD (fatal and non-fatal)	Sex, season, method, energy intake, BMI, smoking, alcohol consumption, leisure-time physical activity, and education
Dalmeijer et al. [27]	EPIC-NL; Netherlands	25.5	48.7	25.6	10	1184 death, 1807 total CVD, 1309 total CHD,	33,625	Total dairy, High-fat dairy, Low-fat dairy, Fermented dairy, Cheese	79-item FFQ (validated)	All-cause mortality, CVD (fatal and nonfatal), CHD (fatal and nonfatal); Register of hospital discharge diagnoses	Gender, age, total energy intake, physical activity, smoking, education, BMI, ethanol, coffee, fruit, vegetables, fish, meat and bread
Kondo et al. [28]	National Integrated Project for Prospective Observation of Non-communicable Disease And its Trends in the Aged, Japan	44	50.3	22.7	24	893 CVD death, 174 CHD death;	9243	Milk	Weighed diet records and dietary interviews	CVD mortality, CHD mortality; follow-up surveys	Age, body mass index, smoking status, alcohol drinking habit, history of diabetes, use of antihypertensive, work category, and total energy intake
Soedamah-Muthu et al. [31]	Whitehall II Study, United Kingdom	72	56	25.9	10	323 CHD; 237 all-cause mortality	4526	Total dairy, High-fat dairy, Low-fat dairy, Milk, Fermented dairy, Cheese, Yogurt	114 item FFQ (validated)	All-cause mortality, CHD (fatal and non-fatal); Death was collected from NHS Central Registry, cases of MI were identified from twelve-lead electrocardiograms	Age, ethnicity and employment grade, smoking, alcohol intake, BMI, physical activity and family history of CHD/ hypertension, fruit and vegetables, bread, meat, fish, coffee, tea and total energy intake

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Louie et al. [29]	The Blue Mountain Eye Study, Australia	44	65.4	26.2	15	1048 death (548 from CVD, 432 from CHD)	2900	Total dairy, High-fat dairy, Low-fat dairy	145-item FF1 (validated)	CVD mortality, CHD mortality; Australian National Death Index	Age, sex, total energy, baseline BMI, change in weight during follow up, physical activity level (METs), previous acute myocardial infarction, previous stroke, smoking status, stage II hypertension, type 2 diabetes status, use of antihypertensive medication, use of statins and change in dairy intake
Ruesten et al. [33]	EPIC-Potsdam Study; German	39	50	–	8	363 CVD	23,531	High-fat dairy, Low-fat dairy, High-fat cheese, Low-fat cheese	FFQ (validated)	CVD (fatal and non-fatal); self-administered follow-up questionnaires and medically verified	Age, sex, smoking status, pack-years of smoking, alcohol consumption, leisure-time physical activity, BMI, waist-to-hip ratio, prevalent hypertension at baseline, history of high blood lipid levels at baseline, education, vitamin supplementation and total energy intake
Van Aerde et al. [32]	The Hoorn Study; Netherlands	43.8	61.1	26.5	12.4	403 death (116 from CVD, 50 from CHD)	1956	Total Dairy, High-fat dairy, Low-fat dairy, Milk, Fermented dairy, Cheese	92-item FFQ (validated)	All-cause Mortality, fatal CVD, fatal CHD; General practitioners and the local hospital	Age, sex, BMI, Smoking, educational level, total energy intake, alcohol consumption, physical activity and intake of meat, fish, bread, vegetables, fruit, coffee, and tea

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Patterson et al. [30]	Swedish Mammography cohort, Sweden	0	61.2	24.9	11.6	1392 MI	33,636	Total dairy, Milk, Fermented dairy, Low-fat fermented dairy, High-fat fermented dairy, Cheese	96-item semi quantitative FFQ (validated)	Incident cases of MI (fatal and nonfatal); Registry and record linkage	Smoking status, physical activity, waist-to-hip ratio, alcohol consumption, diagnosis of hypertension, diagnosis of high cholesterol, family history of myocardial infarction, education, aspirin usage, hormone therapy usage, energy intake, all other dairy food groups, fruit and vegetables and whole-grain foods, use of oils in cooking, and use of low-fat margarine on bread
Huang et al. [35]	Nutrition and Health Survey in Taiwan, Taiwan	–	35.6	22.9	13.7	444 death (87 from CVD)	3810	Total dairy	FFQ (validated)	All-cause mortality, CVD mortality; National death registration	Age, gender, BMI, region, ethnicity, education level, marriage, history of disease (cardiovascular disease and/or cancer), smoking, drinking, chew betel nut, and supplement use, overall Dietary Index–Revised (dairy score excluded), Calcium intake, vitamin D intake

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Haring et al. [34]	Atherosclerosis Risk in Communities Study; USA	44.2	53.8	27.1	22	1147 CHD	12,066	Total dairy, High-fat dairy, Low-fat dairy	FFQ (unvalidated)	CHD (fatal and non-fatal); study visits, yearly telephone follow-up calls, review of hospital discharge lists and medical charts, death certificates, next-of-kin interviews, and physician-completed questionnaires	Age, sex, race, study centre, total energy intake, smoking, education, systolic blood pressure, use of antihypertensive medication, HDL-cholesterol, total cholesterol, use of lipid lowering medication, body mass index, waist-to-hip ratio, alcohol intake, sports-related physical activity, leisure-related physical activity, carbohydrate intake, fibre intake, and magnesium intake
Michaelsson et al. [6]	Swedish Mammography Cohort, Sweden/Cohort of Swedish Men, Sweden	0/100	53.7/60.3	24.7/25.8	20.1/11.2	15,541 death (5278 death from CVD)/10,112 death (4568 death from CVD)	61,433/45,339	Milk, Cheese, Fermented dairy	FFQ (validated)	All-cause mortality and CVD mortality; Swedish cause of death registries	Age, body mass index, height, total energy intake, total alcohol intake, healthy dietary pattern, calcium and vitamin D supplementation, ever use of cortisone, educational level, living alone, physical activity level estimated as metabolic equivalents, smoking status, and Charlson's comorbidity index; and in women additionally for use of oestrogen replacement therapy and nulliparity

Table 1 continued

References	Study, country	Men (%)	Mean age, year	Mean BMI, kg/m^2	Follow-up time	No. of cases	No. of subjects	Dairy types included in meta-analysis	Dietary assessment	Outcome; ascertainment	Main confounders
Bergholdt et al. [36]	Copenhagen General Population Study; Denmark	12	56.7	–	5.4	2777 IHD	74,965	Milk	Self-reported questionnaire	IHD (fatal and nonfatal); National DANISH Patient Registry	Age, sex, physical activity in leisure time and at work, smoking, alcohol intake, use of lipid-lowering therapy, fruit, vegetables, fish, fast food, and soda drinks
Praagman et al. [37]	the Rotterdam Study, Netherlands	38	66.9	26.2	17.3	567 CHD (350 fatal)	4235	Total dairy, High-fat dairy, Low-fat dairy, Fermented dairy, Cheese, Yogurt	FFQ (validated)	Total CHD and fatal CHD; medical record and digital record linkage	Age, gender, and total energy intake, BMI, smoking, education level, and alcohol intake, intakes of vegetables, fruit, meat, bread, fish coffee, and tea
Praagman et al. [38]	EPIC-Netherlands cohort	57	48.9	25.6	15	2436 death (727 from CVD, 253 from CHD)	34,409	Fermented dairy, Yogurt, Cheese	FFQ (validated)	All-cause mortality, CVD mortality, CHD mortality; Record linkage and Central Agency for statistics	Age, sex, total energy intake, smoking habit, BMI, physical activity, education level, hypertension at baseline, intakes of alcohol and energy-adjusted intakes of fruit and vegetables
Wang et al. [39]	Japan Collaborative Cohort Study, Japan	42	56.8	22.7 (men); 22.9 (women)	19	21,775 death (6271 death from CVD)	94,980	Milk	Self-administered questionnaires	All-cause mortality and CVD mortality; The date and cause of death were confirmed with the permission of the Director-General of the Prime Minister's Office	Age categories, smoking status, drinking status, physical activity, sleeping duration, body mass index, education level, participation in health check-ups, green-leafy vegetable intake, and history of hypertension, diabetes, and liver disease

BMI body mass index, *CVD* cardiovascular disease, *CHD* coronary heart disease, *FFQ* food frequency questionnaire

Table 2 Characteristics and results of linear and nonlinear dose response meta-analyses of dairy exposures

Dairy type (increment g/day)	Outcome	No studies (populations)	Mean age (years)	Mean BMI (kg/m^2)	median intake range (g/day)	Total N	No events	RR (95% CI)	Heterogeneity I^2 (%), *P*
Total dairy (per 200 g/day)	Mortality	9 (10)	57.2	25.2	323 (0–713)	175,063	21,222	0.99 (0.96, 1.03)	62.2, 0.005
	CHD	11 (12)	57.4	25.8	360 (20–828)	330,350	8298	0.99 (0.96, 1.02)	38.9, 0.081
	CVD	8	54.4	25.6	339 (0–713)	76,207	5525	0.97 (0.91, 1.02)	59.9, 0.015
High-fat dairy (per 200 g/day)	Mortality	5	56.7	26.0	113 (20–339)	47,126	3407	0.96 (0.88, 1.05)	0.0, 0.603
	CHD	9	55.9	25.9	151 (19–586)	171,627	6661	0.99 (0.93, 1.05)	22.9, 0.240
	CVD	7	57.7	25.9	130 (8–414)	95,242	5408	0.93 (0.84, 1.03)	37.4, 0.143
Low-fat dairy (per 200 g/day)	Mortality	6 (7)	58.5	25.4	217 (0–554)	167,978	19,543	1.01 (0.99, 1.03)	0.0, 0.734
	CHD	9 (10)	55.5	25.7	234 (0–825)	262,228	6244	1.00 (0.97, 1.03)	27.3, 0.193
	CVD	7	57.7	25.9	211 (0–604)	95,242	5408	0.98 (0.95, 1.01)	0.0, 0.769
Milk (per 244 g/day)	Mortality	10 (12)	55.5	24.6	268 (0–878)	268,570	69,355	1.00 (0.93, 1.07)	97.4, <0.001
	CHD	11 (12)	51.1	24.5	227 (0–877)	230,621	8612	1.01 (0.96, 1.06)	45.5, 0.043
	CVD	9 (12)	54.6	24.8	245 (0–878)	249,779	21,580	1.01 (0.93, 1.10)	92.4, <0.001
Fermented dairy (per 20 g/day)	Mortality	11 (19)	57.0	25.2	70 (0–500)	378,058	98,536	0.98 (0.97, 0.99)	94.4, <0.001
	CHD	9 (14)	53.7	25.0	96 (0–417)	256,091	5667	0.99 (0.98, 1.01)	44.6, 0.037
	CVD	9 (17)	54.8	25.8	105 (0–627)	271,071	33,980	0.98 (0.97, 0.99)	87.5, <0.001
Cheese (per 10 g/day)	Mortality	11 (13)	57.2	25.2	25 (1–70)	342,120	54,125	0.99 (0.96, 1.01)	93.3, <0.001
	CHD	9 (10)	53.8	25.0	34 (3–192)	256,091	4022	0.99 (0.97, 1.02)	40.3, 0.089
	CVD	9 (11)	55.3	25.8	34 (0–103)	234,447	15,519	0.98 (0.95, 1.00)	82.6, <0.001
Yogurt (per 50 g/day)	Mortality	3	51.3	25.9	46 (0–145)	40,460	2850	0.97 (0.85, 1.11)	65.8, 0.054
	CHD	3	56.4	25.9	60 (0–145)	98,936	1143	1.03 (0.97, 1.09)	0.0, 0.685
	CVD	3	50.6	26.3	147 (0–627)	36,624	817	1.03 (0.97, 1.09)	0.0, 0.499

$P = 0.005$) and CVD ($I^2 = 59.9\%$, $P = 0.015$) but not CHD ($I^2 = 38.9\%$, $P = 0.081$). In sensitivity analyses, heterogeneity among studies of the mortality could be reduced to 50% ($P = 0.042$) with a RR of 1.00 (95% CI 0.97–1.04) by excluding the study of Soedamah-Muthu et al. [31]; the heterogeneity among studies of CVD was reduced ($I^2 = 11.2$, $P = 0.338$) after removing the study of Hu et al. [16] with a resulting RR of 0.98 (95% CI 0.96–1.00). Sub-group analyses of CHD (Supplemental Table 4) indicated inverse associations for study populations with a mean age >50 years (RR 0.97, 95% CI 0.94–1.00, 8 populations) and also for studies which did not adjust for 7 major confounders defined in methods as age, sex, smoking, alcohol, BMI, physical activity, food energy intake (RR 0.94, 95% CI 0.88–1.00, 3 populations).

High-fat dairy intake (per 200 g/day) showed no association with mortality (Supplemental Figure 4; RR 0.96, 95% CI 0.88–1.05, 5 populations), CHD (Supplemental Figure 5; RR 0.99, 95% CI 0.93–1.05, 9 populations) or CVD (Supplemental Figure 6; RR 0.93, 95% CI 0.84–1.03, 7 populations), and there was no significant heterogeneity. In sensitivity analyses of the association between high-fat dairy and CHD, I-squared was reduced from 22.9% ($P = 0.240$) to 0% ($P = 0.464$) with results of RR 1.01, 95% CI 0.96–1.06) after removing the study of Dalmeijer et al. [27]. Also, sensitivity analyses of the association between high-fat dairy and CVD showed I-squared reduced to 0% ($P = 0.143$) with results of RR 0.98 (95% CI 0.93–1.03) after excluding study Bonthuis et al. [24]. Sub-group analysis of CVD by age showed a stronger inverse association between high-fat dairy intake and CVD risk in the subjects ≤50 years (RR 0.76, 95% CI 0.59–0.97, 3 populations), although the sample size was small. There was no heterogeneity ($I^2 = 31.5\%$, $P = 0.232$).

Low-fat dairy intake (per 200 g/day) was not significantly associated with mortality (Supplemental Figure 7; RR 1.01, 95% CI 0.99–1.03, 7 populations), CHD (Supplemental Figure 8; RR 1.00, 95% CI 0.97–1.03) or CVD (Supplemental Figure 9; RR 0.98, 95% CI 0.95–1.01). No heterogeneity was found in the meta-analysis on low-fat dairy. In the sub-group analysis for CVD (Supplemental Table 5) on subjects whose BMI > 25 kg/m^2, low-fat dairy intake was inversely associated with the risk of CVD (RR 0.97, 95% CI 0.94–1.00, 6 populations).

Milk

Milk intake (per 244 g/day, 12 populations) was not associated with all-cause mortality (Supplemental

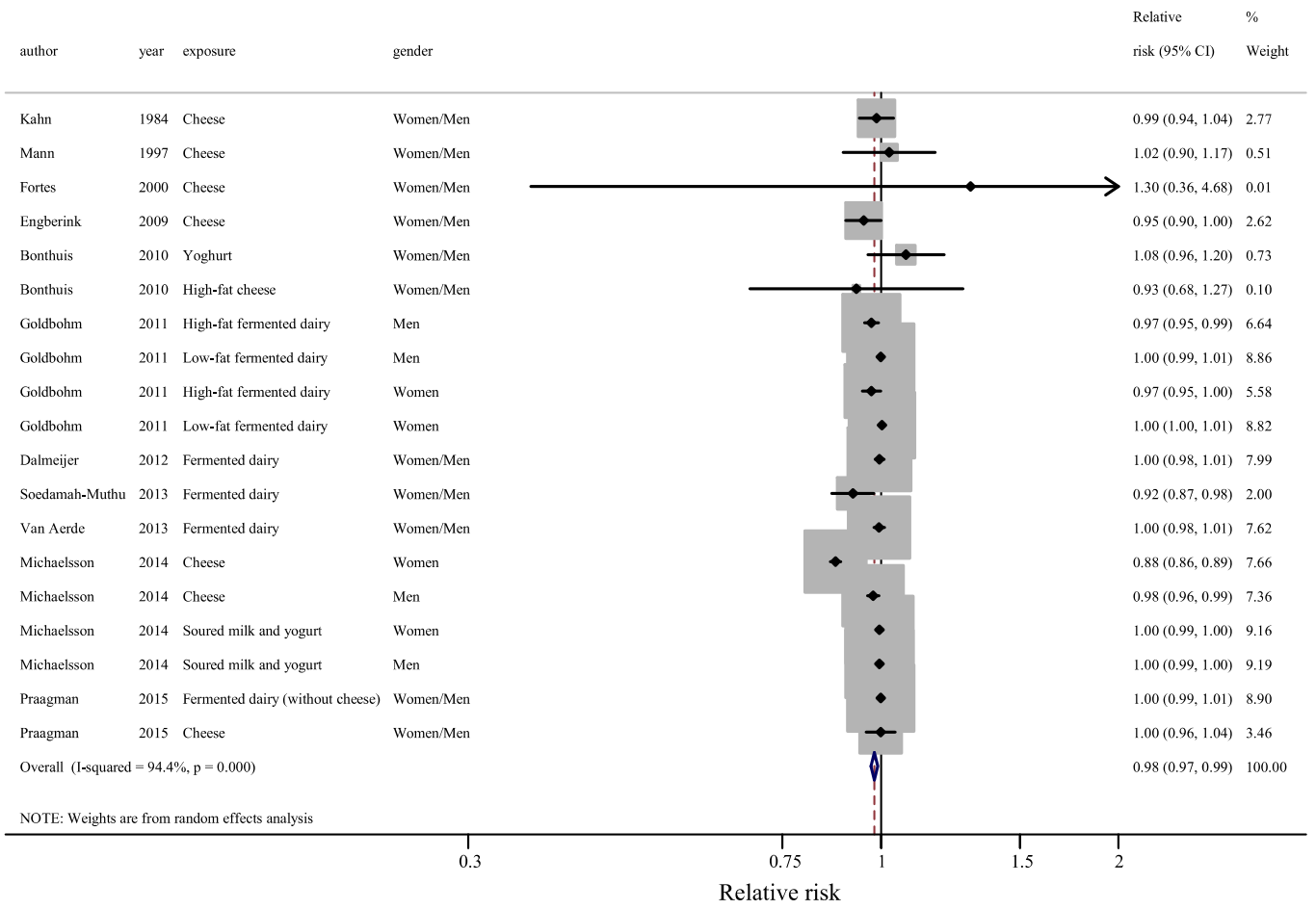

Fig. 2 Relative risk of all-cause mortality for an increment of 20 g/day of fermented dairy intake. *Squares* represent study-specific RR. *Square areas* are proportional to the overall specific-study weight to the overall meta-analysis. *Horizontal lines* represent 95% CIs. *Diamonds* represent the pooled relative risk and 95% CIs. By excluding the Swedish study [6] of women's results for cheese, RR = 1.00 (95% CI 0.99–1.00), $I^2 = 45.2\%$ ($P = 0.02$)

Figure 10; RR 1.00, 95% CI 0.93–1.07), CHD (Supplemental Figure 11; RR 1.01, 95% CI 0.96–1.06) or CVD (Supplemental Figure 12; RR 1.01, 95% CI 0.93–1.10). Significant heterogeneity was present for all-cause mortality ($I^2 = 97.4$, $P < 0.001$), CHD ($I^2 = 45.5$, $P = 0.043$) and CVD ($I^2 = 92.4$, $P < 0.001$). In sensitivity analyses for the association between milk and all-cause mortality by excluding data of Michaelsson et al. [6] for women, I^2 reduced to 70.1% ($P < 0.001$) with RR 0.99 (95% CI 0.96–1.01). By removing Kondo et al. [28] from the meta-analysis of CHD, heterogeneity reduced ($I^2 = 35.10$, $P = 0.118$) with a RR of 1.01 (95% CI 0.97–1.05). Results of high-fat milk or low-fat milk were not reported, as only one study [30] was available for the effect of high-fat milk or low-fat milk in relation to CHD. Sub-group analyses showed an inverse association between milk consumption and mortality (Supplemental Table 3) in the subgroup of studies with a mean age ≤50 years (3 populations without heterogeneity ($I^2 = 0\%$, $P = 0.479$). Also, inverse associations were found between milk intake and CVD (Supplemental Table 5) for the studies which did not adjust for 7 confounders (age, sex, smoking, alcohol, BMI, physical activity, food energy intake) (RR 0.94, 95% CI 0.89–0.99; $I^2 = 28.6$, $P = 0.210$) or for the NOS score <7 (RR 0.95, 95% CI 0.90–1.00; $I^2 = 22.1$, $P = 0.278$).

Total fermented dairy, cheese and yogurt

Total fermented dairy intake (weighted median intake 77 g/day, 19 populations, 11 studies) was non-linearly and marginally associated with lower mortality risk, with a RR of 0.98 (95% CI 0.97–0.99) per 20 g/day but with high heterogeneity ($I^2 = 94.4\%$, $P < 0.001$; Fig. 2). In sensitivity analysis, by excluding the Swedish study [6] of women's results for cheese, I^2 was reduced to 45.2% ($P = 0.02$), with RR of 1.00 (95% CI 0.99–1.00). Similarly, total fermented dairy intake (17 populations, 9 studies) was non-linearly and modestly associated with a 2% lower CVD risk per 20 g/day (RR 0.98, 95% CI 0.97–0.99) (Fig. 3). Significant heterogeneity was present

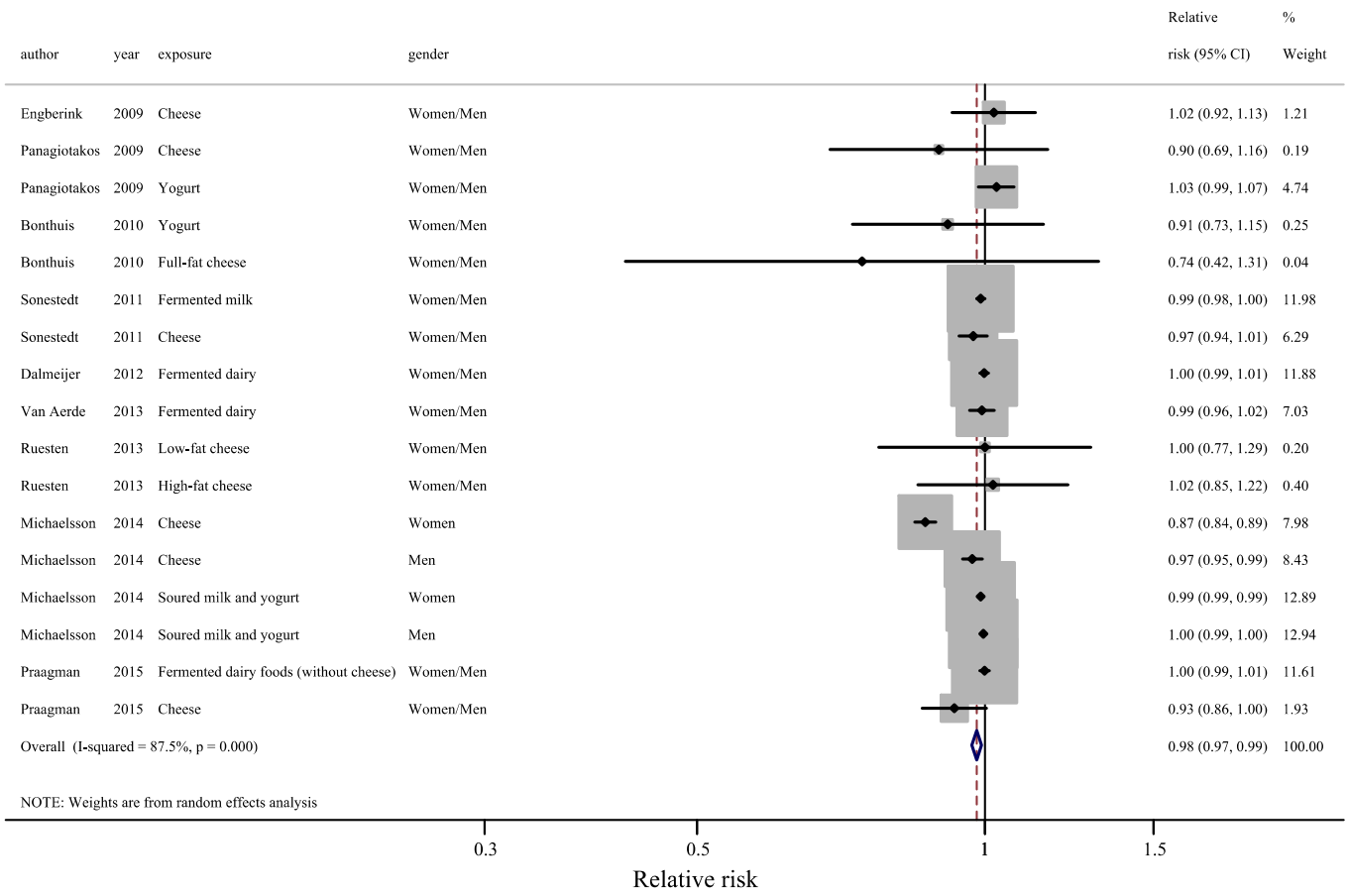

Fig. 3 Relative risk of CVD for an increment of 20 g/day of fermented dairy intake. *Squares* represent study-specific RR. *Square areas* are proportional to the overall specific-study weight to the overall meta-analysis. *Horizontal lines* represent 95% Cis. *Diamonds* represent the pooled relative risk and 95% CIs. By excluding the Swedish study [6] of women's results for cheese, RR = 0.99 (95% CI 0.99–1.00), $I^2 = 23.8\%$ ($P = 0.19$)

($I^2 = 87.5\%$, $P < 0.001$). Again, in a sensitivity test, excluding the Swedish study [6] of women's results for cheese, showed a marked decrease in heterogeneity to 23.8% ($P = 0.19$), with a 1% lower CVD risk (RR 0.99, 95% CI 0.99–1.00). Total fermented dairy intake (14 populations, 9 studies) showed no association with CHD risk, with a RR of 0.99 (95% CI 0.98–1.01) per 20 g/day increment with no indications of a nonlinear association (Supplementary Figure 13). The heterogeneity in the CHD and total fermented dairy data was significant ($I^2 = 44.6\%$, $P = 0.037$). In sensitivity analyses, after excluding the study of Patterson et al. [30], the heterogeneity for cheese was reduced ($I^2 = 32.5\%$; $P = 0.122$), but with results remaining similar with a RR of 1.00 (95% CI 0.99–1.01).

Cheese (per 10 g/day) was marginally non-linearly inversely related to CVD (Fig. 4; RR 0.98, 95% CI 0.95–1.00; 11 populations), but not to risk of mortality (Supplementary Figure 14; RR 0.99, 95% CI 0.96–1.01; 13 populations) or CHD (Supplementary Figure 15; RR 0.99, 95% CI 0.97–1.02). Significant heterogeneity was seen for mortality ($I^2 = 93.3\%$, $P < 0.001$) or CVD ($I^2 = 82.6\%$, $P < 0.001$). In sensitivity analyses, heterogeneity was reduced after removal of the large Swedish study [6] ($I^2 = 11\%$, $P = 0.337$ for mortality; $I^2 = 0\%$, $P = 0.835$ for CVD), with no association for mortality and CVD (RR = 1 for both).

Yogurt (3 populations) was not associated with all-cause mortality ($I^2 = 65.8\%$, $P = 0.054$, RR 0.97, 95% CI 0.85–1.11), CHD ($I^2 = 0\%$, $P = 0.685$, RR 1.03, 95% CI 0.97–1.09) or CVD ($I^2 = 0\%$, $P = 0.499$, RR 1.03, 95% CI 0.97–1.09) (Supplementary Figure 16–18).

Discussion

This meta-analysis combining data from 29 prospective cohort studies showed there were no associations between total dairy, high- and low-fat dairy, milk and the health outcomes including all-cause mortality, CHD or CVD. The modest inverse associations of total fermented dairy were found with all-cause mortality and CVD, but not CHD. By examining different types of fermented food in relation to

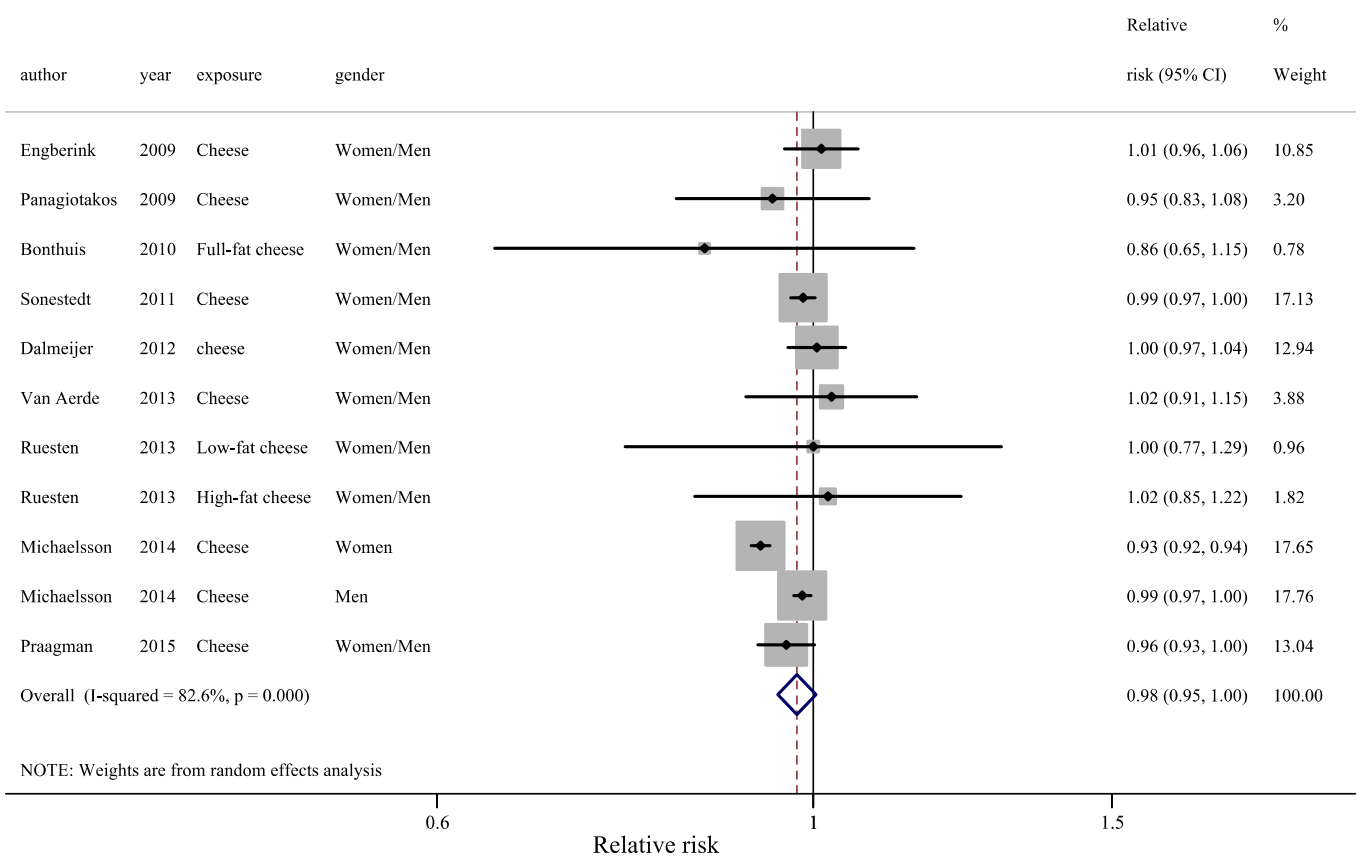

Fig. 4 Relative risks of CVD for an increment of 10 g/day of cheese. *Squares* represent study-specific RR. *Square areas* are proportional to the overall specific-study weight to the overall meta-analysis. *Horizontal lines* represent 95% CIs. *Diamonds* represent the pooled relative risk and 95% Cis. By excluding the Swedish study [6] of women's results for cheese, RR = 0.99 (0.98–0.99), $I^2 = 0\%$ ($P = 0.84$)

CVD, we found marginally inverse association with cheese but not yogurt. However, further sensitivity tests showed the inverse associations of fermented dairy and cheese with all-cause mortality or CVD disappeared after removing the study of Michaelsson et al. [6].

No associations were found between total dairy and milk consumption with all-cause mortality, CHD or CVD in the current study, which is in agreement with several meta-analyses [47, 48]. Larsson et al. [47] reported neutral associations of dairy and milk consumption with mortality or CVD mortality. Mullie et al. [48] reported neutral associations of milk consumption with all-cause mortality or CHD. In addition, the current study is in agreement with a recently published review [49] which indicated neutral associations between the consumption of total dairy and risk of CHD or CVD. Results of sub-group analyses showed the inverse associations were observed between total dairy intake and CHD, or the association between milk consumption and CVD when studies did not adjust for major confounders. Thus, confounders included in statistical analyses in prospective studies have substantial effects on the final findings and conclusions. Furthermore, inverse associations were also found in sub-groups of studies defined by mean age (≤50, >50 years) or BMI (>25 kg/m^2) of the associations between total, high-fat, low-fat dairy and milk with risk of all-cause mortality, CHD or CVD, which indicated the findings and conclusions were also affected by characteristics of the study populations within different studies.

Three US prospective cohort studies described by Chen et al. [50] showed a substantially lower risk of CVD when animal fats, including dairy fat, were replaced by unsaturated fats. Recently, UK National Health Service (NHS) has recommended low-fat milk and dairy products as healthy choices [51]. However, in the current study, high-fat and low-fat dairy consumption were investigated separately and no substitution models replacing high by low-fat dairy products were carried out. We found no significant associations between high-and low-fat dairy and all-cause mortality, CHD or CVD. This supports two previous meta-analyses [5, 52] which also reported no association of high or low-fat dairy and CHD. Furthermore, beneficial effects of high-fat dairy foods on human health were reported by a cross-sectional study [53], which showed an inverse association of full-fat dairy food and the metabolic syndrome. In addition, another US study [54], which reviewed cross-

sectional and prospective cohort studies, showed that 11 of the 16 studies identified that population with higher full-fat dairy intake had less adiposity. It is also noteworthy that butter as a high fat dairy food containing 80% fat [55], a recent meta-analysis on the effects of butter [10] showed that whilst consumption was weakly associated with all-cause mortality (per 14 g/day: RR 1.01, 95% CI 1.00–1.03), there was no significant association with CHD, CVD or stroke and there was an inverse association with incidence of diabetes (RR 0.96, 95% CI 0.93–0.99). Therefore, the effect of dairy fat on CVD is complex and may be influenced by the nature of the fat containing food vehicle, which needs confirmation in further studies.

Despite their fat content and composition, milk and dairy products are naturally rich in various minerals (e.g. calcium, potassium), protein and vitamins (e.g. vitamin A and vitamin B_{12}) [56]. Nutrients including calcium, potassium and magnesium have been suggested to be associated with lower risk of stroke [57, 58]. Short-term human intervention studies [59, 60] also indicated that subjects who have high-fat diets enriched with dairy minerals or calcium have significantly lower total cholesterol and LDL-cholesterol levels than those on a control diet. This may explain in part why total dairy consumption has a neutral role in terms of the effect on health outcomes.

The current study also showed total fermented dairy and cheese intake to be marginally inversely associated with mortality and CVD risk, respectively, and large heterogeneity was present. However, by removing the study of Michaelsson et al. [6], heterogeneity of the associations of total fermented dairy and mortality or CVD, cheese and mortality or CVD were markedly reduced. Also, the marginally inversely associations were disappeared. To our knowledge, the present study is the first dairy meta-analysis to include the large Swedish cohort results [6]. The markedly reduced heterogeneity after removing the results of the Swedish female cohort [6] indicated the heterogeneous nature of the Swedish study, which may be related to the diet and lifestyle characteristics of the study participants, as they had a relatively low education level (80 and 70% for women and men were educated for $\leq$9 years, respectively), also the highest milk drinkers had highest percentage of smokers and those living alone.

Cheese consumption based on 11 populations was found to be modestly and inversely associated with CVD risk, with a 2% lower risk of CVD per 10 g/day of cheese, however, the significant association disappeared after removing the study of Michaelsson et al. [6]. Compared with other meta-analyses on cheese, Alexander et al. [4] has reported 11% lower risk of CVD per 35 g/day (95% CI 0.78–1.01), while Chen et al. [61] presented 10% lower risk of CHD per 50 g/day (95% CI 0.84–0.95). However, the analysis of the associations between cheese and CVD in studies of Alexander et al. [4] and Chen et al. [61] were based on 3 and 8 populations, respectively, which was less than our current study of 11 populations.

Furthermore, total fermented dairy and cheese were modestly inversely associated with risk of CVD but not CHD in the current meta-analysis, so perhaps both dairy types play a role in reducing the risk of stroke. This is supported by the evidence of another recent meta-analysis [9], which found a 9% lower risk of stroke (RR 0.91, 95% CI 0.82–1.01) associated with higher total fermented dairy intake and a 3% lower risk of stroke (RR 0.97, 95% CI 0.94–1.01) with higher cheese consumption, although none of these associations were statistically significant. As there was limited information of the different sub-types of the CVD events, the understanding of the association of fermented dairy products with varied CVD types remains unclear. In addition, unlike the result for cheese, the association of yogurt with disease outcomes was neutral. However, a previous review of randomised trials suggested that yogurt is associated with lower risk of CVD [62]. Our null results for yogurt intake and CVD may be due to the limited number of participants from only 3 populations. In addition, a very recent meta-analysis showed a 14% lower risk of type 2 diabetes for 80 g/day yogurt intake (RR 0.86, 95% CI 0.83–0.90) based on 11 prospective cohort studies [63].

The mechanism of the beneficial association of fermented dairy products and reduced CVD risk and mortality is uncertain. Evidence from randomised controlled trials suggests that the reason, at least in part, may be an effect of the food matrix reducing lipid absorption and short chain fatty acids produced by the bacteria in the large intestine [64]. Moreover, omics-techniques have suggested that some of the beneficial effects of cheese can be accounted for by microbial fermentation producing short chain fatty acids such as butyrate [65].

Strengths of our study include the use of dose–response meta-analysis, the inclusion of more studies than in previous meta-analyses and the consideration of examination the individual dairy products separately such as dairy products in terms of fat content (high-fat, low-fat) or processing method (fermented or non-fermented). However, investigation of total dairy or total fermented dairy consumption with disease outcomes by combining dairy foods, high and low-fat dairy foods, solids and liquids, simply adding these up is a limitation which should be addressed in future studies by collecting and analyzing more detailed data. In addition, limitations of the study include sub-group analyses that lack statistical power, such as for Asian studies and effects of gender. We have 9 studies with scores of 7 or less by using the Newcastle–Ottawa Scale (NOS) [43]. Study quality could explain some heterogeneity but not all. For example, NOS scores of all studies

containing high-fat dairy or low-fat dairy were ≥7, which could have resulted in lower heterogeneity for those analyses. Furthermore, residual confounding is a limitation of prospective cohort studies. The background diet should be taken into account in the statistical analyses as major confounders, which was done in 15 out of 29 cohort studies. Comparisons of dairy products with other foods in replacement models were not possible from the available data. The neutral risks of dairy products with mortality and CVD risk could be because of replacement by other foods, for example, those with high intake of dairy products may consume less sugar sweetened beverages which could lead to lower CVD mortality [66] or consume more processed meat which could lead to higher CVD risks [67, 68]. For future studies it is important to investigate in more detail how dairy products can be replaced by other foods.

Conclusions

The current meta-analysis of 29 prospective cohort studies suggested neutral associations of total, high and low-fat dairy, milk and yogurt with risk of all-cause mortality, CHD and CVD. In addition, a possible role of fermented dairy was found in CVD prevention, but the result was driven by a single study.

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Authors' contributions JG, AA, DIG, JAL, and SSSM designed the research. JG performed the literature search, extracted data. JG, SSSM checked data. JG performed the analyses and drafted the paper. AA, DIG, JAL, SSSM critically reviewed and improved it. JG is guarantor.

Compliance with ethical standards

Conflict of interest SSSM received funding from the Global Dairy Platform, Dairy Research Institute and Dairy Australia for a meta-analysis on cheese and blood lipids (2012) and this meta-analysis of dairy and mortality (2015). SSSM has also received the Wiebe Visser International Dairy Nutrition Prize from the Dutch Dairy Association's (NZO) Utrecht Group. AA is recipient of research grants from Arla Foods, DK; Danish Dairy Research Foundation; Global Dairy Platform; Danish Agriculture and Food Council; GEIE European Milk Forum, France. He is member of advisory boards for Dutch Beer Knowledge Institute, NL; IKEA, SV; Lucozade Ribena Suntory Ltd, UK; McCain Foods Limited, USA; McDonald's, USA; Weight Watchers, USA. He is consultant for Nestlé Research Center, Switzerland; Nongfu Spring Water, China. Astrup receives honoraria as Associate Editor of American Journal of Clinical Nutrition, and for membership of the Editorial Boards of Annals of Nutrition and of Metabolism and Annual Review of Nutrition. He is recipient of travel expenses and/or modest honoraria (<$2000) for lectures given at meetings supported by corporate sponsors. He received financial support from dairy organizations for attendance at the Eurofed Lipids Congress (2014) in France and the meeting of The Federation of European Nutrition Societies (2015) in Germany; DIG and JG received funding from the Global Dairy Platform, DIG and JAL have received funding from The Dairy Council and AHDB Dairy for dietary pattern analysis of diets defined by dairy food content (2012–2015).

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Online Supporting Material

CONTENT

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Supplemental Methods

Search strategy (PubMed) – updated until Sep 2016

EMBASE (http://www.embase.com) and SCOPUS (http://www.scopus.com) search strategies were based on the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) query syntax which is shown below.

PubMed (http://www.ncbi.nlm.nih.gov/pubmed)

Action 1 Determinants

#1 dairy [Title/Abstract]) OR milk*[Title/Abstract]) OR cheese*[Title/Abstract]) OR yogurt*[Title/Abstract]) OR yogurt*[Title/Abstract]) OR butter [Title/Abstract]) OR buttermilk [Title/Abstract]) OR dietary pattern*[Title/Abstract]

#2 dairy products [MeSH Terms]) OR milk [MeSH Terms]) OR cheese [MeSH Terms]) OR yogurt[MeSH Terms]) OR butter[MeSH Terms]) OR cultured milk products[MeSH Terms]

#3 custard*[Title/Abstract]) OR pudding*[Title/Abstract]) OR cream*[Title/Abstract]) OR cream[Title/Abstract]) OR ice cream[Title/Abstract]) OR ice-cream[Title/Abstract]) OR curd*[Title/Abstract]) OR porridge[Title/Abstract]

#4 diet[Title/Abstract]) OR diets[Title/Abstract]) OR dietary[Title/Abstract]) OR intake*[Title/Abstract]) OR suppl*[Title/Abstract]) OR consumption[Title/Abstract]) OR food*[Title/Abstract]) OR drink*[Title/Abstract]) OR meal[Title/Abstract]) OR nutrition[Title/Abstract]) OR nutrient*[Title/Abstract]) OR products[Title/Abstract]

#5 [(#1 OR #2 OR #3) AND #4]

Action 2 Outcome

#6 diet[Title/Abstract]) OR diets[Title/Abstract]) OR dietary[Title/Abstract]) OR intake*[Title/Abstract]) OR suppl*[Title/Abstract]) OR consumption[Title/Abstract]) OR food*[Title/Abstract]) OR drink*[Title/Abstract]) OR meal[Title/Abstract]) OR nutrition[Title/Abstract]) OR nutrient*[Title/Abstract]) OR products[Title/Abstract]

#7 cardiovascular [Title/Abstract]) OR vascular [Title/Abstract]) OR CVD [Title/Abstract]) OR Cardiovascular Diseases [Mesh:NoExp]

#8 coronary[Title/Abstract]) OR cardiac[Title/Abstract]) OR heart[Title/Abstract]) OR infarction*[Title/Abstract]) OR infarct*[Title/Abstract]) OR ischaemic[Title/Abstract]) OR ischemic[Title/Abstract]) OR ischaemia[Title/Abstract]) OR ischemia[Title/Abstract]) OR CHD[Title/Abstract]) OR CAD[Title/Abstract]) OR MI[Title/Abstract]) OR myocard*[Title/Abstract]) OR Coronary Artery Disease[Mesh:NoExp]) OR coronary disease[Mesh:NoExp]

#9 cerebrovascular*[Title/Abstract]) OR stroke[Title/Abstract]) OR CVA[Title/Abstract]) OR Cerebrovascular disease[Mesh:NoExp]) OR stroke[Mesh:NoExp]

#10 (#6 OR #7 OR #8 OR #9)

Action 3 Combine exposure and outcome

#11 (#5 AND #10)

Action 4 Limits

#12 Rats[Mesh:NoExp]) OR Mice[Mesh:NoExp]) OR rat[Title/Abstract]) OR rats[Title/Abstract]) OR mouse[Title/Abstract]) OR mice[Title/Abstract]) OR vivo[Title/Abstract]) OR vitro[Title/Abstract])

#13 (#11 NOT #12)

NEWCASTLE – OTTAWA QUALITY ASSESSMENT SCALE
COHORT STUDIES

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Outcome categories. A maximum of two stars can be given for Comparability.

Selection

1) Representativeness of the exposed cohort

a) truly representative of the average *healthy adults* in the community★

b) somewhat representative of the average *healthy adults* in the community ★

c) selected group of users *e.g. nurses, volunteers, vegetarian*

d) no description of the derivation of the cohort

2) Selection of the non-exposed cohort

a) drawn from the same community as the exposed cohort★

b) drawn from a different source

c) no description of the derivation of the non-exposed cohort

3) Ascertainment of exposure

a) secure record (*e.g. 7 day food diary)* ★

b) structured interview/≥ 2 *dietary recalls/diet history/ food frequency questionnaire validated for dairy components* ★

c) written self-report (*e.g. <2 dietary recalls/non-validated food frequency questionnaire or not reported whether food frequency questionnaire was validated*)

d) no description

4) Demonstration that outcome of interest was not present at start of study

a) yes★

b) no

Comparability

1) Comparability of cohorts on the basis of the design or analysis

a) study controls for *age, sex, smoking, total energy intake, and body mass index*★

b) study controls for any additional factor (*e.g. physical activity, alcohol intake, family history of diabetes, dietary factors*) ★

Outcome

1) Assessment of outcome

a) independent blind assessment (e.g. clinical diagnosis/complete medical information available).★

b) record linkage/*medical record or validated self-report* ★

c) non-validated self-report

d) no description

2) Was follow-up long enough for outcomes to occur

a) yes/ *follow up period for outcome of interest is 10 years or over* ★

b) no

3) Adequacy of follow-up of cohorts

a) complete follow-up - all subjects accounted for ★

b) subjects lost to follow-up unlikely to introduce bias - small number lost ≤*20%* follow-up, or description provided of those lost ★

c) follow-up rate <*80%* or no description of those lost

d) no statement

Supplemental Table 1. Quality assessment of cohorts studies on dairy intake, risk of CHD, CVD or all-cause mortality.

	Selection				**Comparability**	**Outcome**			
	Representativeness of the exposed cohort	**Selection of the non-exposed cohort**	**Ascertainment of exposure**	**Outcome not present at start of study**	**Comparability of cohorts on the basis of the design or analysis**	**Assessment of outcome**	**Follow-up long enough for outcomes to occur**	**Adequacy of follow-up of cohorts**	**Total score**
Kahn et al, 1984 [1]	B★	A★	C	B	-	B★	A★	B★	5
Mann et al, 1997 [2]	C	A★	C	A★	-	B★	A★	D	4
Hu et al, 1999 [3]	C	A★	B★	A★	A★ B★	B★	A★	B★	8
Appleby et al, 1999 [4]	C	A★	B★	B	-	B★	A★	B★	6
Bostick et al, 1999 [5]	C	A★	B★	A★	A★ B★	B★	B	B★	9
Fortes et al, 2000 [6]	C	A★	B★	A★	-	B★	B	B★	6
Ness et al, 2001 [7]	B★	A★	D	A★	-	B★	A★	D	5
Al-Delaimy et al, 2003 [8]	C	A★	B★	A★	A★ B★	B★	A★	B★	8
Elwood et al, 2004 [9]	B★	A★	A★	A★	A★ B★	A★	A★	B★	8
Knoops et al, 2006 [10]	B★	A★	B★	A★	-	D	A★	D	5
Paganini-Hill et al, 2007 [11]	B★	A★	C	A★	-	B★	A★	B★	6
Panagiotakos et al, 2009 [12]	B★	A★	B★	A★	-	B★	B	C	5
Engberink et al, 2009 [13]	A★	A★	B★	A★	A★ B★	A★	A★	D	8
Bonthuis et al. 2010 [14]	B★	A★	B★	A★	A★ B★	B★	A★	B★	8
Goldbohm et al. 2011 [15]	A★	A★	B★	A★	A★ B★	B★	A★	B★	9

	Selection				Comparability	Outcome			
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Outcome not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total score
Sonestedt et al. 2011 [16]	B★	A★	A★	A★	A★ B★	A★	A★	A★	9
Kondo et al. 2012 [17]	A★	A★	B★	A★	A★ B★	B★	A★	B★	9
Dalmeijer et al, 2012 [18]	A★	A★	B★	A★	A★ B★	B★	A★	B★	9
Patterson et al. 2013 [19]	B★	A★	B★	A★	-	B★	A★	C	6
Soedamah-Muthu et al. 2013 [20]	C	A★	B★	A★	A★ B★	A★	A★	D	7
Louie et al. 2013 [21]	C	A★	B★	A★	A★ B★	B★	A★	B★	9
von Ruesten et al, 2013 [22]	A★	A★	B★	A★	A★ B★	B★	B★	A★	8
Van Aerde et al, 2013 [23]	B★	A★	B★	A★	A★ B★	B★	A★	B★	9
Michaelsson et al. 2014 [24]									
-Swedish Mammography Cohort	B★	A★	A★	A★	A★ B★	B★	A★	C	8
-The Cohort of Swedish Men	B★	A★	A★	A★	A★ B★	B★	A★	C	8
Praagman et al. 2014 (Rotterdam) [25]	A★	A★	B★	A★	A★ B★	A★	A★	B★	8
Haring et al, 2014 [26]	B★	A★	C	A★	A★ B★	B★	A★	C	7
Huang et al. 2015 [27]	A★	A★	B★	A★	-	B★	A★	D	6
Bergholdt et al, 2015 [28]	A★	A★	B★	A★	-	A★	B★	B★	6
Praagman et al. 2015 [29]	B★	A★	B★	A★	A★ B★	A★	A★	B★	9

	Selection				Comparability	Outcome			
	Representativeness of the exposed cohort	Selection of the non-exposed cohort	Ascertainment of exposure	Outcome not present at start of study	Comparability of cohorts on the basis of the design or analysis	Assessment of outcome	Follow-up long enough for outcomes to occur	Adequacy of follow-up of cohorts	Total score
Wang et al. 2015 [30]	B★	A★	C	A★	-	B★	A★	A★	6

Supplemental Table 2. Definition of dairy products as described in the paper of 29 prospective cohort studies included in the meta-analysis.

Exposure category in original paper	Exposure category in meta-analysis	Definition (if available)
Kahn et al, 1984 [1]		
Milk	Milk	Not further defined
Cheese	Cheese	Not further defined
Mann et al, 1997 [2]		
Milk	Milk	Not further defined
Cheese	Cheese	Cheese (excluding cottage)
Hu et al, 1999 [3]		
Total dairy	Total dairy	Not further defined
High-fat dairy	High-fat dairy	Whole milk, hard or cream cheese, ice cream, and butter
Low-fat dairy	Low-fat dairy	Skim or low-fat milk, yogurt, and cottage cheese
Milk	Milk	Not further defined
Appleby et al, 1999 [4]		
Milk	Milk	Not further defined
Cheese	Cheese	Not further defined
Bostick et al, 1999 [5]		
Total dairy	Total dairy	Milk products excluding butter
Fat-containing dairy intake	High-fat dairy	Milk products other than butter containing fat (exclude skim milk)
Fortes et al, 2000 [6]		
Cheese	Cheese	Not further defined
Ness et al, 2001 [7]		
Milk	Milk	Milk
Al-Delaimy et al, 2003 [8]		
Total dairy	Total dairy	Not further defined
High-fat dairy	High-fat dairy	Not further defined
Low-fat dairy	Low-fat dairy	Not further defined

Milk	Milk	Not further defined
Elwood et al, 2004 [9]		
Milk	Milk	Liquid milk, not milk used in food preparation
Knoops et al, 2006 [10]		
Milk and milk products	Total dairy	Not further defined
Paganini-Hill et al, 2007 [11]		
Milk	Milk	Milk
Panagiotakos et al, 2009 [12]		
Dairy products	Total dairy	Not further defined
Cheese	Cheese	Not further defined
Yogurt	Yogurt	Not further defined
Milk	Milk	Not further defined
Bonthuis et al. 2010 [14]		
Total dairy	Total dairy	Skim milk, low-fat milk, low-fat yogurt, cottage or ricotta cheese, whole milk, cream, ice cream, yogurt, full-fat cheese and custard
Low-fat dairy	Low-fat dairy	Skim milk, low-fat milk, low-fat yogurt, cottage or ricotta cheese
Full-fat dairy	Full-fat dairy	Whole milk, cream, ice cream, yogurt, full-fat cheese and custard
Milk	Milk	Whole milk, skimmed and low-fat milk
Yogurt	Yogurt	Not further defined
Full-fat cheese	Full-fat cheese	Not further defined
Goldbohm et al. 2011 [15]		
Milk products	Total dairy	Milk, yogurt, buttermilk, quark, and dishes in which these foods were used
Nonfermented full-fat milk	High-fat dairy	Whole milk (3.7% fat), cream (36%, 20% fat), condensed whole milk, whole-milk cocoa, pudding, and ice cream
Nonfermented low-fat milk	Low-fat dairy	Low-fat milk (1.5% fat), skim milk (0.1% fat), condensed low-fat milk, and low-fat and skim cocoa
Fermented full-fat milk	High-fat fermented dairy	Yogurt (3.5% fat), full-fat quark (fresh cheese), and sour cream
Fermented low-fat milk	Low-fat fermented dairy	Buttermilk, skim yogurt (0.1% fat), and non-fat quark (fresh cheese)
Cheese	Cheese	Not further defined

butter	Butter	Not further defined
Low-fat dairy	Low-fat dairy	Not further defined
Sonestedt et al. 2011 [16]		
Total dairy	Total dairy	Milk, cheese (>10% fat), cream, butter (including the milk-based spread Bregott)
Milk	Milk products	Fermented (yogurt and processed sour milk), non-fermented milk products
Fermented milk	Fermented dairy	Yogurt and processed sour milk
Low-fat milk	Low-fat dairy	Milk and milk products≤2.4% fat
high-fat milk	High-fat dairy	Milk and milk products>2.4% fat
cheese	Cheese	Cheese>10% fat
Kondo et al. 2012 [17]		
Milk and dairy product consumption	Milk	93% was in the form of milk
Dalmeijer et al, 2012 [18]		
Total dairy intake	Total dairy intake	All dairy food products except for butter and ice cream.
High-fat dairy	High-fat dairy	Milk and milk products with a fat content ≥2 g/100 g (whole milk products) or cheese products with a fat content ≥20 g/100 g.
Low-fat dairy	Low-fat dairy	Milk and milk products with a fat content<2 g/100 g (skimmed or semi-skimmed milk products) or cheese with a fat content< 20 g/100 g
Cheese	Cheese	All types of cheese except for curd
Fermented dairy	Fermented dairy	Buttermilk, yogurts, and cheese
Patterson et al. 2013 [19]		
Total dairy foods	Total dairy	Total dairy intake was the sum of milk [full-fat (≥3.0% fat), semi-skimmed (≤1.5% fat), skimmed (0.5% fat), and pancakes], cultured milk/yogurt [full-fat (≥3.0% fat) and low-fat (≤1.5% fat)], cheese [full-fat (>17% fat), low-fat (≤17% fat), and cottage cheese/quark], cream and crème fraiche (full-fat and low-fat) intakes.
Milk	Milk	Full-fat (≥3.0% fat), semi-skimmed (≤1.5% fat), skimmed (0.5% fat), and pancakes (A serving of pancakes contributed one serving of total milk)

Low-fat milk	Low-fat milk	Semi-skimmed (≤1.5% fat) and skimmed (0.5% fat)
Full-fat milk	Full-fat milk	Milk (≥3.0% fat)
Cultured milk /yogurt	Fermented dairy	Not further defined
Low-fat cultured milk/ yogurt	Low-fat fermented dairy	Cultured milk/yogurt (≤1.5% fat)
Full-fat cultured milk/yogurt	High-fat fermented dairy	Cultured milk/yogurt (≥3.0% fat)
Cheese	cheese	Full-fat (>17% fat), low-fat (≤17% fat), and cottage cheese/quark
Low-fat cheese	Low-fat cheese	Low-fat varieties (10–17%) and excluded very-low-fat cheese (i.e., cottage cheese/quark (4% fat))
High-fat cheese	Full-fat cheese	Cheese (>17% fat)
Soedamah-Muthu et al. 2013 [20]		
Total dairy	Total dairy	All dairy products, except butter and ice cream
High-fat dairy	High-fat dairy	Full-fat cheese, yogurt, milk puddings, whole and Channel Islands milk
Low-fat dairy	Low-fat dairy	Cottage cheese, semi-skimmed, skimmed milk and milk-based hot drinks
Total milk	Total milk	Whole and low-fat milk
Fermented dairy	Fermented dairy	Yogurt and total cheese
Cheese	Cheese	Full-fat cheese and cottage
Yogurt	Yogurt	Not further defined
Louie et al. 2013 [21]		
Total dairy	Total dairy	Whole fat milk, reduced fat/skim milk, low fat cheese, whole fat cheese, reduced fat dairy dessert (e.g., low fat yogurt), and medium fat dairy dessert (e.g., custard and whole fat yogurt).
Low/reduced fat dairy	Low-fat dairy	Reduce fat/ skim milk, reduced fat dairy dessert and low fat cheese
Whole fat dairy	Full-fat dairy	Whole fat milk, whole fat cheese and medium fat dairy dessert
von Ruesten et al, 2013 [22]		
Low-fat dairy	Low-fat dairy	Fat-reduced variants of: milk/milkshake (1.5% fat or less), yogurt, fruit yogurt (1.5% fat or less), soured milk/kefir, curd/curd with herbs
High-fat dairy	High-fat dairy	Normal- or high-fat variants of: milk/milkshake, yogurt, fruit yogurt, soured milk/kefir, curd/curd with herbs
Low-fat cheese	Low-fat cheese	Fat-reduced variants of: Cream cheese, hard cheese (for example, gouda,

		Emmental cheese, Tilsiter cheese), soft cheese (for example, camembert, brie, gorgonzola)
High-fat cheese	High-fat cheese	Normal- or high-fat variants of: Cream cheese, processed cheese, hard cheese (for example, gouda, Emmental cheese, Tilsiter cheese), soft cheese (for example, camembert, brie, gorgonzola), whipped cream
Van Aerde et al, 2013 [23]		
Total dairy	Total dairy	Includes all dairy products, except butter
High-fat dairy	High-fat dairy	All milk products with a fat content>2.0/100 g or cheese products with a fat content>20 g/100 g
Low-fat dairy	Low-fat dairy	All milk products with a fat content<2.0/100 g or cheese products with a fat content<20/100 g
Milk	Milk	All milk: skimmed, semi-skimmed, and whole milk
Fermented dairy	Fermented dairy	All fermented products, such as yogurt, buttermilk, curds, and cheese products
Cheese	Cheese	Soft cheese and hard cheese (both low-fat and high-fat)
Michaelsson et al. 2014 [24]		
Milk	Milk	Not further defined
Cheese	Cheese	Not further defined
Fermented milk products	Fermented dairy	Yogurt and other soured milk products
Praagman et al. 2014 [25] *and Engberink et al. 2009* [13]		
Total dairy	Total dairy	Milk, buttermilk, yogurt, coffee creamer, curd, pudding, porridge, custard, whipped cream, ice cream, and cheese, but not butter
Low-fat dairy	Low-fat dairy	Milk and milk products with a fat content <2.0/100 g and cheese products with a fat content <20/100 g
High-fat dairy	High-fat dairy	Milk and milk products with a fat content ≥2.0/100 g and cheese products with a fat content >20/100 g
Fermented dairy	Fermented dairy	All types of buttermilk, yogurt, curd and cheese
Cheese	Cheese	All types of cheese, excluding curd
Yogurt	Yogurt	Not further defined
Haring et al, 2014 [26]		

Total dairy intake	Total dairy intake	Not further defined
High-fat dairy	High-fat dairy	Not further defined
Low-fat dairy	Low-fat dairy	Not further defined
Huang et al. 2015 [27]		
Total dairy	Total dairy	Liquid milk and fat-free, low-fat, high-fat, and flavoured dairy products
Bergholdt et al, 2015 [28]		
Milk	Milk	whole milk (3.5% fat), semi-skimmed (0.5-1.5% fat) and skimmed milk (0.1-0.3% fat)
Praagman et al. 2015 [29]		
Fermented dairy foods	Fermented dairy	Butter milk, yogurt (fat and skim), yogurt drink, curd
Yogurt	Yogurt	Not further defined
Cheese	Cheese	Cheese 'Goudse', cheese 'Edammer' 40+, cheese 'Leidse', cheese 'brie' 50+, cheese 'Trenta', cheese on pizza
Wang et al. 2015 [30]		
Milk	Milk	Not further defined

Supplemental Table 3. Association between dairy foods and all-cause mortality by subgroups[1].

Dairy food	Subgroup	No. study populations	Relative risk (95% CI)[2]	Heterogeneity test	
				I^2 (%)	P-value
Total dairy	Overall	10	0.99 (0.96, 1.03)	62.2	0.005
Per 200 g/d	Age (y)				
	≤50	3	0.90 (0.73, 1.11)	62.4	0.070
	>50	7	0.99 (0.96, 1.04)	67.4	0.005
	Follow-up time (y)				
	≤10	5	1.02 (0.99, 1.05)	51.6	0.083
	>10	5	0.93 (0.87, 1.01)	54.0	0.069
	Gender				
	Men	1	1.01 (0.99, 1.03)		
	Women	1	1.02 (0.99, 1.06)		
	Men and Women	8	0.96 (0.90, 1.03)	65.5	0.005
	Continent				
	Europe	8	1.00 (0.97, 1.03)	62.0	0.010
	Australia	1	0.80 (0.65, 1.00)		
	Asia	1	0.69 (0.35, 1.33)		
	Confounding factors[2]				
	Yes	6	0.99 (0.96, 1.03)	61.3	0.024
	No	4	0.95 (0.76, 1.21)	70.1	0.018
	BMI				
	≤25	3	1.01 (1.00, 1.03)	0.0	0.458
	>25	6	0.95 (0.89, 1.01)	65.5	0.013
	Newcastle-Ottawa quality score				
	<7	3	0.78 (0.42, 1.45)	76.9	0.013
	≥7	7	0.99 (0.96, 1.02)	57.9	0.027
Low-fat dairy	Overall	7	1.01 (0.99, 1.03)	0.0	0.734
Per 200 g/d	Age (y)				
	≤50	2	1.01 (0.95, 1.08)	0.0	0.753
	>50	5	1.01 (0.99, 1.03)	0.0	0.483
	Follow-up time (y)				
	≤10	3	1.02 (0.99, 1.04)	0.0	0.813
	>10	4	0.97 (0.93, 1.02)	0.0	0.896
	Gender				
	Men	1	1.01 (0.98, 1.04)		
	Women	1	1.03 (0.99, 1.06)		
	Men and Women	5	0.99 (0.95, 1.02)	0.0	0.823
	Continent				
	Europe	6	1.01 (0.99, 1.03)	0.0	0.624
	Australia	1	0.97 (0.74, 1.27)		
	Confounding factors[2]				

	Yes	6	1.01 (0.99,1.03)	0.0	0.816
	No	1	0.98 (0.92, 1.03)		
	BMI				
	≤25	2	1.02 (0.99, 1.04)	0.0	0.522
	>25	5	0.99 (0.95, 1.02)	0.0	0.823
	Newcastle-Ottawa quality score				
	<7	0			
	≥7	7	1.01 (0.99, 1.03)	0.0	0.734
Milk	Overall	12	1.00 (0.93, 1.07)	97.4	<0.001
Per 244 g/d	Age (y)				
	≤50	3	0.95 (0.92, 0.98)	0.0	0.479
	>50	8	1.02 (0.93, 1.11)	97.8	<0.001
	Follow-up time (y)				
	≤10	0			
	>10	12	1.00 (0.93, 1.07)	97.4	<0.001
	Gender				
	Men	4	0.99 (0.94, 1.04)	85.9	<0.001
	Women	2	1.09 (0.83, 1.43)	98.6	<0.001
	Men and Women	6	0.99 (0.96, 1.03)	43.4	0.116
	Continent				
	Europe	7	1.01 (0.91, 1.13)	98.2	<0.001
	Australia	1	0.91 (0.68, 1.22)		
	Asia	2	0.95 (0.91, 0.99)	0.0	0.934
	USA	2	1.01 (0.98, 1.05)	70.9	0.064
	Confounding factors[2]				
	Yes	5	1.03 (0.93, 1.07)	98.3	<0.001
	No	7	0.98 (0.95, 1.01)	71.2	0.002
	BMI				
	≤25	5	1.01 (0.88, 1.16)	98.3	<0.001
	>25	6	0.99 (0.94, 1.04)	71.8	0.003
	Newcastle-Ottawa quality score				
	<7	6	0.97 (0.94, 1.01)	74.7	0.001
	≥7	6	1.04 (0.92,1.17)	97.9	<0.001
Total fermented dairy	Overall	19	0.98 (0.97, 0.99)	94.4	<0.001
Per 20 g/d	Age (y)				
	≤50	6	1.00 (0.99, 1.00)	0.0	0.816
	>50	12	0.97 (0.96, 0.99)	96.5	<0.001
	Follow-up time (y)				
	≤10	6	0.99 (0.99, 1.00)	59.7	0.030
	>10	13	0.97 (0.96, 0.99)	96.1	<0.001
	Gender				
	Men	4	0.99 (0.98, 1.00)	72.0	0.013
	Women	4	0.96 (0.92, 1.00)	99.0	<0.001
	Men and Women	11	0.99 (0.99, 1.00)	20.3	0.250
	Continent				
	Europe	16	0.98 (0.97, 0.99)	95.3	<0.001

	Australia	2	1.06 (0.95, 1.18)	0.0	0.389
	USA	1	0.99 (0.94, 1.04)		
	Confounding factors[2]				
	Yes	15	0.98 (0.97, 0.99)	95.6	<0.001
	No	4	0.97 (0.94, 1.01)	0.0	0.609
	BMI				
	≤25	7	0.97 (0.95, 1.00)	98.0	<0.001
	>25	11	0.99 (0.99, 1.00)	42.2	0.068
	Newcastle-Ottawa quality score				
	<7	3	0.99 (0.95, 1.04)	0.0	0.805
	≥7	16	0.98 (0.97, 0.99)	95.3	<0.001
Cheese	Overall	13	0.99 (0.96, 1.01)	93.3	<0.001
Per 10 g/d	Age (y)				
	≤50	4	1.00 (0.98, 1.02)	0.0	0.784
	>50	8	0.98 (0.95, 1.01)	95.8	<0.001
	Follow-up time (y)				
	≤10	4	1.00 (0.99, 1.02)	21.1	0.284
	>10	9	0.98 (0.96, 1.01)	93.4	<0.001
	Gender				
	Men	2	1.00 (0.98, 1.02)	88.5	0.003
	Women	2	0.96 (0.91, 1.02)	97.5	<0.001
	Men and Women	9	0.99 (0.98, 1.00)	0.0	0.918
	Continent				
	Europe	11	0.99 (0.96, 1.01)	94.3	<0.001
	Australia	1	0.96 (0.83, 1.13)		
	USA	1	0.99 (0.97, 1.02)		
	Confounding factors[2]				
	Yes	9	0.99 (0.96, 1.01)	95.4	<0.001
	No	4	0.99 (0.97, 1.00)	0.0	0.600
	BMI				
	≤25	4	0.99 (0.94, 1.03)	97.8	<0.001
	>25	8	0.99 (0.98, 1.00)	0.0	0.906
	Newcastle-Ottawa quality score				
	<7	2	0.99 (0.97, 1.02)	0.0	0.675
	≥7	11	0.99 (0.96, 1.01)	94.3	<0.001

[1] Insufficient studies to split results for high-fat dairy and yogurt.
[2] Confounding factors adjusted for age, sex, BMI, smoking, alcohol, leisure activity and total energy intake.

Supplemental Table 4. Association between dairy foods and CHD by subgroups[1].

Dairy food	Subgroup	No. study populations	Relative risk (95% CI)[2]	Heterogeneity test	
				I^2 (%)	P-value
Total dairy	Overall	12	0.99 (0.96, 1.02)	38.9	0.081
Per 200 g/d	Age (y)				
	≤50	4	1.02 (0.98, 1.06)	33.1	0.214
	>50	8	0.97 (0.94, 1.00)	16.5	0.300
	Follow-up time (y)				
	≤10	5	0.99 (0.96, 1.02)	0.0	0.733
	>10	7	0.99 (0.94, 1.03)	62.4	0.014
	Gender				
	Men	2	0.99 (0.95, 1.03)	0.0	0.403
	Women	4	1.00 (0.94, 1.06)	70.2	0.018
	Men and Women	6	0.98 (0.94, 1.02)	21.6	0.272
	Continent				
	Europe	7	0.98 (0.95, 1.02)	14.9	0.317
	Australia	1	0.87 (0.77, 0.98)		
	USA	4	1.01 (0.97, 1.04)	30.8	0.227
	Confounding factors[2]				
	Yes	9	1.01 (0.98, 1.03)	3.4	0.406
	No	3	0.94 (0.88, 1.00)	37.7	0.201
	BMI				
	≤25	2	0.99 (0.89, 1.10)	88.8	0.003
	>25	10	0.99 (0.96, 1.01)	0.0	0.487
	Newcastle-Ottawa quality score				
	<7	1	0.94 (0.89, 0.99)		
	≥7	11	1.00 (0.97, 1.03)	25.8	0.198
High-fat dairy	Overall	9	0.99 (0.93, 1.05)	22.9	0.240
Per 200 g/d	Age (y)				
	≤50	2	0.89 (0.61, 1.29)	76.1	0.041
	>50	7	0.98 (0.92, 1.05)	0.0	0.474
	Follow-up time (y)				
	≤10	3	0.94 (0.77, 1.14)	39.1	0.194
	>10	6	1.00 (0.93, 1.07)	25.0	0.246
	Gender				
	Men	1	0.98 (0.88, 1.09)		
	Women	2	1.03 (0.97, 1.10)	0.0	0.800
	Men and Women	6	0.92 (0.80, 1.06)	40.3	0.137
	Continent				
	Europe	4	0.86 (0.68, 1.08)	30.9	0.227
	Australia	1	0.86 (0.70, 1.05)		
	USA	4	1.02 (0.97, 1.08)	0.0	0.793
	Confounding factors[2]				
	Yes	7	1.00 (0.94, 1.07)	25.5	0.235
	No	2	0.89 (0.75, 1.07)	0.0	0.451

	BMI				
	≤25	1	1.04 (0.97, 1.11)		
	>25	8	0.97 (0.89, 1.05)	19.7	0.274
	Newcastle-Ottawa quality score				
	≥7	9	0.99 (0.93, 1.05)	22.9	0.240
Low-fat dairy	Overall	10	1.00 (0.97, 1.03)	27.3	0.193
Per 200 g/d	Age (y)				
	≤50	2	0.97 (0.91, 1.04)	38.3	0.203
	>50	8	1.01 (0.97, 1.05)	26.8	0.215
	Follow-up time (y)				
	≤10	4	1.00 (0.96, 1.06)	14.5	0.320
	>10	6	1.00 (0.95, 1.05)	43.5	0.115
	Gender				
	Men	2	1.01 (0.97, 1.05)	0.0	0.848
	Women	2	1.00 (0.87, 1.15)	75.1	0.045
	Men and Women	6	1.00 (0.95, 1.05)	35.7	0.169
	Continent				
	Europe	6	1.02 (0.97, 1.08)	32.7	0.191
	Australia	1	0.94 (0.73, 1.21)		
	USA	3	0.98 (0.93, 1.03)	39.6	0.191
	Confounding factors[2]				
	Yes	8	0.99 (0.96, 1.03)	34.4	0.154
	No	2	1.04 (0.96, 1.12)	0.0	0.407
	BMI				
	≤25	3	1.00 (0.92, 1.09)	54.0	0.114
	>25	7	1.00 (0.97, 1.04)	24.9	0.239
	Newcastle-Ottawa quality score				
	≥7	10	1.00 (0.97, 1.03)	27.3	0.193
Milk	Overall	12	1.01 (0.96, 1.06)	45.5	0.043
Per 244 g/d	Age (y)				
	≤50	4	1.02 (0.94, 1.12)	54.6	0.086
	>50	8	1.00 (0.94, 1.07)	48.5	0.059
	Follow-up time (y)				
	≤10	2	0.97 (0.91, 1.04)	0.0	0.676
	>10	10	1.02 (0.60, 1.08)	53.1	0.024
	Gender				
	Men	4	0.98 (0.93, 1.04)	34.4	0.206
	Women	3	1.04 (0.93, 1.17)	64.2	0.061
	Men and Women	5	1.00 (0.93, 1.08)	9.0	0.355
	Continent				
	Europe	8	1.00 (0.95, 1.04)	25.4	0.227
	Asia	2	0.99 (0.19, 5.05)	87.2	0.005
	USA	2	1.03 (0.97, 1.08)	23.1	0.254
	Confounding factors[2]				
	Yes	4	1.02 (0.97, 1.08)	20.3	0.288
	No	8	1.00 (0.93, 1.08)	54.3	0.032

	BMI				
	≤25	6	1.08 (0.97, 1.20)	46.0	0.099
	>25	5	0.98 (0.94, 1.01)	0.0	0.537
	Newcastle-Ottawa quality score				
	<7	5	1.01 (0.95, 1.07)	43.3	0.133
	≥7	7	1.00 (0.93, 1.09)	52.6	0.049
Total fermented dairy Per 20 g/d	Overall	14	0.99 (0.98, 1.01)	44.6	0.037
	Age (y)				
	≤50	5	1.00 (0.97, 1.04)	65.7	0.020
	>50	9	0.99 (0.98, 1.00)	21.9	0.249
	Follow-up time (y)				
	≤10	6	1.00 (0.98, 1.02)	9.6	0.355
	>10	8	0.99 (0.98, 1.01)	53.3	0.036
	Gender				
	Men	2	0.92 (0.77, 1.10)	57.3	0.126
	Women	4	0.99 (0.97, 1.01)	53.7	0.090
	Men and Women	8	1.00 (0.98, 1.02)	41.3	0.103
	Continent				
	Europe	14	0.99 (0.98, 1.01)	44.6	0.037
	Confounding factors[2]				
	Yes	9	1.00 (0.99, 1.01)	0.0	0.447
	No	5	0.99 (0.97, 1.02)	72.5	0.006
	BMI				
	≤25	8	0.99 (0.96, 1.02)	63.2	0.008
	>25	6	1.00 (0.99, 1.01)	0.0	0.727
	Newcastle-Ottawa quality score				
	<7	4	0.99 (0.96, 1.03)	78.5	0.003
	≥7	10	1.00 (0.99, 1.01)	0.0	0.535
Cheese Per 10 g/d	Overall	10	0.99 (0.97, 1.02)	40.3	0.089
	Age (y)				
	≤50	4	1.06 (0.98, 1.15)	61.7	0.05
	>50	6	0.99 (0.98, 0.99)	0.0	0.809
	Follow-up time (y)				
	≤10	4	0.99 (0.95, 1.02)	27.1	0.249
	>10	6	1.01 (0.97, 1.05)	54.4	0.052
	Gender				
	Men	1	0.97 (0.93, 1.02)		
	Women	2	0.99 (0.98. 1.00)	0.0	0.648
	Men and Women	7	1.01 (0.97, 1.06)	48.9	0.068
	Continent				
	Europe	10	0.99 (0.97, 1.02)	40.3	0.089
	Confounding factors[2]				
	Yes	6	0.99 (0.96, 1.01)	0.0	0.461
	No	4	1.03 (0.97, 1.10)	71.2	0.015
	BMI				

	≤25	5	1.00 (0.95, 1.05)	63.3	0.028
	>25	5	1.00 (0.97, 1.03)	0.0	0.543
	Newcastle-Ottawa quality score				
	<7	3	1.14 (0.93, 1.40)	80.3	0.006
	≥7	7	0.99 (0.97, 1.01)	0.0	0.561

[1] Insufficient studies to split results for high-fat dairy and yogurt.
[2] Confounding factors adjusted for age, sex, BMI, smoking, alcohol, leisure activity and total energy intake.

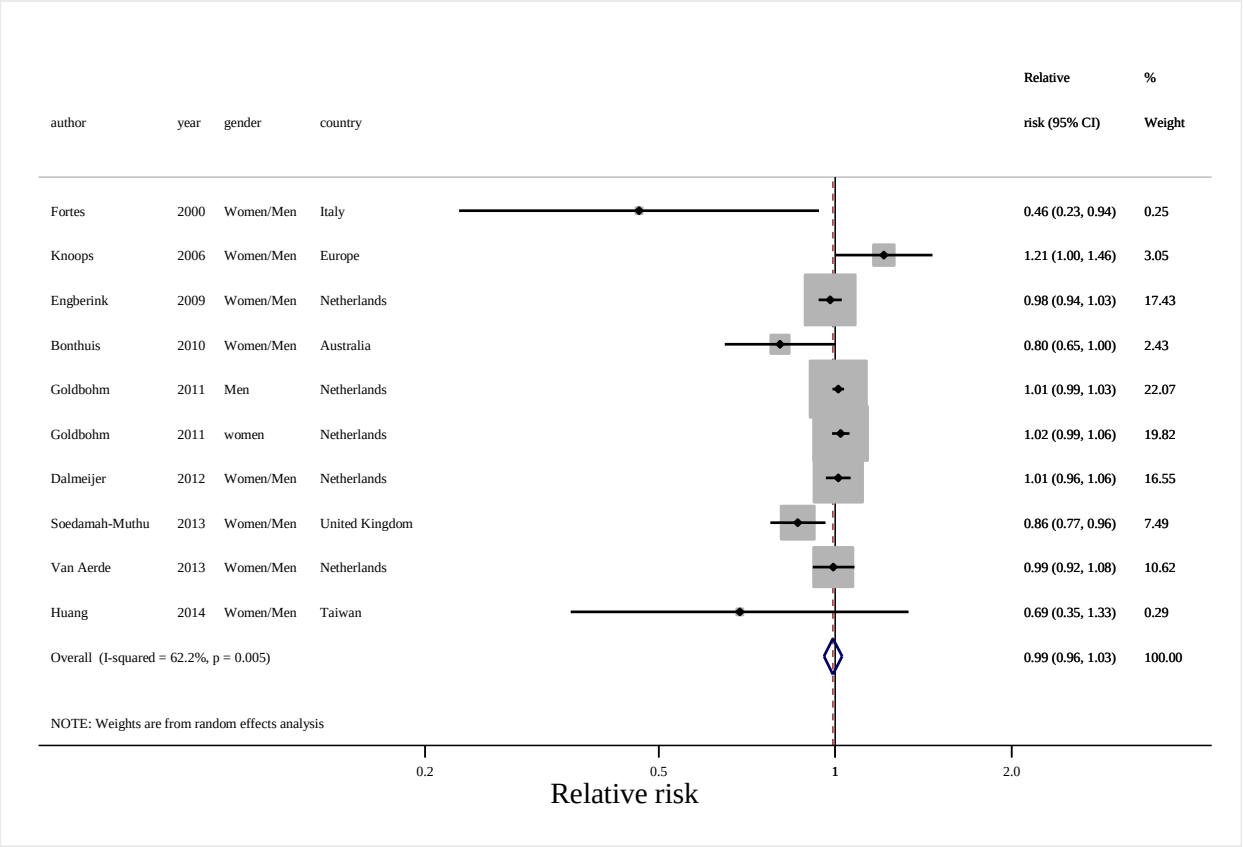
Supplemental Table 5. Association between dairy foods and CVD by subgroups[1].

Dairy food	Subgroup	No. study populations	Relative risk (95% CI)[2]	Heterogeneity test	
				I^2 (%)	P-value
Total dairy	Overall	8	0.97 (0.91, 1.02)	59.9	0.015
Per 200 g/d	Age (y)				
	≤50	3	0.68 (0.39, 1.19)	77.5	0.012
	>50	5	0.97 (0.91, 1.04)	52.6	0.077
	Follow-up time (y)				
	≤10	2	1.08 (0.81, 1.44)	59.4	0.116
	>10	6	0.95 (0.87, 1.03)	64.9	0.014
	Gender				
	Men and Women	8	0.97 (0.91, 1.02)	59.9	0.015
	Continent				
	Europe	5	0.98 (0.94, 1.02)	36.5	0.178
	Australia	2	0.81 (0.60, 1.09)	52.1	0.149
	Asia	1	0.19 (0.04, 0.76)		
	Confounding factors[2]				
	Yes	4	0.98 (0.92, 1.03)	58.3	0.066
	No	4	0.95 (0.80, 1.12)	68.1	0.024
	BMI				
	≤25	1	0.19 (0.04, 0.76)		
	>25	7	0.97 (0.92, 1.01)	50.9	0.057
	Newcastle-Ottawa quality score				
	<7	2	0.56 (0.91, 1.02)	59.9	0.015
	≥7	6	0.96 (0.92, 1.01)	47.7	0.088
High-fat dairy	Overall	7	0.93 (0.84, 1.03)	37.4	0.143
Per 200 g/d	Age (y)				
	≤50	3	0.76 (0.59, 0.97)	31.5	0.232
	>50	4	0.99 (0.93, 1.04)	0.0	0.797
	Follow-up time (y)				
	≤10	2	0.82 (0.67, 1.01)	0.0	0.570
	>10	5	0.96 (0.86, 1.07)	39.4	0.159
	Gender				
	Men and Women	7	0.93 (0.84, 1.03)	37.4	0.143
	Continent				
	Europe	5	0.98 (0.93, 1.04)	0.0	0.450
	Australia	2	0.72 (0.41, 1.28)	75.1	0.045
	Confounding factors[2]				
	Yes	5	0.88 (0.74, 1.04)	53.9	0.070
	No	2	0.97 (0.85, 1.11)	0.0	0.338
	BMI				
	≤25	1	0.86 (0.66, 1.13)		
	>25	6	0.94 (0.83, 1.05)	43.6	0.115
	Newcastle-Ottawa quality score				

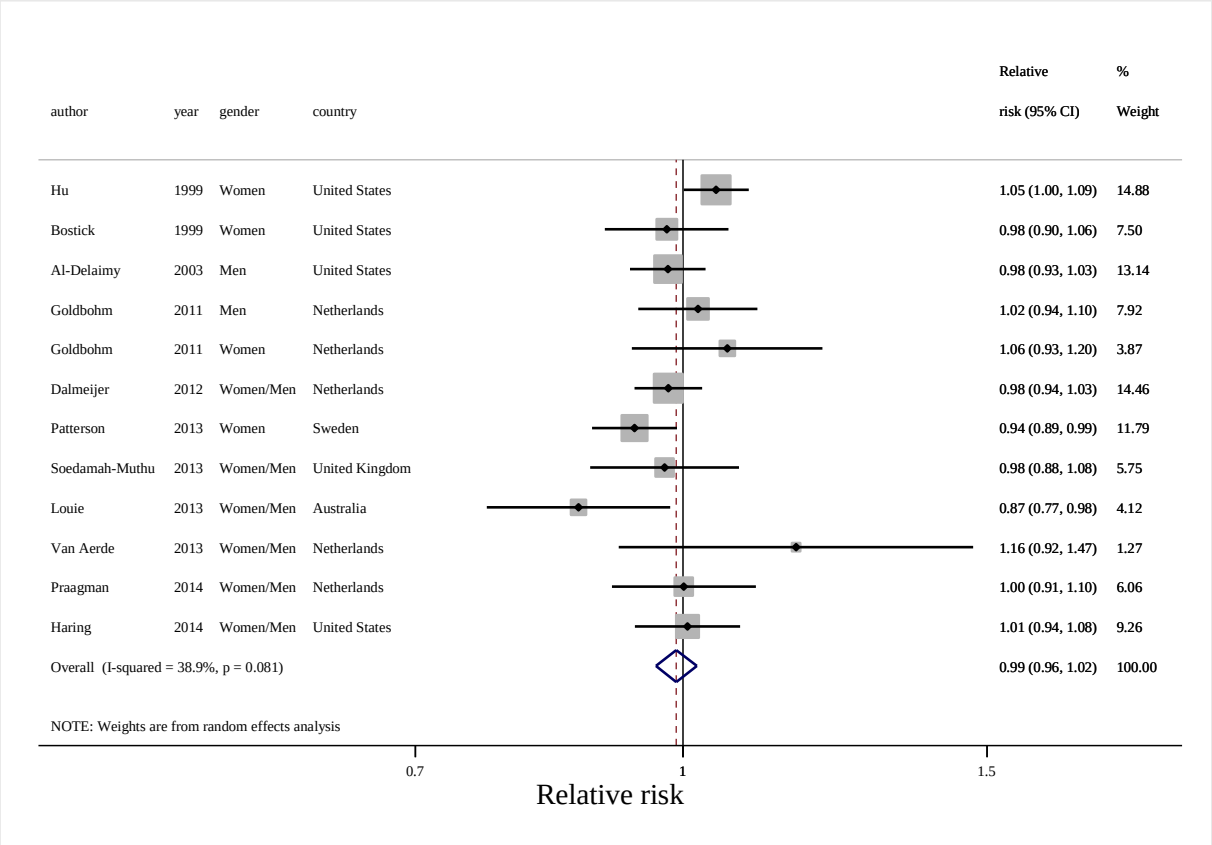
	≥7	7	0.93 (0.84, 1.03)	37.4	0.143
Low-fat dairy	Overall	7	0.98 (0.95, 1.01)	0.0	0.769
Per 200 g/d	Age (y)				
	≤50	3	0.99 (0.94, 1.04)	0.0	0.577
	>50	4	0.97 (0.93, 1.01)	0.0	0.584
	Follow-up time (y)				
	≤10	2	0.98 (0.94, 1.03)	0.0	0.599
	>10	5	0.97 (0.94, 1.01)	0.0	0.579
	Gender				
	Men and Women	7	0.98 (0.95, 1.01)	0.0	0.769
	Continent				
	Europe	5	0.98 (0.95, 1.01)	0.0	0.888
	Australia	2	0.96 (0.69, 1.35)	43.8	0.182
	Confounding factors[2]				
	Yes	5	0.98 (0.95, 1.01)	0.0	0.742
	No	2	0.95 (0.86, 1.04)	0.0	0.345
	BMI				
	>25	6	0.97 (0.94, 1.00)	0.0	0.715
	Newcastle-Ottawa quality score				
	≥7	7	0.98 (0.95, 1.01)	0.0	0.769
Milk	Overall	12	1.01 (0.93, 1.10)	92.4	<0.001
Per 244 g/d	Age (y)				
	≤50	2	0.96 (0.92, 1.01)	0.0	0.399
	>50	10	1.03 (0.93, 1.13)	92.5	<0.001
	Follow-up time (y)				
	≤10	1	1.67 (0.75, 3.72)		
	>10	11	1.01 (0.92, 1.09)	93.0	<0.001
	Gender				
	Men	5	0.97 (0.91, 1.05)	80.2	<0.001
	Women	2	0.99 (0.76, 1.28)	93.4	<0.001
	Men and Women	4	1.11 (0.84, 1.47)	91.2	0.052
	Continent				
	Europe	7	1.06 (0.96, 1.17)	95.0	<0.001
	Australia	1	0.77 (0.46, 1.29)		
	Asia	4	0.91 (0.82, 1.02)	39.8	0.173
	Confounding factors[2]				
	Yes	5	1.10 (0.98-1.25)	95.3	<0.001
	No	7	0.94 (0.89-0.99)	28.6	0.210
	BMI				
	≤25	5	0.99 (0.80, 1.22)	93.5	<0.001
	>25	7	1.00 (0.94, 1.06)	70.9	0.002
	Newcastle-Ottawa quality score				
	<7	4	0.95 (0.90, 1.00)	22.1	0.278
	≥7	8	1.05 (0.94, 1.16)	93.0	<0.001
Total fermented dairy	Overall	17	0.98 (0.97, 0.99)	87.5	<0.001
Per 20 g/d	Age (y)				
	≤50	7	1.00 (0.99, 1.01)	0.0	0.541

	>50	10	0.98 (0.96, 0.99)	92.5	<0.001
	Follow-up time (y)				
	≤10	5	1.00 (0.99, 1.01)	0.0	0.659
	>10	12	0.97 (0.96, 0.99)	91.1	<0.001
	Gender				
	Men	2	0.99 (0.96, 1.01)	77.3	0.036
	Women	2	0.93 (0.81, 1.06)	99.0	<0.001
	Men and Women	13	0.99 (0.99, 1.00)	0.0	0.476
	Continent				
	Europe	15	0.98 (0.97, 0.99)	88.9	<0.001
	Australia	2	0.89 (0.72, 1.10)	0.0	0.510
	Confounding factors[2]				
	Yes	14	0.98 (0.97, 0.99)	89.5	<0.001
	No	3	1.02 (0.98, 1.06)	0.0	0.593
	BMI				
	≤25	2	0.93 (0.81, 1.06)	99.0	<0.001
	>25	13	0.99 (0.99, 1.00)	25.0	0.191
	Newcastle-Ottawa quality score				
	<7	2	1.02 (0.97, 1.08)	4.1	0.307
	≥7	15	0.98 (0.97, 0.99)	88.8	<0.001
Cheese	Overall	11	0.98 (0.95, 1.00)	82.6	<0.001
Per 10 g/d	Age (y)				
	≤50	5	0.98 (0.96, 1.01)	0.0	0.528
	>50	6	0.98 (0.94, 1.01)	90.5	<0.001
	Follow-up time (y)				
	≤10	4	1.00 (0.96, 1.04)	0.0	0.853
	>10	7	0.97 (0.85, 1.00)	88.7	<0.001
	Gender				
	Men	1	0.99 (0.97, 1.00)		
	Women	1	0.93 (0.92, 0.94)		
	Men and Women	9	0.99 (0.97, 1.00)	0.0	0.764
	Continent				
	Europe	10	0.98 (0.95, 1.00)	84.1	<0.001
	Australia	1	0.86 (0.65, 1.15)		
	Confounding factors[2]				
	Yes	9	0.98 (0.95, 1.00)	85.2	<0.001
	No	2	1.00 (0.96, 1.05)	0.0	0.354
	BMI				
	≤25	1	0.93 (0.92, 0.94)		
	>25	8	0.99 (0.98, 0.99)	0.0	0.679
	Newcastle-Ottawa quality score				
	<7	1	0.95 (0.83, 1.08)		
	≥7	10	0.98 (0.95, 1.01)	84.3	<0.001

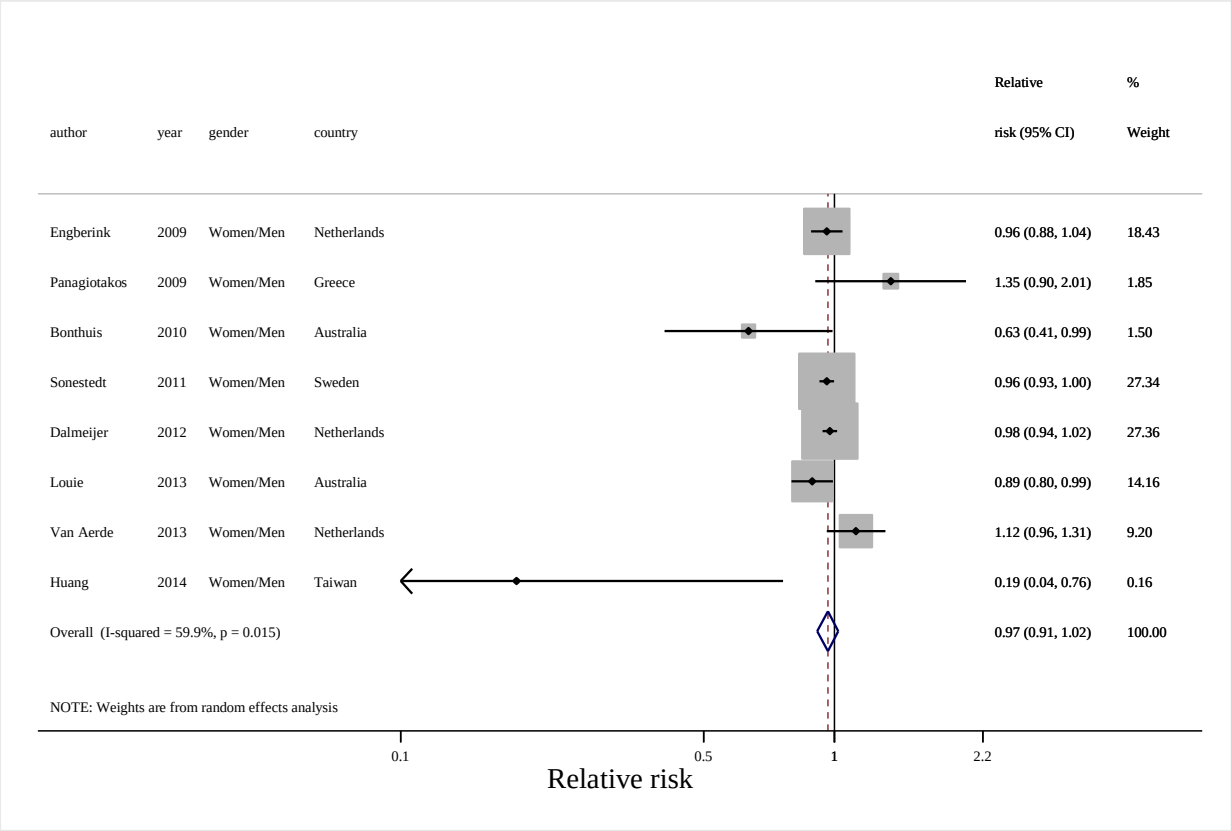
[1] Insufficient studies to split results for high-fat dairy and yogurt. [2] Confounding factors adjusted for age, sex, BMI, smoking, alcohol, leisure activity and total energy intake.



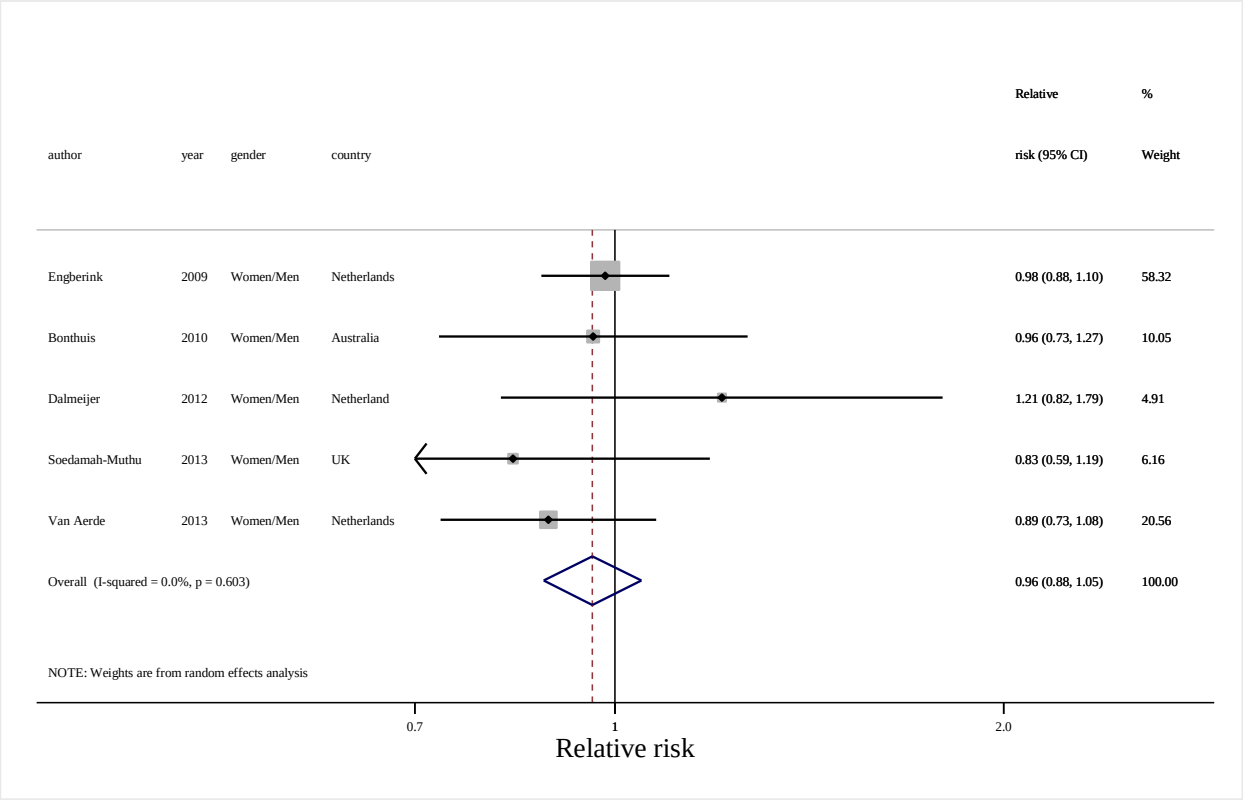
Supplemental Figure 1. Forest plot for the association between total dairy intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between total dairy and all-cause mortality (per increment of 200 g/d), including 10 populations (n=175,063 individuals). Heterogeneity (I^2) of between-study variations is 62.2%.



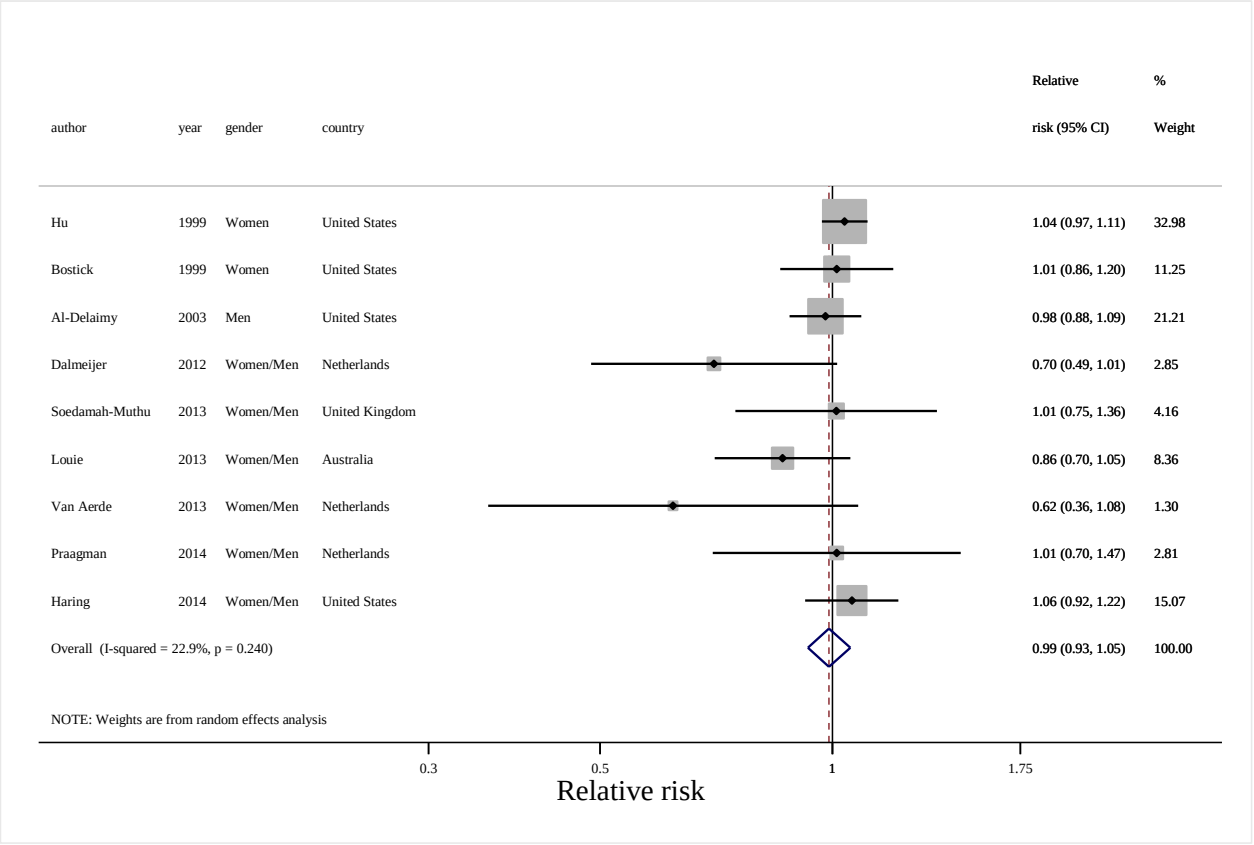
Supplemental Figure 2. Forest plot for the association between total dairy intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between total dairy and CHD (per increment of 200 g/d), including 12 populations (n=330,350 individuals). Heterogeneity (I^2) of between-study variations is 38.9%.



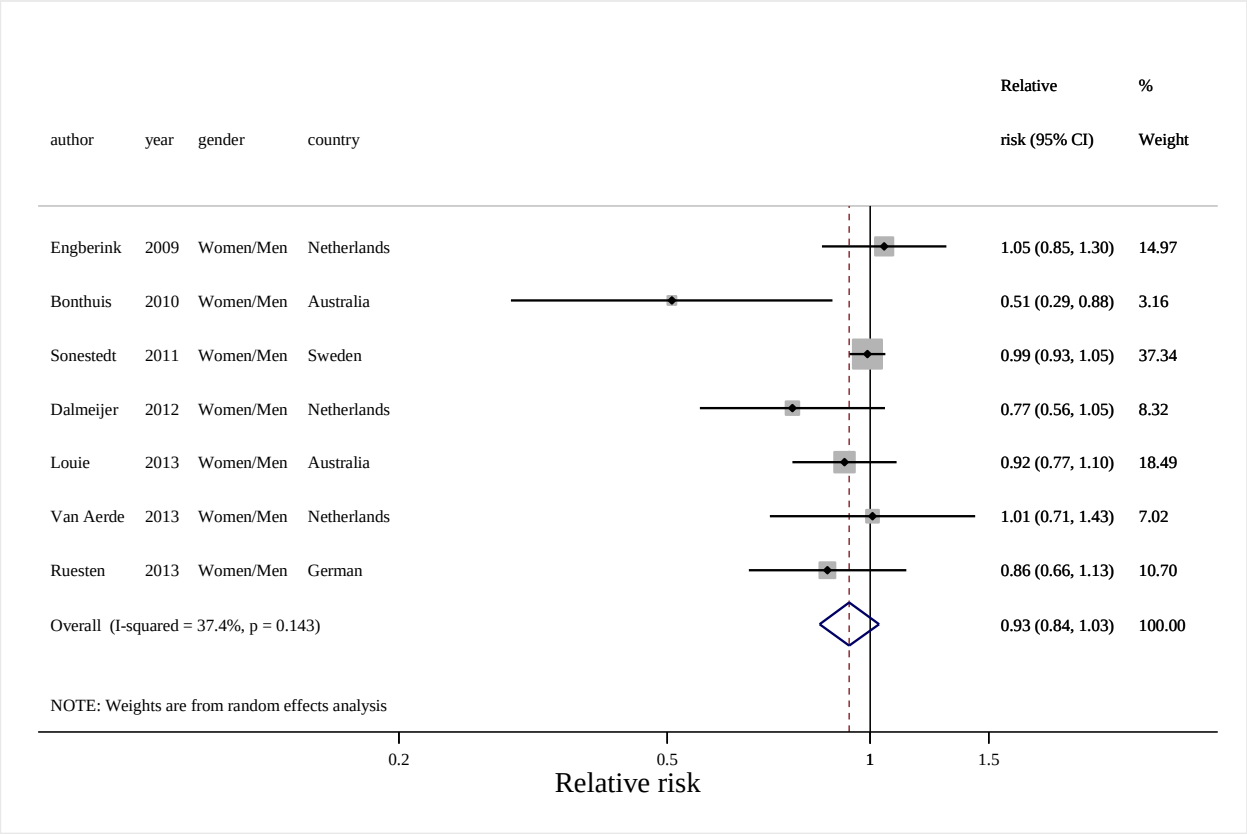
Supplemental Figure 3. Forest plot for the association between total dairy intake and CVD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between total dairy and CVD (per increment of 200 g/d), including 8 populations (n=76,207 individuals). Heterogeneity (I^2) of between-study variations is 59.9%.



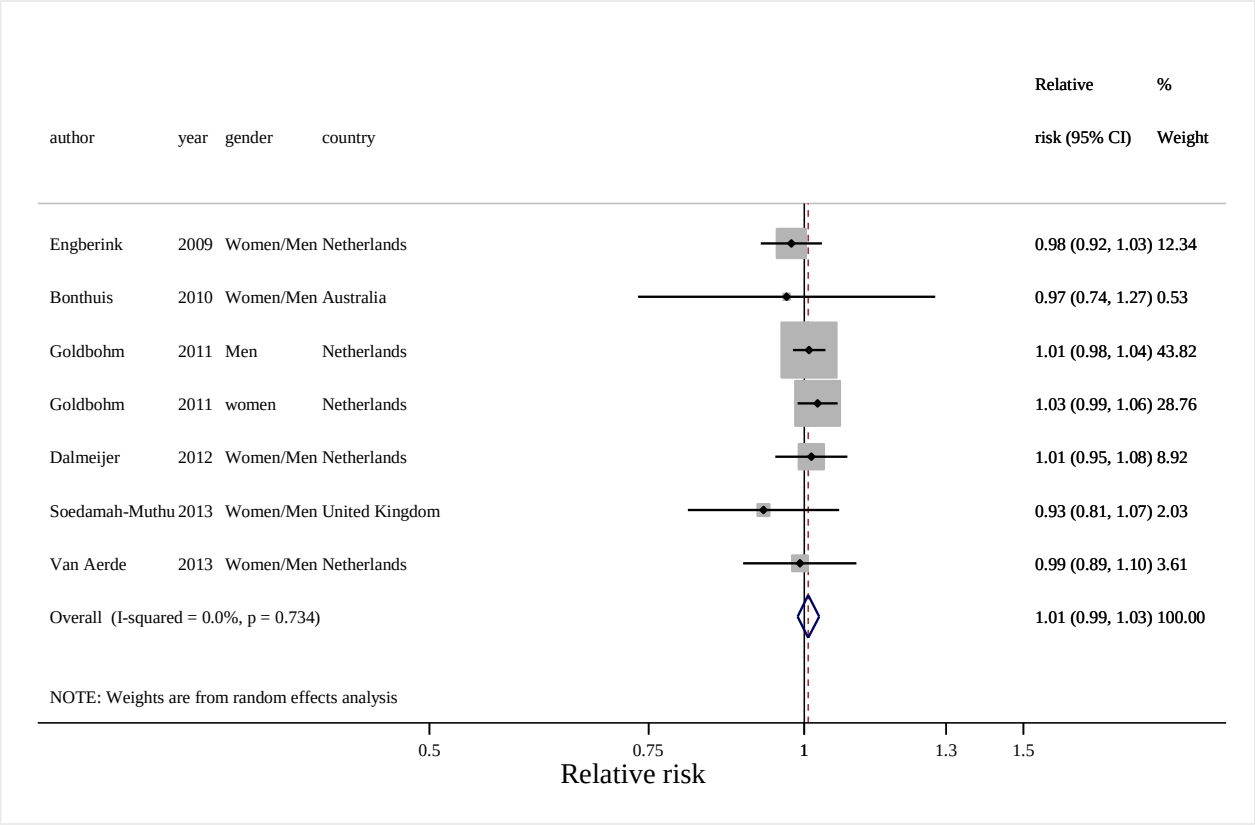
Supplemental Figure 4. Forest plot for the association between high-fat dairy intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between high-fat dairy and all-cause mortality (per increment of 200 g/d), including 5 populations (n=47,126 individuals). Heterogeneity (I^2) of between-study variations is 0%.



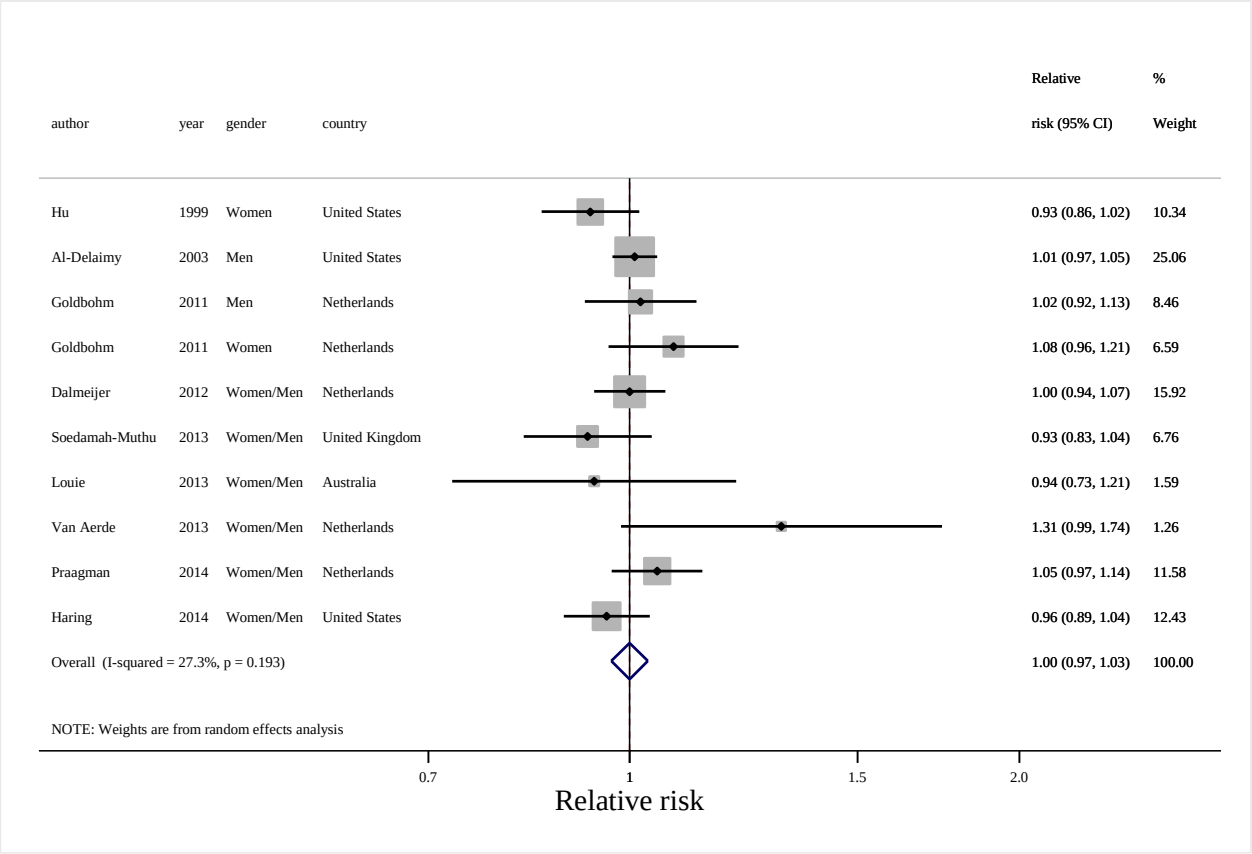
Supplemental Figure 5. Forest plot for the association between high-fat dairy intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between high-fat dairy and CHD (per increment of 200 g/d), including 9 populations (n=171,627 individuals). Heterogeneity (I^2) of between-study variations is 22.9%.



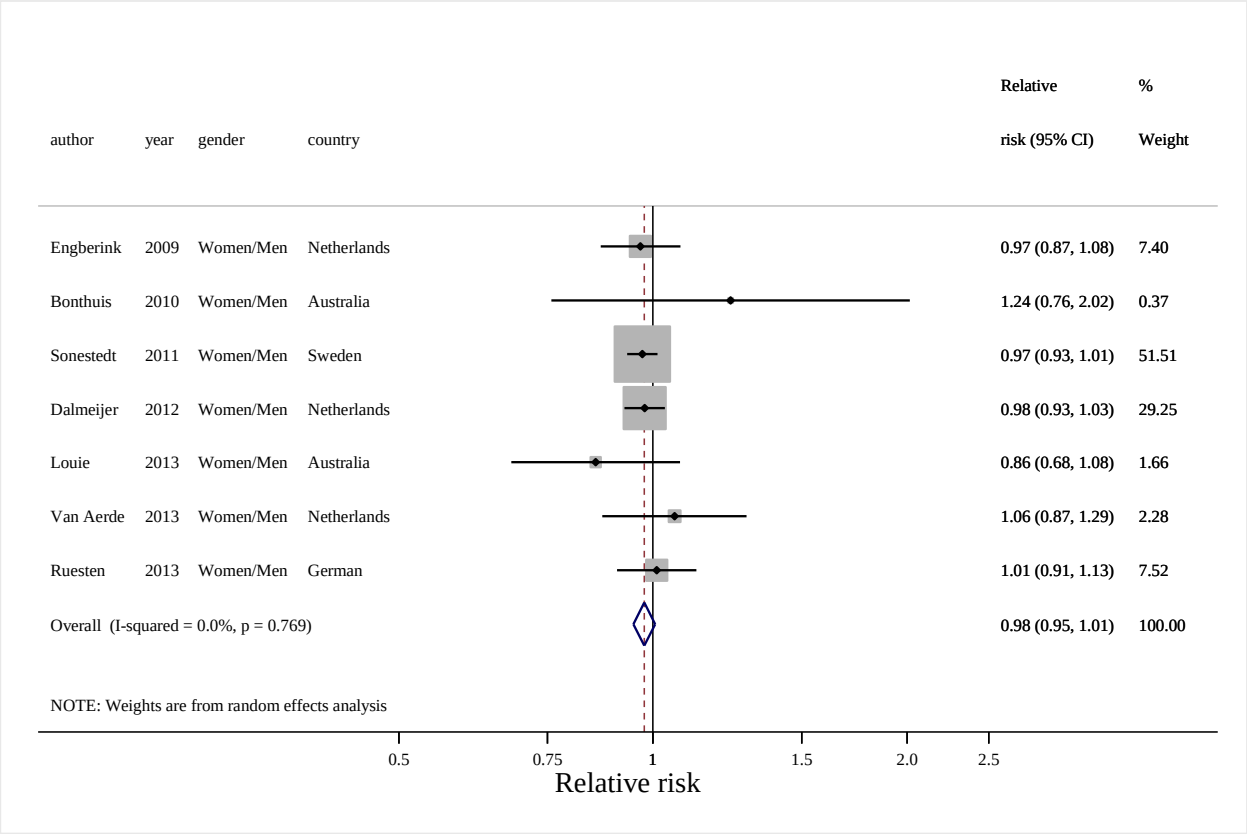
Supplemental Figure 6. Forest plot for the association between high-fat dairy intake and CVD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between high-fat dairy and CVD (per increment of 200 g/d), including 7 populations (n=95,242 individuals). Heterogeneity (I^2) of between-study variations is 37.4%.



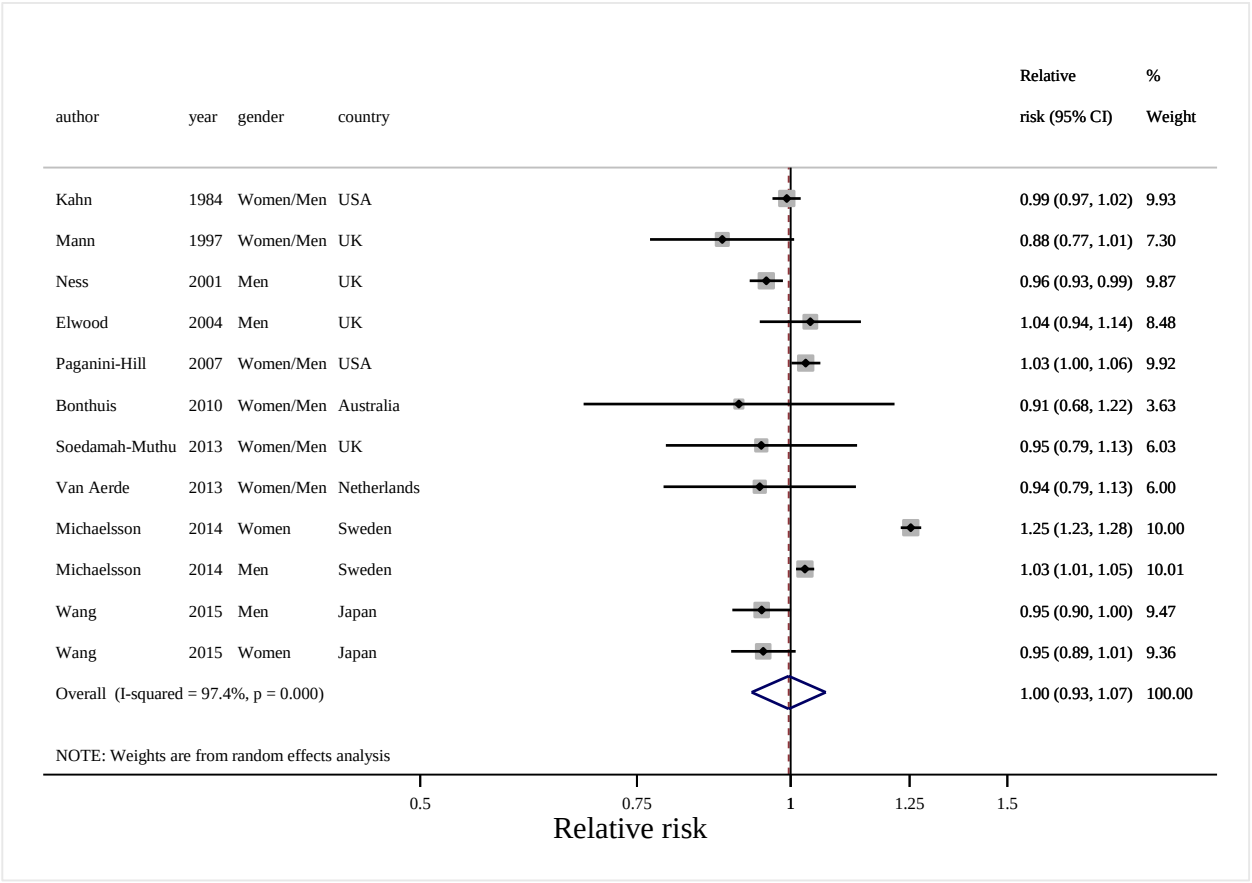
Supplemental Figure 7. Forest plot for the association between low-fat dairy intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between low-fat dairy and all-cause mortality (per increment of 200 g/d), including 7 populations (n=167,978 individuals). Heterogeneity (I^2) of between-study variations is 0%.



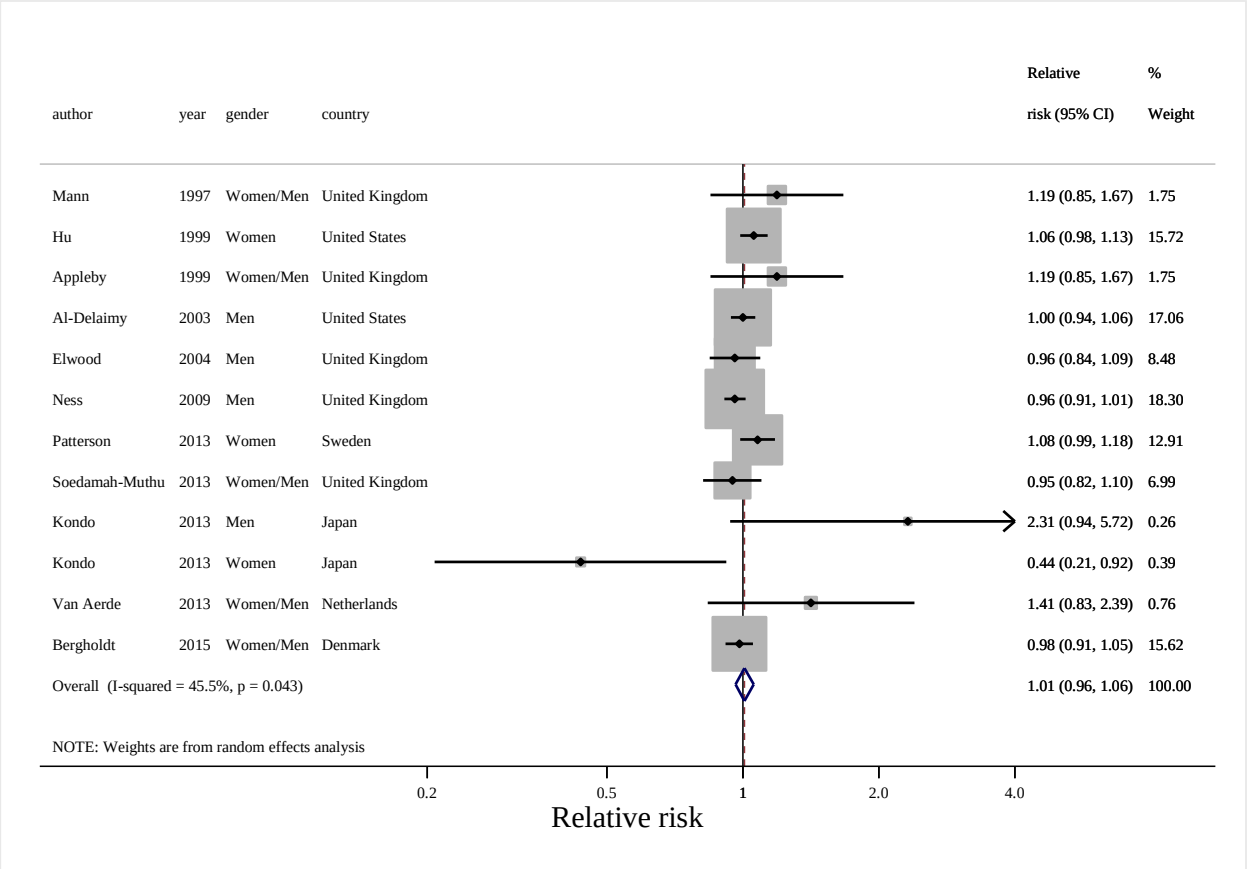
Supplemental Figure 8. Forest plot for the association between low-fat dairy intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between low-fat dairy and CHD (per increment of 200 g/d), including 10 populations (n=262,228 individuals). Heterogeneity (I^2) of between-study variations is 27.3%.



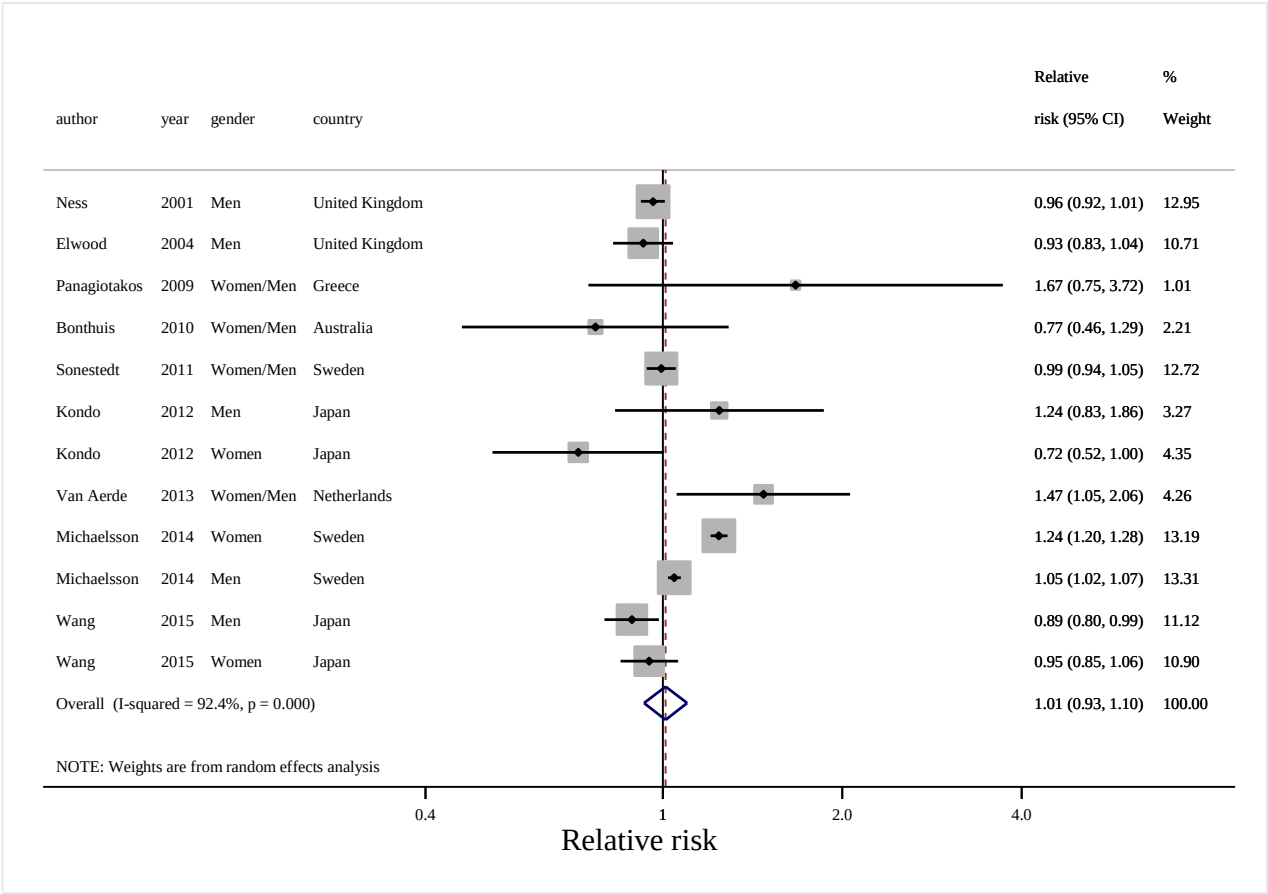
Supplemental Figure 9. Forest plot for the association between low-fat dairy intake and CVD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between low-fat dairy and CVD (per increment of 200 g/d), including 7 populations (n=95,242 individuals). Heterogeneity (I^2) of between-study variations is 0%.



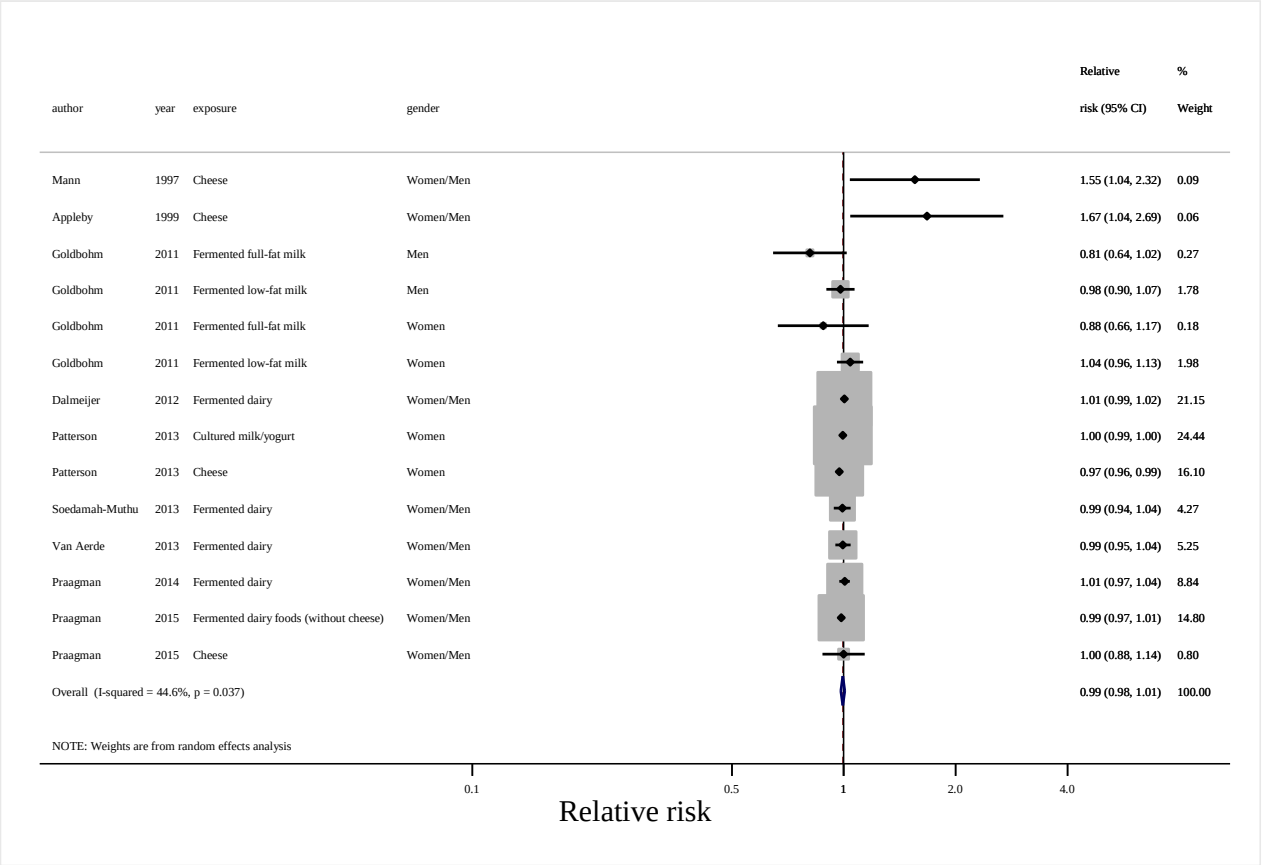
Supplemental Figure 10. Forest plot for the association between milk intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between milk and all-cause mortality (per increment of 244 g/d), including 12 populations (n=268,570 individuals). Heterogeneity (I^2) of between-study variations is 97.4%.



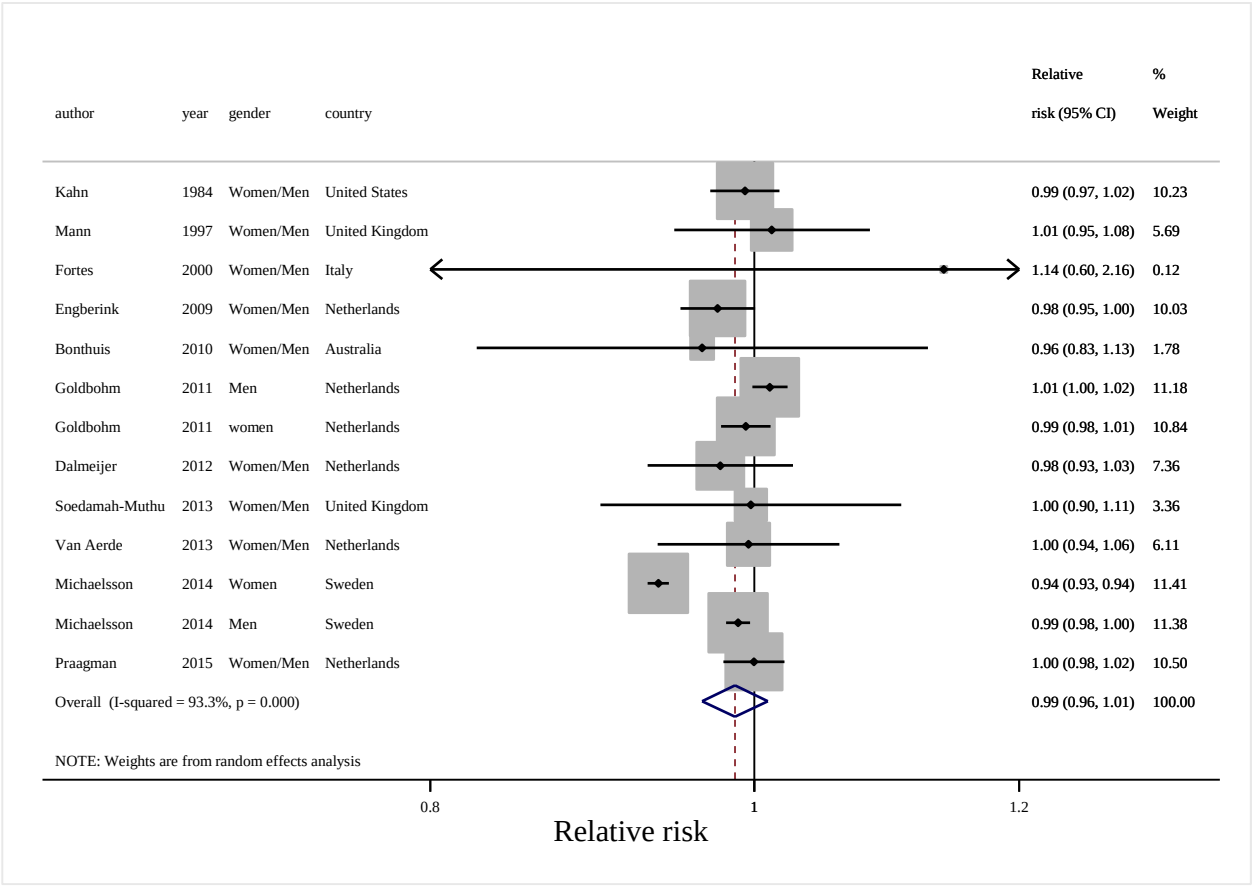
Supplemental Figure 11. Forest plot for the association between milk intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between milk and CHD (per increment of 244 g/d), including 12 populations (n=230,621 individuals). Heterogeneity (I^2) of between-study variations is 45.5%.



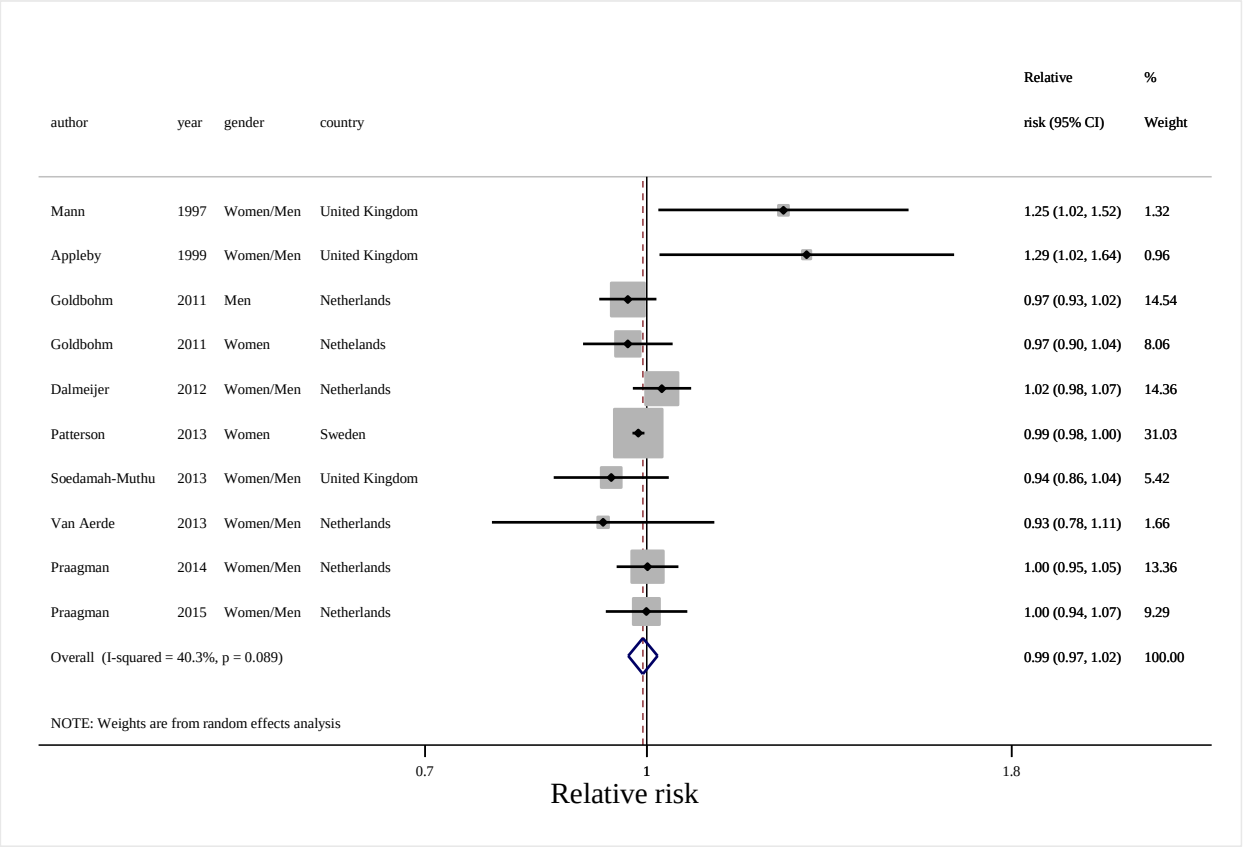
Supplemental Figure 12. Forest plot for the association between milk intake and CVD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between milk and CVD (per increment of 244 g/d), including 12 populations (n=249,779 individuals). Heterogeneity (I^2) of between-study variations is 92.4%.



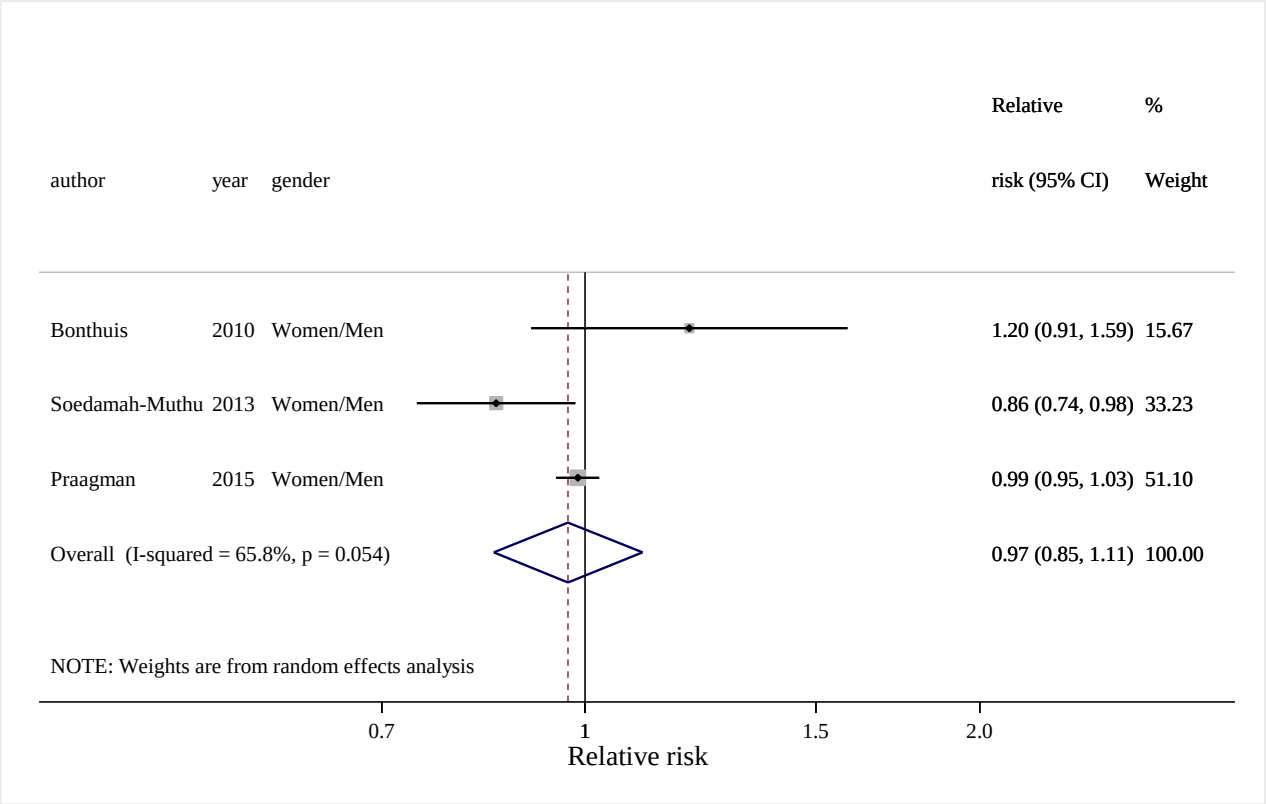
Supplemental Figure 13. Forest plot for the association between total fermented dairy intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between total fermented dairy and CHD (per increment of 20 g/d), including 14 populations (n=256,091 individuals). Heterogeneity (I^2) of between-study variations is 44.6%.



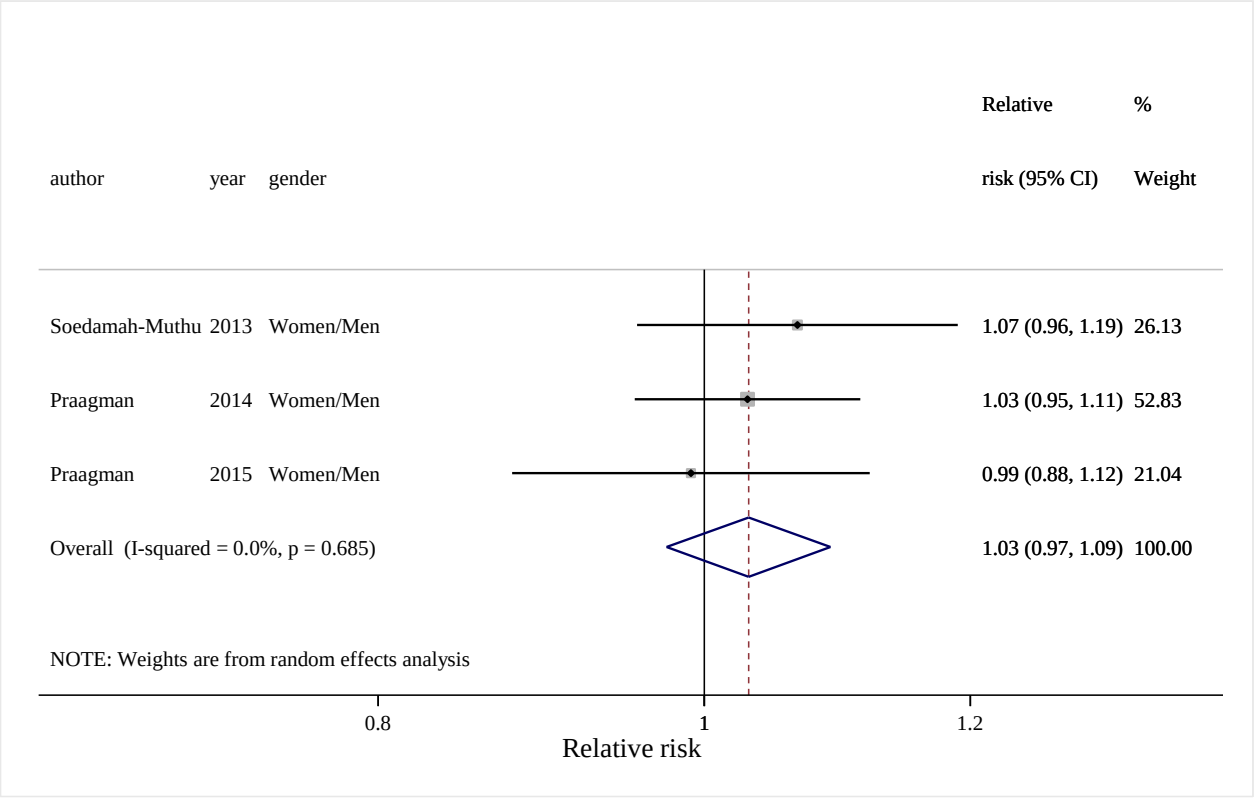
Supplemental Figure 14. Forest plot for the association between cheese intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between cheese and all-cause mortality (per increment of 10 g/d), including 13 populations (n=342,120 individuals). Heterogeneity (I^2) of between-study variations is 93.3%.



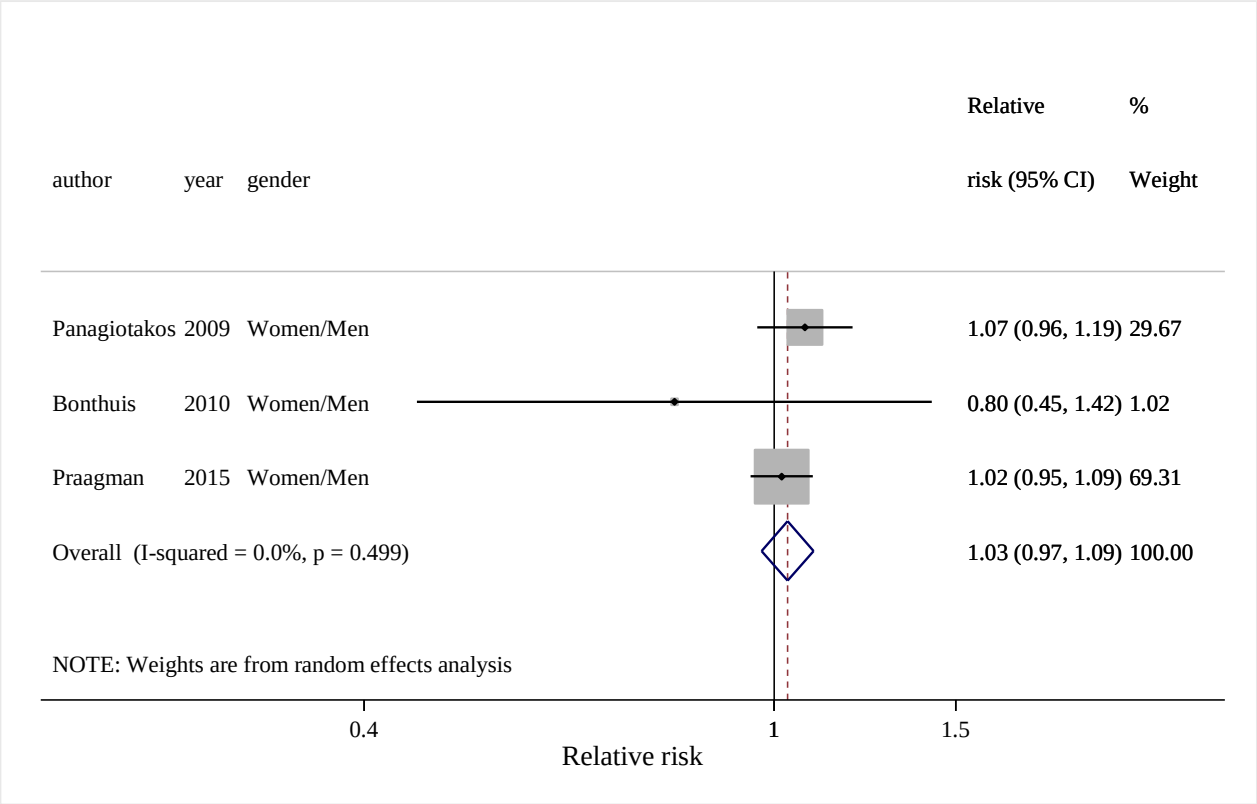
Supplemental Figure 15. Forest plot for the association between cheese intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between cheese and CHD (per increment of 10 g/d), including 10 populations (n=256,091 individuals). Heterogeneity (I^2) of between-study variations is 40.3%.



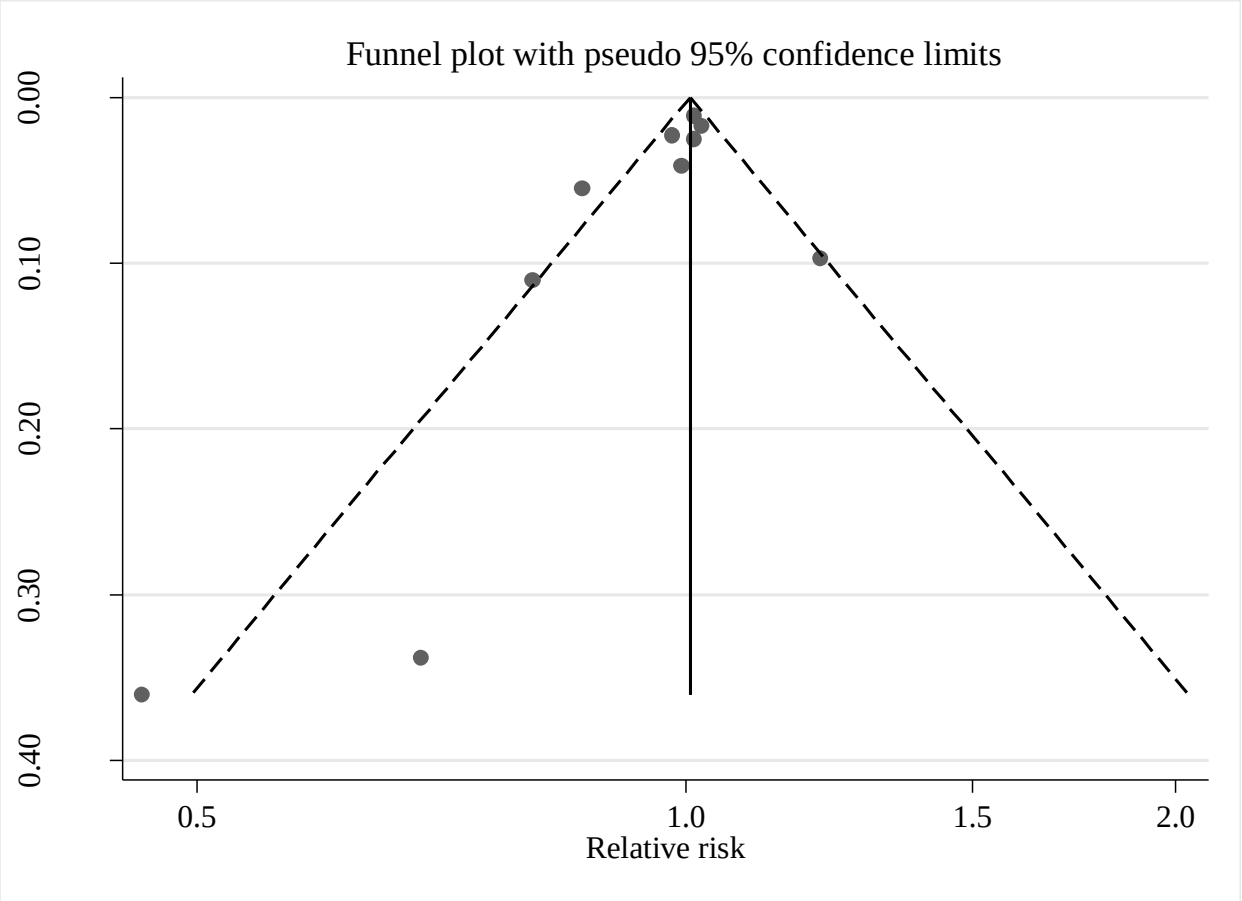
Supplemental Figure 16. Forest plot for the association between yogurt intake and all-cause mortality. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between yogurt and all-cause mortality (per increment of 50 g/d), including 3 populations (n=40,460 individuals). Heterogeneity (I^2) of between-study variations is 65.8%.



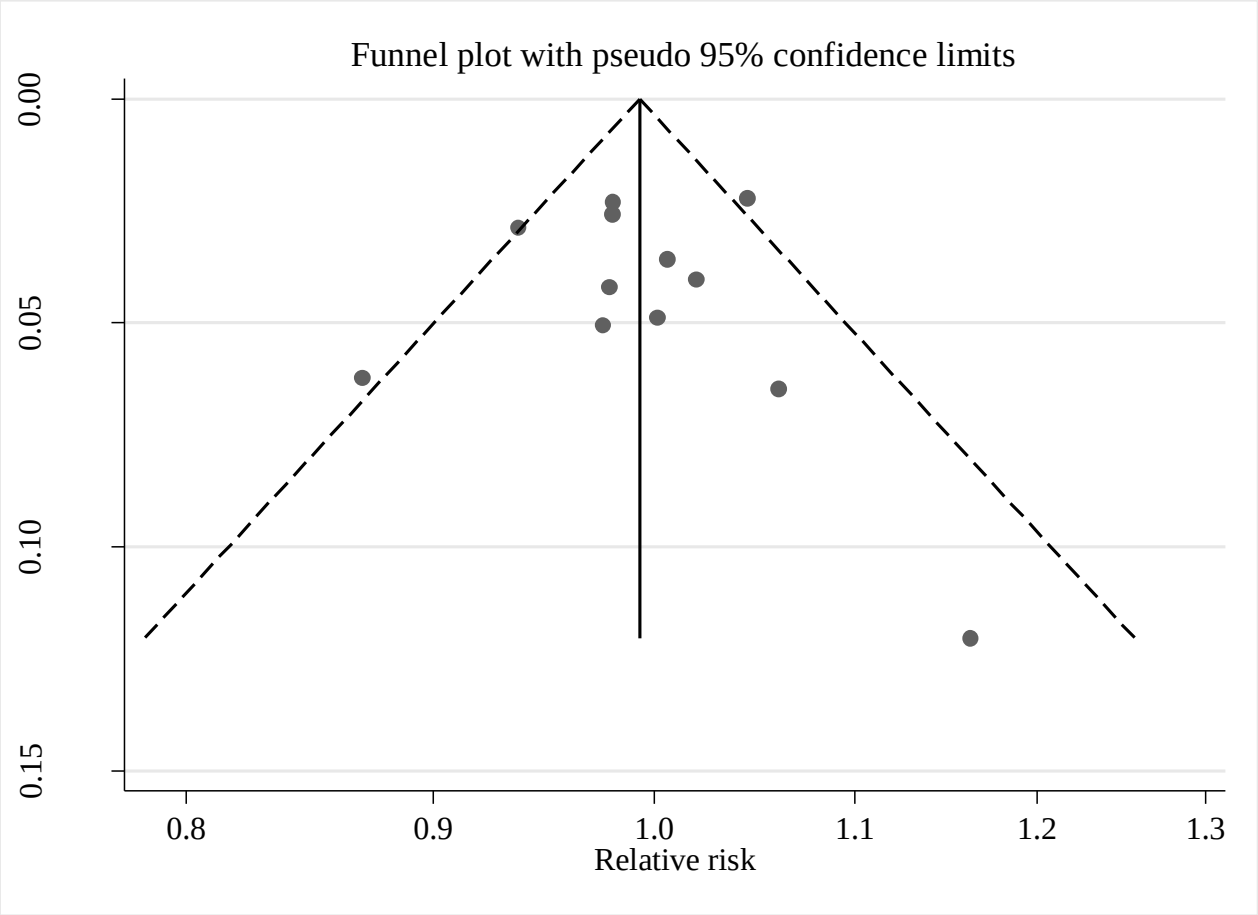
Supplemental Figure 17. Forest plot for the association between yogurt intake and CHD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between yogurt and CHD (per increment of 50 g/d), including 3 populations (n=98,936 individuals). Heterogeneity (I^2) of between-study variations is 0%.



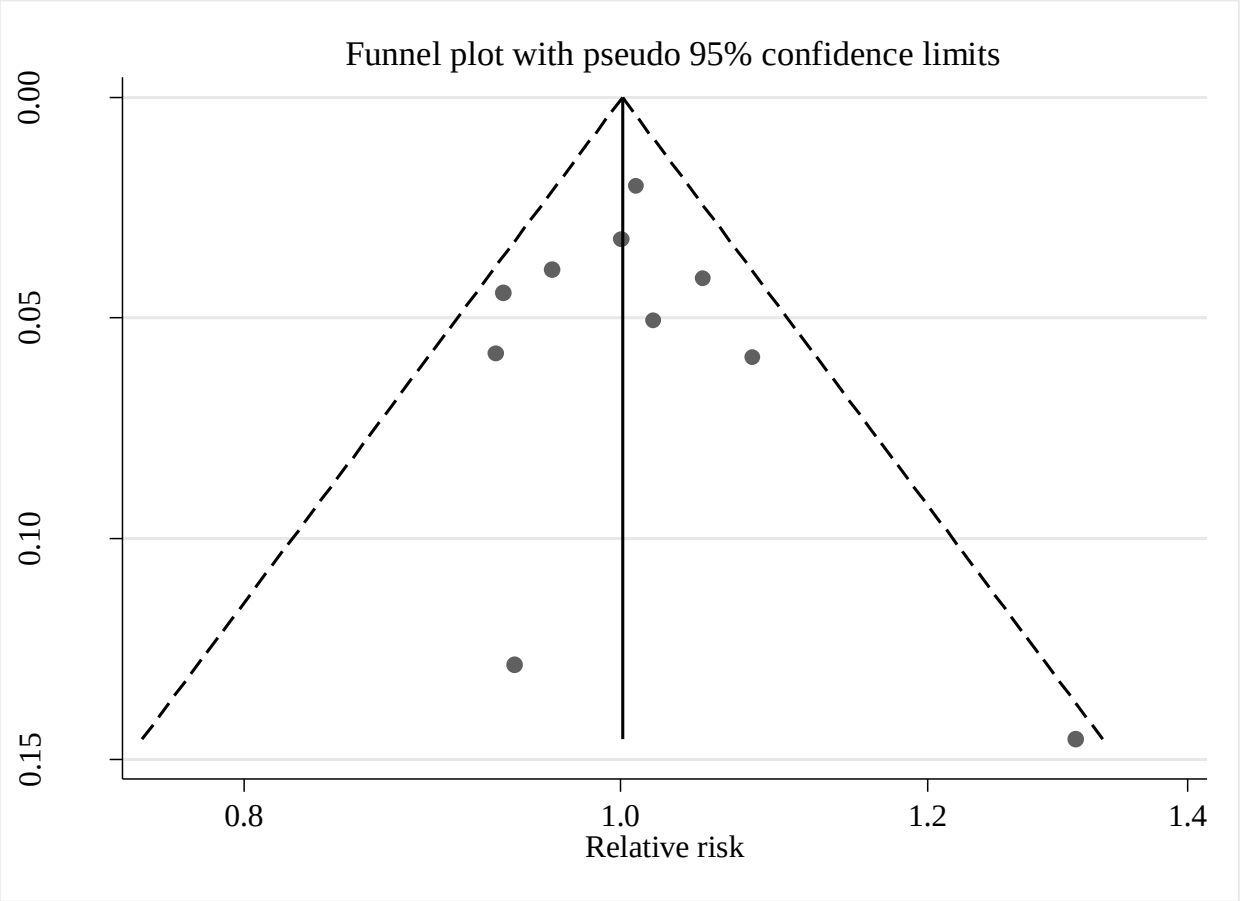
Supplemental Figure 18. Forest plot for the association between yogurt intake and CVD. Squares represent study-specific RRs. Square areas are proportional to the overall specific-study weight to the overall meta-analysis. Horizontal lines represent 95% CIs. Diamonds represent the pooled relative risk and 95% CIs. Overall no association between yogurt and CVD (per increment of 50 g/d), including 3 populations (n=36,624individuals). Heterogeneity (I^2) of between-study variations is 0%.



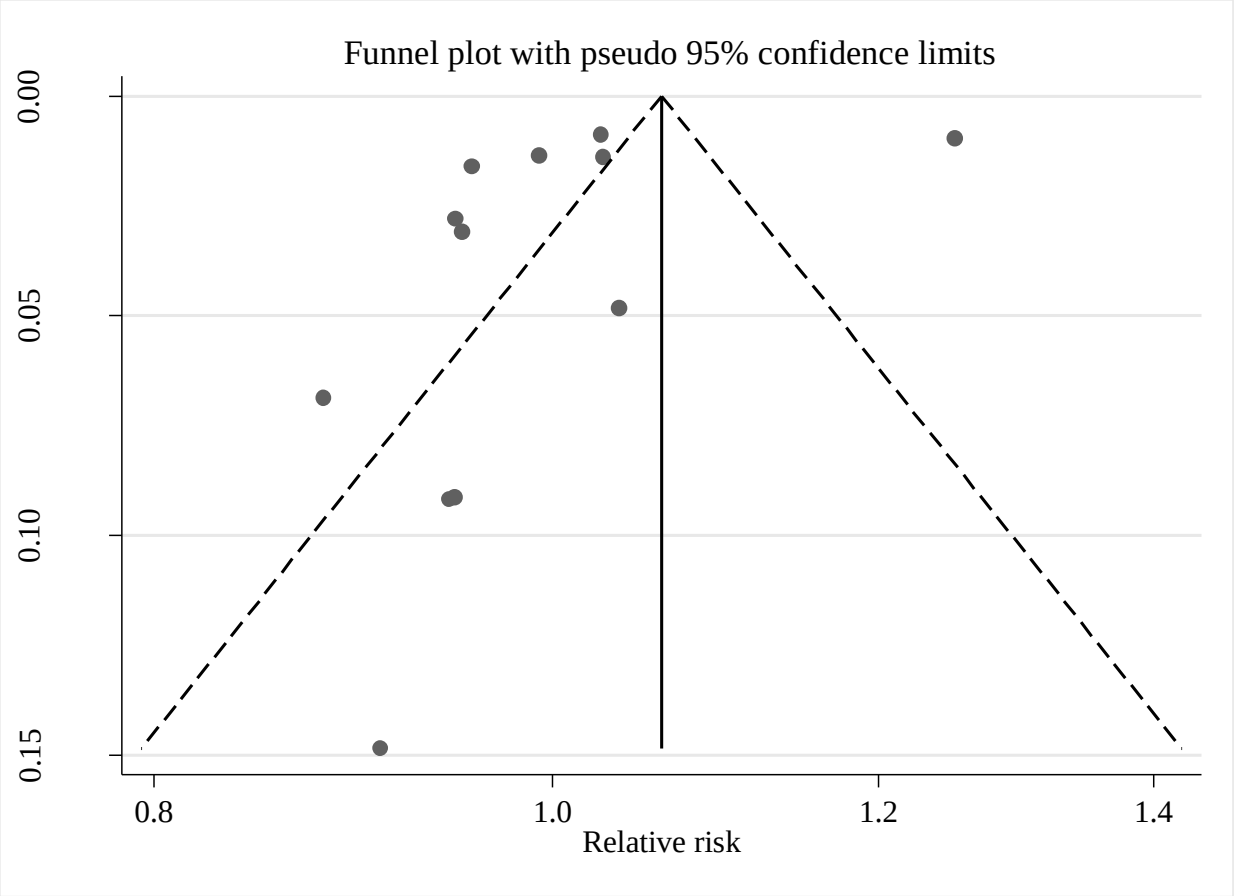
Supplemental Figure 19. Funnel plot for studies of the association between total dairy intake and all-cause mortality based on linear dose-response slopes (no. of cases=21,222; total n=175,063). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test P = 0.086, symmetry indicates no evidence of publication bias.



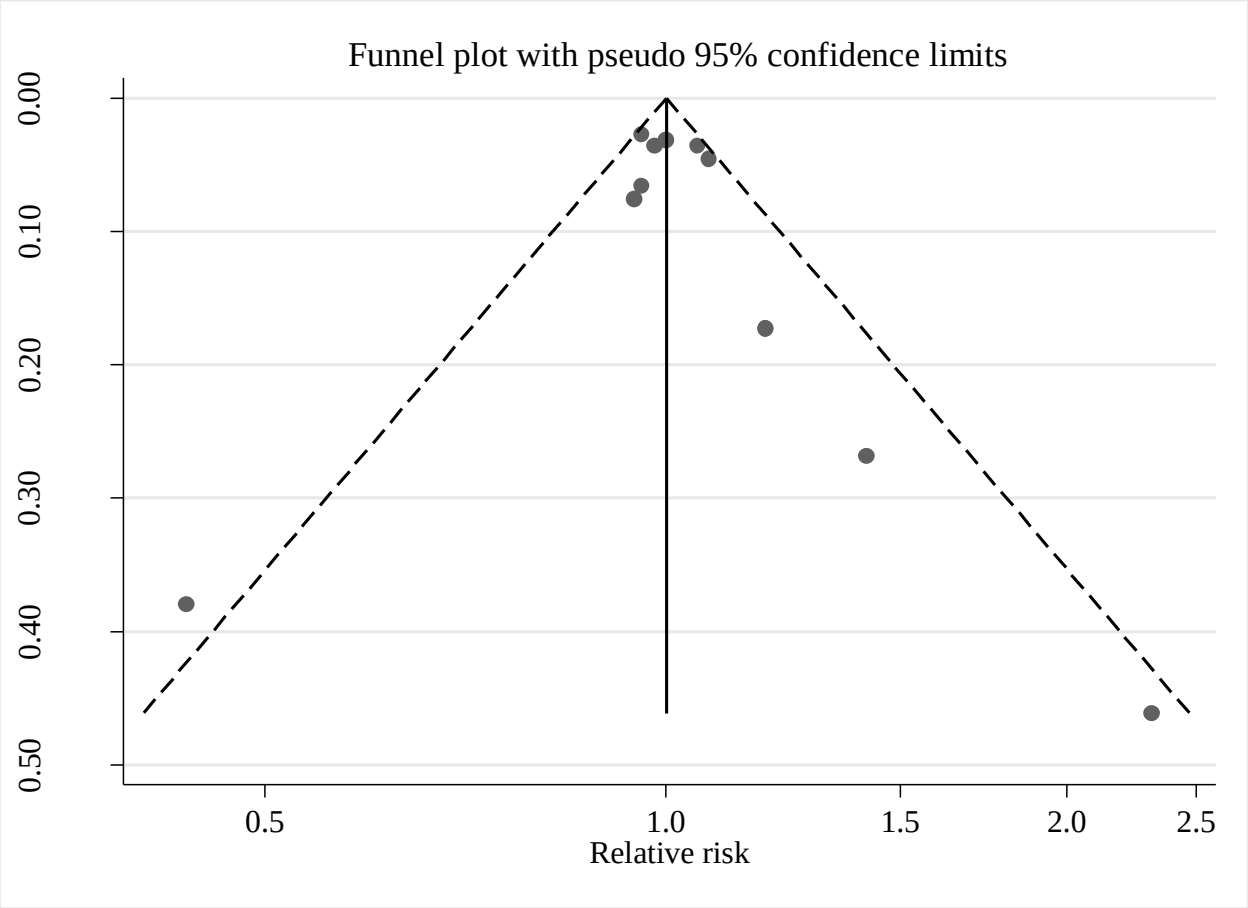
Supplemental Figure 20. Funnel plot for studies of the association between total dairy intake and CHD based on linear dose-response slopes (no. of cases=8,298; total n=330,350). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test *P* = 1.000, symmetry indicates no evidence of publication bias.



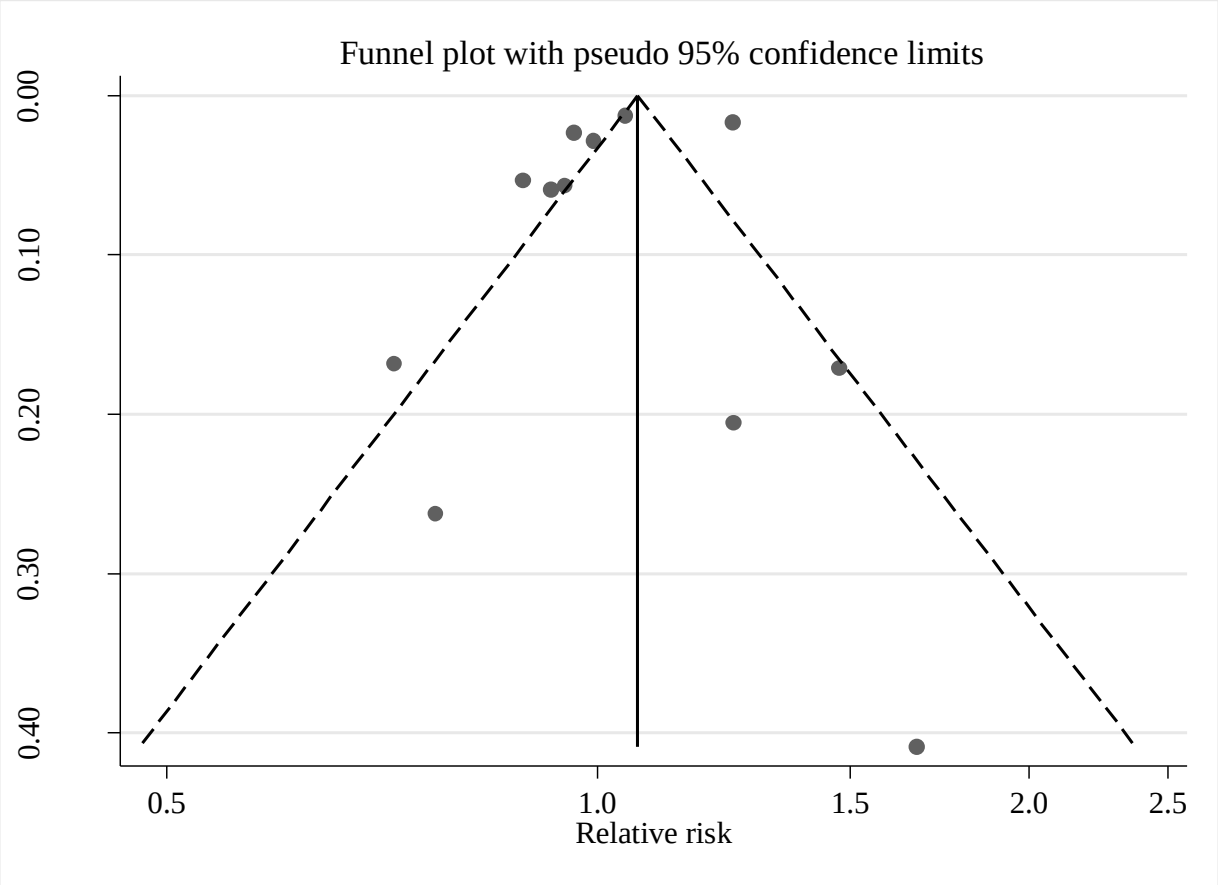
Supplemental Figure 21. Funnel plot for studies of the association between low-fat dairy intake and CHD based on linear dose-response slopes (no. of cases=6,244; total n=262,228). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test P = 0.747, symmetry indicates no evidence of publication bias.



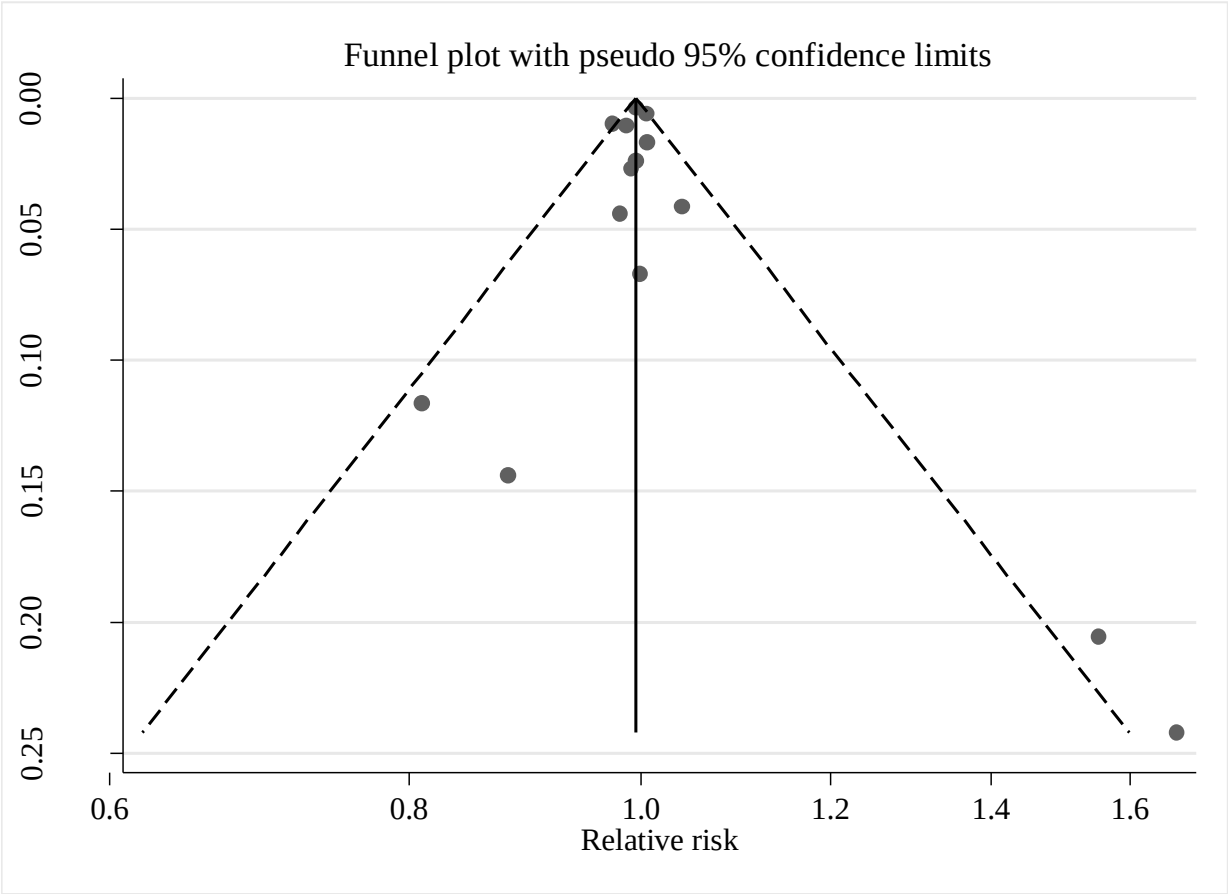
Supplemental Figure 22. Funnel plot for studies of the association between milk intake and all-cause mortality based on linear dose-response slopes (no. of cases=69,355; total n=268,570). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR).Test for publication bias: Egger's test *P* = 0.254, symmetry indicates no evidence of publication bias.



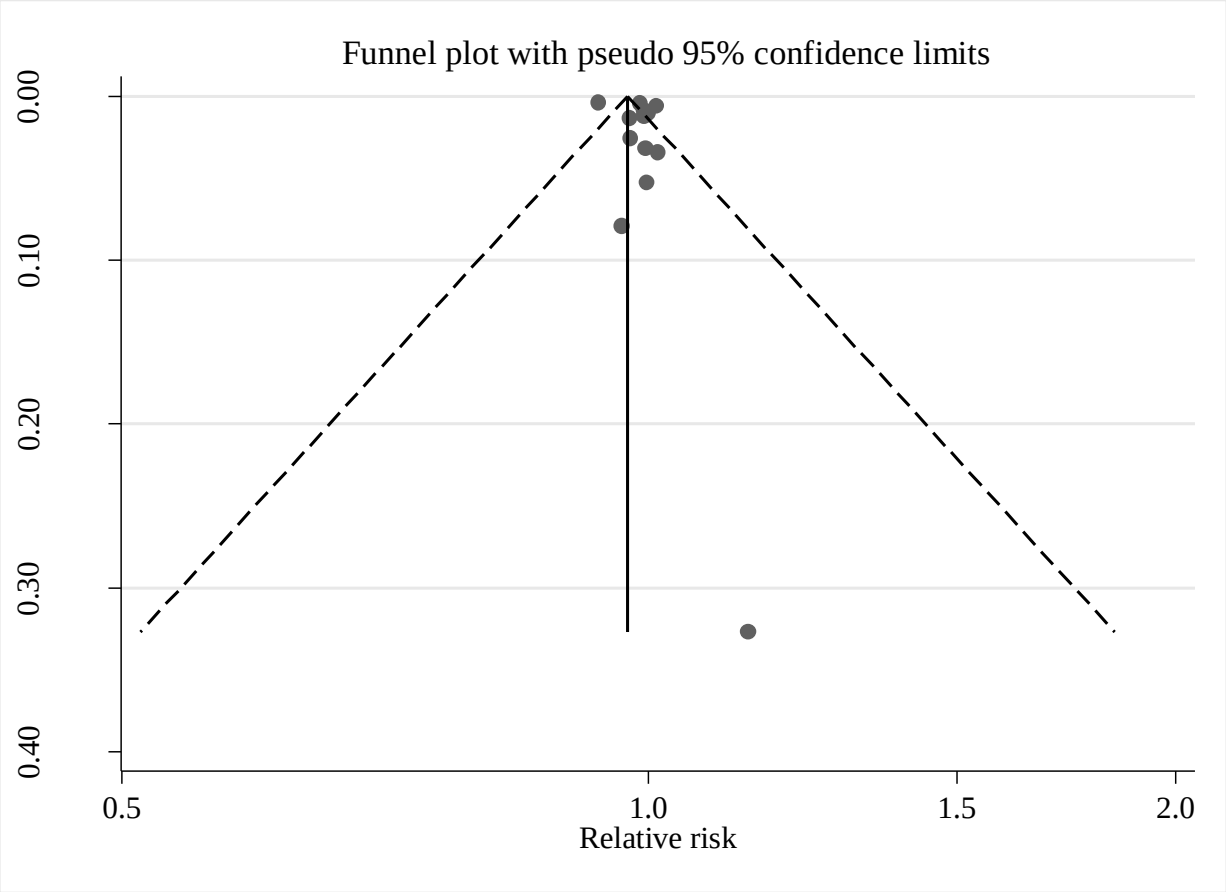
Supplemental Figure 23. Funnel plot for studies of the association between milk intake and CHD based on linear dose-response slopes (no. of cases=8,612; total n=230,621). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test $P = 0.397$, symmetry indicates no evidence of publication bias.



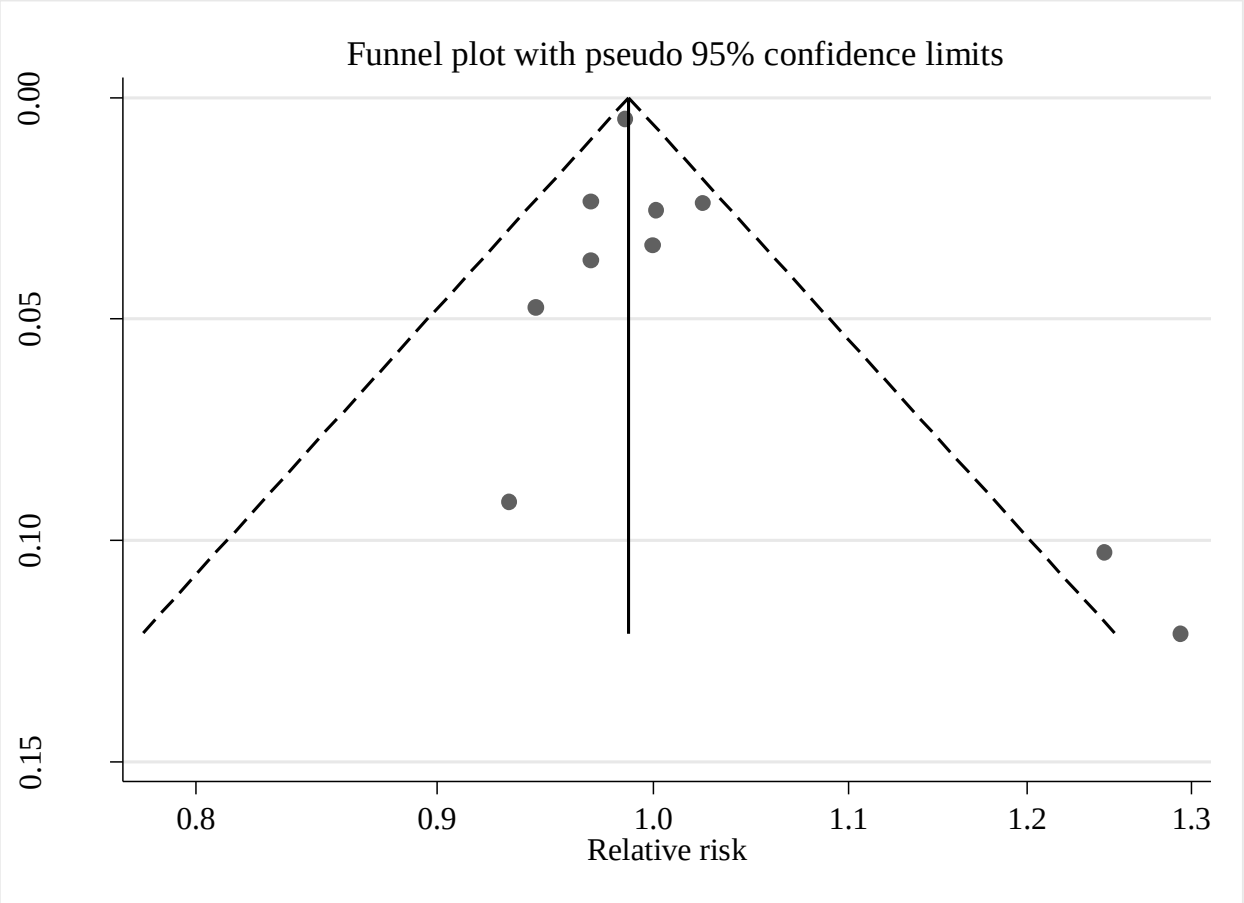
Supplemental Figure 24. Funnel plot for studies of the association between milk intake and CVD based on linear dose-response slopes (no. of cases=21,580; total n=249,779). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test $P = 0.449$, symmetry indicates no evidence of publication bias.



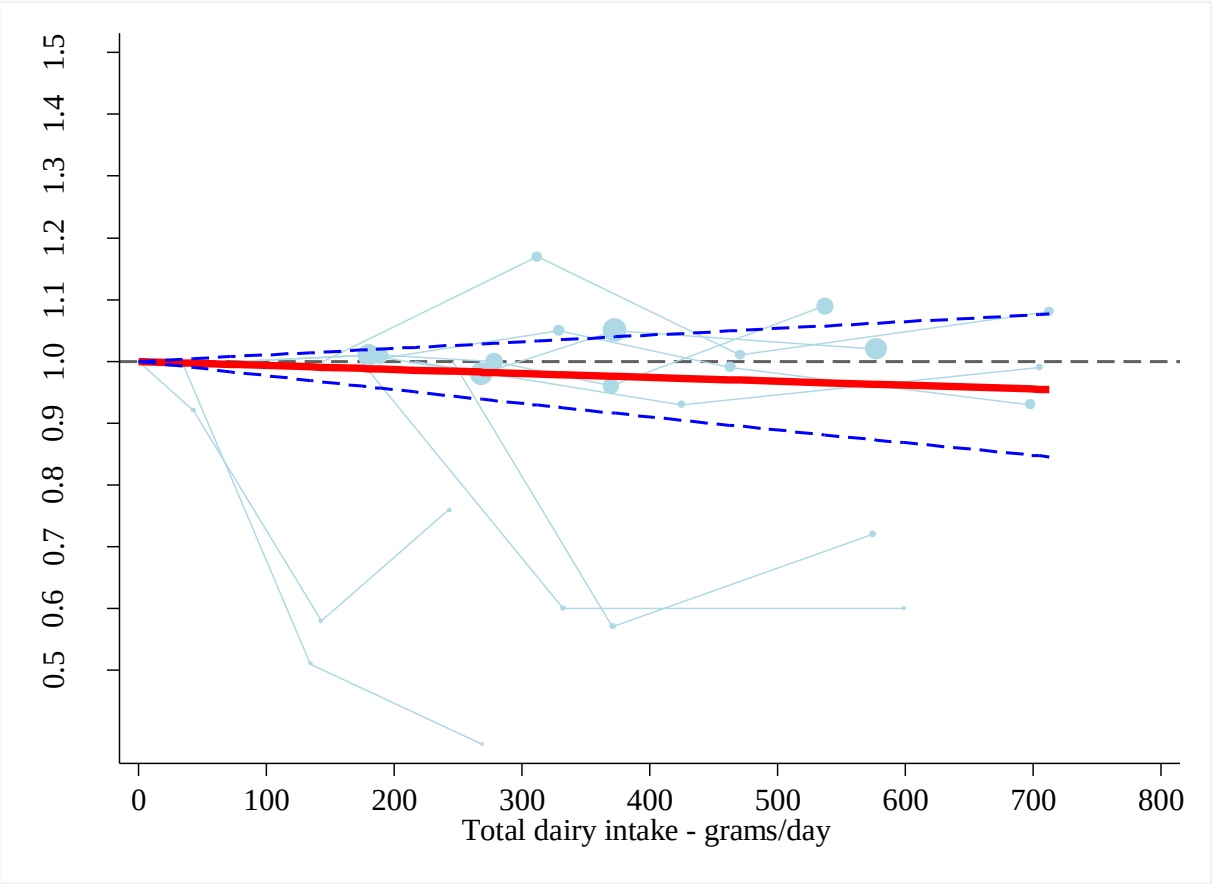
Supplemental Figure 25. Funnel plot for studies of the association between fermented dairy intake and CHD based on linear dose-response slopes (no. of cases=5,667; total n=256,091). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test P = 0.726, symmetry indicates no evidence of publication bias.



Supplemental Figure 26. Funnel plot for studies of the association between cheese intake and all-cause mortality based on linear dose-response slopes (no. of cases=54,125; total n=342,120). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test *P* = 0.310, symmetry indicates no evidence of publication bias.



Supplemental Figure 27. Funnel plot for studies of the association between cheese intake and CHD based on linear dose-response slopes (no. of cases=4,022; total n=256,091). Each dot indicates a study population with its relative risk (RR). The y-axis represents the SEs of the log (RR). Test for publication bias: Egger's test $P = 0.273$, symmetry indicates no evidence of publication bias.



Supplemental Figure 28. Spaghetti plot for the association between total dairy intake and all-cause mortality. Each light blue line represents a study population. Circles are placed at the study-specific RRs that are related to the corresponding quantity of the intake. Circles area is proportional to the study-specific overall weight. Solid red line represents the pooled RR at each quantity of intake and the two dashed dark blue lines are the corresponding 95% CI.

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Chapter 7 - Effect of dietary vitamin D_3 and 25(OH) D_3 on concentrations of 25(OH) D3 in blood plasma and milk of dairy cows

The present chapter aims to investigate the effect of feeding cows different rates and forms of vitamin D on vitamin D forms and concentration in milk.

DIG, BJ and JAL designed the study, technicians in CEDAR of University of Reading conducted the research. JG received statistics training from KEK. JG analysed the data and wrote the manuscript.

Effect of dietary vitamin D_3 and 25(OH) D_3 on concentrations of 25(OH) D_3 in blood plasma and milk of dairy cows

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[5]Interpretive summary: supplementation with 25-hydroxyvitmain D_3 is more effective in raising vitamin D status of dairy cows.

[6]Running title: 25(OH) D_3 supplementation to dairy cows

Abstract

Milk enriched with vitamin D by supplementing dairy cow diets could provide a valuable dietary source of vitamin D, but information on the feasibility of this approach is limited. In the current study, the effect of supplementing dairy cows with either vitamin D_3 or 25(OH) D_3 over the transition/early lactation period on plasma and milk vitamin D concentrations were compared. Sixty dairy cows were randomly allocated to one of four dietary treatments from 14 days before calving to early lactation (56 days): a control diet (Control) for both transition and early lactation containing 0.625 mg Vitamin D_3; HyD pre-calving had same diet with Control at early lactation, but the transition diet supplemented with 6 mg 25(OH) D_3 during pre-calving in addition to Control diet; HyD post-calving had same transition diet with Control, but early lactation diet included 1.5 mg 25(OH) D_3 supplements in addition to Control; D3max had same transition diet with Control, but with supplemented 2 mg vitamin D_3 in addition to Control diet. The results showed no treatment effect on milk yield, composition or 25(OH) D_3 concentration. However there was an interaction of treatment and time for plasma 25(OH) D_3 concentration; this increased within two weeks of supplementation for the HyD pre-calving group (peaking just after calving, 202 ng/ml), whereas that of the HyD post-calving group had a slower response following supplementation, continuing to increase at 56 days. There were correlations between plasma and milk 25(OH) D_3 concentrations at days 4 and 14 of lactation, but not at later sampling points. The D3max treatment group did not increase 25(OH) D_3 concentration in plasma or milk. Overall, results from this study indicate that supplemental 25(OH) D_3 is an effective means of enhancing dairy cow plasma 25(OH) D_3 concentrations than vitamin D_3 supplementation. However, vitamin D content of typical milk consumption (200 ml) would contribute 0.02 to 0.66 µg, which was not sufficient to achieve dietary recommended levels.

Key words: vitamin D_3, 25(OH) D_3, milk, enrichment.

Introduction

Vitamin D is important for bone health, and mounting evidence demonstrates that vitamin D status is inversely associated with risk of chronic disease, such as cardiovascular disease, diabetes and cancers (Borradale and Kimlin, 2009; Holick and Chen, 2008). There is increasing evidence that vitamin D deficiency is prevalent through the world, including UK (Hilger et al., 2014; Cashman and Kiely, 2016), mainly due to lifestyle changes over time (Holick, 1995; Tsiaras and Weinstock, 2011). Dietary sources have therefore become more important in sustaining adequate vitamin D status (Spiro and Buttriss, 2014). However, few types of foods are naturally high in vitamin D (Schmid and Walther, 2013). Therefore, vitamin D food fortification has been recommended as a strategy to increase vitamin D intake across the population (Cashman, 2015).

Vitamin D concentrations of milk and dairy products are naturally low (McDermott et al., 1985). However, because milk and dairy products are widely consumed, a fortification programme has been instigated in some countries. Different food standard policies prevent fortification in other countries (Samaniego-Vaesken et al., 2012), so increasing milk vitamin D concentration via supplementation of dairy cow diets is an alternative strategy.

In practice, vitamin D_3 is the form of vitamin D usually used for fortification. However, it is now clear the metabolically-active form, 25(OH) D_3, is more effective in raising serum 25 (OH) D_3 concentrations than vitamin D_3, and also may be absorbed faster than vitamin D_3 from the human digestive tract (Barger-Lux et al., 1998; Cashman et al., 2012; Jetter et al., 2013). Previous studies investigating the effect of supplementing dairy cow diets with vitamin D_3 (Hollis et al., 1981; McDermott et al., 1985; Thompson, 1983) suggest concentrations in milk following supplementation remain relatively low compared with the RNI of vitamin D (SACN, 2016). To date, only a few studies (Weiss et al., 2015; Wilkens et al., 2012) have examined the effect of supplementing cow diets with 25(OH) D_3 on plasma or milk vitamin D

concentration. However, the main hypotheses of these studies were focused around reducing prevalence of hypocalcaemia in the cow.

The main objective of the present study was to investigate the effect of supplemental vitamin D_3 or 25(OH) D_3 in dairy cow diets on the 25(OH) D_3 concentration of both plasma and milk over the transition period. Vitamin D_3 was included as a measurement in the milk as it is the precursor form of 25(OH) D_3 (McDermott et al., 1985). We hypothesized that supplementing cows with 25(OH) D_3 would be more efficient at increasing 25(OH) D_3 concentrations of plasma and milk than vitamin D_3 supplementation. As hypocalcaemia in dairy cows frequently occurs after the initiation of milk production (DeGaris et al., 2008), the secondary objective of the study was to investigate the effect of supplemental 25(OH) D_3 during the pre-calving period on plasm calcium concentration during the periparturient and milk initiation production periods. Furthermore, as hypocalcaemia is often accompanied by decreased plasma phosphorus and magnesium concentrations (Klimiene et al., 2005), the balance between these is crucial during the calving period (Reinhardt et al., 1988), thus, the effects of treatments on plasma and milk phosphorus and magnesium concentrations in cows were also studied.

Materials and methods

Animals and management

All licensed procedures were conducted according to Scientific Procedures Act 1986 under the authority of Home Office Project Licence 70/7727. Sixty non-lactating (parity 2 or greater) Holstein-Friesian dairy cows with previous lactation yield (305 d) of 10,141 kg (SE=177) and initial weight of 725kg (SE=7.5) at the start of the study were randomly allocated to 1 of 4 experimental diets using a continuous design, at 14 days prior to calving (average duration of the pre-calving period was 14 days (SE=0.5)). When not restrained for measurements, cows were loose-housed in a straw yard in the late gestation phase and in a

cubicle yard with washed sand bedding and automatic alley scrapers during the lactation phase. For the period immediately around parturition, cows were housed in straw-bedded maternity pens.

Cows were group fed during the pre-calving period and for the first week post-calving. From day 7 of lactation onwards all cows were fed individually using Calan gates (Calan Broadbent Feeding System, American Calan) for the remainder of the study. Cows were milked twice daily in the morning and afternoon at unequal intervals (0500 and 1500 h) through a 50-point Dairymaster rotary parlour. All cows were housed at the University of Reading's Centre for Dairy Research during the winter period of October 2013 to March 2014 to avoid the confounding factor of in vivo vitamin D synthesis due to ultraviolet radiation.

Treatment diets, experimental design and blocking

The four treatment diets were as follows: The control group (Control) was fed a basal transition cow diet from 14 days before calving, and a basal early lactation diet until 56 days post-calving (Table 1), both supplemented with 0.625 mg vitamin D_3 (DSM Nutritional Products, Basel, Switzerland) per cow per day (Table 2; NRC, 2001). Treatment "HyD pre-calving" received an additional 6 mg 25(OH) D_3 per cow per day (ROVIMIX® HyD®, DSM Nutritional Products, Basel, Switzerland) to the transition diet, and the lactating diet remained the same. Treatments "HyD post-calving" and "D3 max" received the basal transition diet up to calving. HyD post-calving then received an additional 1.5 mg 25(OH) D_3 (ROVIMIX® HyD® 1.25%, DSM Nutritional Products, Basel, Switzerland) per cow per day, and D3max received 2 mg vitamin D_3 (maximum permitted EU level, EC, 2004) post-calving until 56 days (Table 2).

All supplements were formulated to provide daily required dose of vitamin D_3 and/or 25(OH) D_3 within 250 g ground wheat. Each group was blocked in group four according to expected calving date. Within each group and block, cows were allocated at random to the

treatments. The composition and estimated nutritive value of the transition and early lactation basal diets are described in Table 1. All of diets were formulated to meet animal nutritional requirements as determined using the UK Feed into Milk model (Thomas, 2004). Both diets were fed as total mixed rations (TMR) and were offered *ad libitum* to achieve 5% refusals. The non-forage component of each diet was combined into a concentrate blend (Table 1). Oven DM (temperature and time in oven?) of the TMR, silages and concentrate blend were measured three times and once (for concentrate) per week. Feed offered were adjusted once per week according to the mean of the last three forage DM results. Diet was prepared daily and feed was dispensed between 0730 h and 0900 h. Milk yield were recorded daily through the whole study.

Experimental sampling

Blood samples were collected from tail-vein of each cow on day 14 and 7 before expected calving date, on the day of calving (within the first 24 hour after parturition) and days 4, 7, 14, 21, 28, 35 and 42 of lactation. Two samples were collected in 10 ml vacutainers containing EDTA (Beckton Dickinson?) from each cow at each sampling time. Each collected sample was immediately centrifuged at 3,000 rpm for 15 minutes at 15 °C to separate plasma, and plasma then stored at -80 °C.

Milk samples were collected on days 4, 14, 28, 35 and 42 of lactation and analysed for vitamin D concentrations (Vitamin D_3 and 25(OH) D_3). Two milk samples (am and pm) were pooled (100 ml) and then were split into 2 × 50 ml samples and immediately frozen at -80 °C. In addition, two milk samples (am and pm) were collected at each day of 4, 28 and 42 and were pooled for mineral analysis).

Chemical analysis

Analysis of plasma and milk vitamin D_3 and 25 (OH) D_3 analyses were conducted by DSM Nutritional Products Ltd (Basel, Switzerland). Food and Drug Administration (FDA; 2013) and European Medicines Agency (EMEA; 2011) bioanalytical guidelines were used to validate the method.

In brief, for plasma vitamin D_3 and 25(OH) D_3, after the addition of a deuterated internal standard solution, proteins were precipitated with a mixture of tetrahydrofuran, acetonitrile and methanol. The supernatant was evaporated and the residue reconstituted with acetonitrile-methanol solution after centrifugation. An aliquot of plasma sample was injected on the LC-MS/MS system.

For milk vitamin D_3 quantification, after addition of internal standard solution, saponification with methanol, ethanol and potassium hydroxide was conducted. Water was added and vitamin D_3 was extracted by liquid/liquid extraction twice successively with cyclohexane. The cyclohexane phase was evaporated to dryness under a stream of nitrogen. The residue was reconstituted in methanol and acetonitrile solution. After the final extraction was filtered, an aliquot was injected on the LC-MS/MS system.

For milk 25 (OH) D_3 quantification, after addition of internal standard solution, a saponification is performed with adding methanol and potassium hydroxide, a liquid/liquid extraction with tert-butyl methyl ether is used to extract 25(OH) D_3. After evaporation of the extract, the sample is cleaned by solid phase extraction technique. With elution with a mixture of acetonitrile and methanol, the eluate is evaporated until dryness under a gentle stream of nitrogen. Final residue is reconstituted in methanol/acetonitrile solvent and filtrated before injection into the LC-MS/MS system.

Concentrations of vitamin D_3 and 25(OH)D_3 in all samples were determined by LC-MS/MS system (Agilent 1290) using reverse phase column, coupled with APPI source (ABSciex 4000) using an atmospheric pressure photospray ionization (APPI) source in

positive mode. The detection of the specific fragment ions is performed by using multiple reactions monitoring mode (MRM).

To assess the daily and long-term laboratory performance of the method, dedicated standard and quality control samples were analyzed daily with the unknown samples to ensure accuracy and precision. Data acquisition of extracted ion chromatograms, integration and quantification were performed by Analyst® software from ABSciex.

Analysis of milk for calcium, phosphate and magnesium in milk were measured by National Milk Laboratories, UK. Milk samples are homogenized by vigorous shaking, 10 g of the milk sample is transferred into a 50 ml polypropylene digestion vessel. By adding 30 ml of nitric acid (Romilk High Purity SpA), the samples are placed in a hotblock at 110°C for 4 hours, after cool to room temperature, each sample is mixed with deionized water and cap to make up 50 ml for the further a 10 times dilution with deionized water before ICP-MS analysis.

Analysis of concentrations of calcium, phosphate and magnesium in plasma were measured by Veterinary Laboratories Agency, UK with using Olympus AU 400 analyzer by using standard kits with appropriate quality control by Animal and Plant Health Agency in the UK.

Milk composition measurements including fat, protein, lactose, casein, urea and somatic cell count were analysed by infrared spectroscopy (Foss Electric Ltd., York, UK), the method as described elsewhere (Reynolds et al., 2014) and the analyses was conducted by National Milk Laboratories, UK.

Statistical analysis

Results were averaged for each cow and sampling period, and were analysed using mixed procedure of SAS (Statistical Analysis System software package version 9.4, SAS Institute, Cary, NC, USA), including fixed effects of dietary treatments and time, and random effects of

cow, with time (day) being the repeated measure within cows. Milk yield and composition were only analysed for the post-calving period, whereas other data were analysed for the whole study. Compound symmetry, heterogeneous compound symmetry, first-order autoregressive or a heterogeneous first-order regressive covariance structure were used for repeated measures analysis based on goodness of the fit criteria for each analysed variable. Orthogonal contrasts were applied to investigate the difference between treatments: control vs all other diets; HyD pre-calving vs HyD post-calving; D3max vs HyDpost-calving. Least square means (SEMs) were reported, and treatment effects were considered significant at $P<0.05$.

Area under curve (AUC) for plasma and milk vitamin D concentrations over time were calculated according to trapezium rule as the summation measure for each treatment, which was analysed by one-way ANOVA in STATA (version 13.0; STATA Corporation, 2014), Bonferroni correction was used subsequently to compute the multiple pairwise comparisons if there was significant effect of the investigated variables between treatments. Furthermore, in order to assess transfer of 25(OH) D_3, calcium, phosphorus or magnesium from plasma to milk at each time point, correlation of 25(OH) D_3, calcium, phosphorus and magnesium of plasma and milk were conducted (across all treatments) by using general linear regression model in STATA.

Results

Characteristics of dairy cows, and mineral of plasma and milk

There was no interaction effect of treatment and time on milk yield, milk composition, or characteristics of the cow (Table 4). In addition, there was no interaction effect of treatment and time on mineral concentrations of calcium, phosphorus and magnesium in both plasma and milk (Table 5).

There was no interaction effect of treatment and time on milk yield, milk composition, or characteristics of the cow (Table 4). In addition, there was no interaction effect of treatment and time on mineral concentrations of calcium, phosphorus and magnesium in both plasma and milk (Table 5).

Vitamin D in plasma

The HyD pre-calving treatment resulted in a greater (P<0.001) mean concentration of 25(OH) D_3 in plasma across the whole study (Table 4; Figure 1). The peak 25(OH) D_3 concentration of 202 ng/ml was achieved by day 1 of lactation, before decreasing gradually. In comparison, HyD_3 post-calving resulted in an increase in plasma 25(OH) D_3 concentration following 14 days of supplementation, and at day 56 reached 179 ng/ml. There was a difference (P<0.001) between treatment on summary exposure Area Under Curve (AUC). $AUC_{\text{-14 to 56 days}}$ of HyD pre-calving (8274 ± 630 ng/mL) and HyD_3 post-calving (6806 ± 356 ng/mL) was significant higher than Control (2964 ± 304 ng/mL) and D3max post-calving (2619 ± 207 ng/mL) treatments. There was no difference between treatments of HyD pre-calving and HyD post-calving, or between Control and D3max.

Vitamin D in milk

There was no overall effect of treatment on 25(OH) D_3 concentration in milk, but there was an effect of time (P=0.004) and a treatment by time interaction (P<0.001) (Table 5; Figure 2). In addition, 25(OH) D_3 concentrations in the milk of HyD pre-calving had a decreasing trend (Figure 2). Vitamin D_3 was measured in milk but 87% of values were below limit of quantification of 60 ng/kg, so data analysis could not be conducted. There was no difference (P=0.14) of treatment effect on $AUC_{\text{4-42 days}}$. $AUC_{\text{4-42 days}}$ of Control, HyD pre-calving, D3 max and HyD post-calving were (33.7 ± 3.7) × 10^3, (42.8 ± 3.7) × 10^3, (31.3 ± 3.3) × 10^3 and (34.8 ± 4.9) × 10^3 ng/kg, respectively.

Nutrient correlations of plasma and milk

Concentrations of 25(OH) D_3 in plasma and in milk were correlated at day 4 (R^2=0.25; *P*=0.009) and 14 (R^2=0.24; *P*=0.01) of lactation, but not at day 28, 35 or 42 (Figure 3). There was no correlation between concentrations of calcium, phosphorus or magnesium in plasma and milk (*P*>0.05).

Discussion

Vitamin D metabolite form of 25(OH) D_3 is more effective than vitamin D_3 in raising human serum 25(OH) D_3 concentrations (Barger-Lux et al., 1998; Cashman et al., 2012; Jetter et al., 2013). To our knowledge, the current study is the first study to compare the effect of supplementing cows of both pre-calving and post-calving with 25(OH) D_3 and vitamin D_3 on 25(OH) D_3 concentrations in plasma and milk. The current study demonstrated that a daily oral supplementation of 6 mg 25(OH) D_3 during two weeks pre-calving or 1.5 mg 25(OH) D_3 during 8 weeks after-calving is more effective in raising plasma 25(OH) D_3 concentrations than vitamin D_3 supplementation.

Supplementing cows with 25(OH) D_3 for two weeks pre-calving increased plasma 25(OH) D_3 concentration, which reached a peak just after calving (day 1; 202 ng/ml) when supplementation stopped. This result is consistent with previous studies (Wilkins et al., 2012; Weiss et al. 2015) who reported that pre-calving 25(OH) D_3 supplementation is effective at increasing plasma concentrations, peaking at the same time. The daily 25(OH) D_3 supplementation dose (6 mg) in the current study was the same as that used by Weiss et al. (2015), and yet the earlier study resulted in a higher peak concentration (274 ng/ml). Wilkens et al. (2012) supplemented with less 25(OH) D_3 (3 mg/day) but the peak plasma concentration was similar to that of the current study (198 ng/ml). One possible reason may due to the influence of vitamin D-binding protein (DBP), previous study (Powe et al., 2013) showed

DBP has influence on 25(OH) D level. Unfortunately, DBP concentrations were not measured in the current study or studies of Weiss et al. (2015) and Wilkens et al. (2012).

Supplementation of vitamin D_3 up to 2 mg /day after-calving for 8 week did not increase plasma 25(OH) D_3 concentration compared with control with 0.625 mg/d. McDermott et al., (1985) compared three daily doses of vitamin D_3 supplements (0.25 mg, 1.25 mg or 6.25 mg) to dairy cows for 14 weeks, and results demonstrated that only the 6.25 mg dose significantly enhanced plasma 25(OH) D_3 concentration. Therefore it is perhaps not surprising that the supplementation level of 2 mg/day used in the current study did not increase plasma 25(OH) D_3 concentrations.

Vitamin D (vitamin D_3 and 25(OH) D_3) concentrations in milk were not affected by treatments, and the mean concentration of 25(OH) D_3 concentration in milk through whole study was 8.75×10^{-4} mg/L. Hollis et al., (1981) has fed cows with daily 0.1 mg or 10 mg vitamin D_3, but this 10-fold elevated supplementation level only resulted in a 2-fold increase in milk 25(OH) D_3 concentration (from 3.72×10^{-4} mg/L increased to 6.85×10^{-4} mg/L). McDermott et al., (1985) supplemented cow diets with a higher daily dose of vitamin D_3 (1.25 mg or 6.25 mg) for 14 weeks, and reported that milk 25(OH) D_3 concentration only slightly increased from 7.5×10^{-4} mg/L to 9.25×10^{-4} mg/L. In agreement with Weiss et al. (2015), the current study demonstrated that milk concentrations of 25(OH) D_3 were highest earlier in lactation compared with later. Furthermore, the current study found a correlation between plasma and milk 25(OH) D_3 concentrations up to 14 days post-calving but not after, which is also in agreement with earlier study of Weiss et al., (2015). This may due to colostrum containing greater concentrations of vitamin D binding protein than milk later in lactation, which facilitates greater transfer of 25(OH) D_3 from the plasma to milk in early lactation (Larson and Jorgensen, 1974).

Concentrations of 25(OH) D_3 in milk from the current study ranged from 0.1 to 3.3 µg/kg, which, for a typical milk serving of 200 ml (FSA, 2005) would contribute 0.02 to 0.66 µg,

well below the current UK recommended intake level of 10 μg/day (SACN, 2016). Nevertheless, supplementing cow diets with 25(OH) D_3 is more effective than supplementing with vitamin D_3 in raising plasma 25(OH) D_3 concentrations in dairy cows, and the plasma 25(OH) D_3 concentrations of all treatments in current study are within the physiological range (Horst et al., 1981). Therefore, the higher effective in raising plasma 25(OH) D_3 may resulted in which can be used as a better food additive than vitamin D_3 to dairy cows in the future.

Mineral concentrations of calcium, phosphorous and magnesium were not stimulated by vitamin D_3 or 25(OH) D_3 supplementations in the current study. Study Okura et al., (2004) shown mineral concentrations is associated with vitamin D biologically form 1,25$(OH)_2$ D3 which is produced in kidney (Okura et al., 2004). Thus, the possible reason maybe because the 1,25$(OH)_2D_3$ was not significant affected by dietary vitamin D_3 or 25(OH) D_3 supplementation in current study. Unfortunately, 1, 25$(OH)_2$ D_3 was not measured in current study, which needs further exploration. Furthermore, because the limitation of the vitamin D concentration in the diets were not tested, thus, it is unknown the actual vitamin D dose that dairy cows received, which should be enhanced in the future studies.

Conclusions

Supplementing dairy cows with 25(OH) D_3 was a successful strategy for increasing circulating concentrations of 25(OH) D_3 in the cow. Transfer of this into milk appeared to be greater during early lactation (0-14 days). Therefore, supplementation of cow diets at this supplementation level may not be an effective dietary strategy for increasing 25(OH) D_3 content of milk in order to address vitamin D deficiency within the general population.

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Table 1. Composition and estimated nutritive value of basal transition and early lactation cow diet.

	Basal transition diet	Basal early lactation diet
Ingredient, g/kg		
Grass silage	244	224
Maize silage	344	242
Wheat straw	160	18
Grass hay	-	39
Megalac	-	12
Minerals[1]	14	10
Sodium chloride	-	4
Ammonium chloride	14	-
Magnesium chloride	14	-
Concentrate blend[2]	210	458
Estimated nutritive value		
Crude protein, g/kg	144	173
Metabolisable energy, MJ/kg	10.2	11.8
Starch, g/kg	169	203
Neutral detergent fibre, g/kg	450	351
Oil, g/kg	44	58
Ash	109	83
Water-soluble carbohydrate	25	48

[1]Containing (mg/kg) Calcium 270,000; Phosphorus 40,000; Magnesium 60,000; Sodium 40,000; Selenium (sodium selenite) 30; Cobalt (cobalt carbonate) 50; Iodine (calcium iodate) 500; Manganese (manganese oxide) 4,000; Zinc (zinc oxide) 5,000; Copper (cupric sulphate) 1,500; Vitamin A (retinyl acetate) 12.5; Vitamin E (di-alpha tocopheryl acetate) 0.01.

[2]Containing (g/kg Dry Matter) Rolled wheat 313; Hipro soyabean meal 159; Soya hulls 60; Palm kernel meal 120; Rapeseed meal 170; Wheatfeed 129; Megalac 16; Molasses 33.

Table 2. Details of experimental treatments.

		Daily feeding for each cow	
Treatment	Cow no.	14 days of pre-calving until calving	Feeding from calving to early lactation of 56 days
Control	15	Basal transition cow diet[1] plus vitamin D supplementation: 0.625 mg vitamin D_3 no 25(OH) D_3	Basal early lactation diet[1] plus vitamin D supplementation: 0.625 mg vitamin D_3 no 25(OH) D_3
HyD pre-calving	15	Basal transition cow diet plus vitamin D supplementation: 0.625 mg vitamin D_3 6 mg 25(OH) D_3	Basal early lactation diet plus vitamin D supplementation: 0.625 mg vitamin D_3 no 25(OH) D_3
D3max	15	Basal transition cow diet plus vitamin D supplementation: 0.625 mg vitamin D_3 no 25(OH) D_3	Basal early lactation diet plus vitamin D supplementation: 2 mg vitamin D_3 no 25(OH) D_3
HyD post-calving	15	Basal transition cow diet plus vitamin D supplementation: 0.625 mg vitamin D_3 no 25(OH) D_3	Basal early lactation diet plus vitamin D supplementation: 0.625 mg vitamin D_3 1.5 mg 25(OH) D_3

[1] Composition and estimated nutritive value of basal transition and early lactation cow diet described in Table 1.

Table 3. Effect of supplements on milk yield, milk composition and dry matter intake of cows (least square means).

	Treatment					P[2]			Contrast P[3]		
Characteristics	Control	HyD pre-calving	D3max	HyD post-calving	SEM[1]	Treatment	Time	Treatment × time	1	2	3
Milk yield											
Milk yield (kg/d)	43.0	42.4	42.3	44.2	1.39	0.735	<0.001	0.320	0.993	0.355	0.311
Fat-corrected milk (kg/d)	46.5	44.3	45.7	46.6	1.71	0.718	0.024	0.183	0.604	0.313	0.680
Energy-corrected milk (kg/d)	45.2	43.2	44.4	45.4	1.60	0.745	0.071	0.210	0.629	0.328	0.634
Fat (g/d)	1722	1587	1689	1697	74.6	0.625	0.001	0.182	0.447	0.332	0.937
Protein (g/d)	1292	1267	1266	1311	42.2	0.841	0.601	0.645	0.819	0.457	0.441
Lactose (g/d)	1926	1895	1901	1979	62.2	0.747	<0.001	0.313	0.993	0.324	0.358
Milk composition											
Fat (%)	4.03	3.73	4.01	3.85	0.116	0.187	<0.001	0.055	0.198	0.479	0.290
Protein (%)	3.02	3.01	2.99	2.97	0.042	0.842	<0.001	0.439	0.582	0.476	0.814
Lactose (%)	4.48	4.47	4.49	4.48	0.025	0.946	0.001	0.779	0.916	0.784	0.755
Casein (%)	2.29	2.29	2.30	2.27	0.045	0.946	<0.001	0.613	0.844	0.569	0.764
Urea (mg/l)	244	239	231	244	13.1	0.762	0.389	0.618	0.620	0.728	0.352
Somatic cell count (×10000/ml)	4.2	7.5	5.5	8.8	0.1	0.293	<0.001	0.257	0.120	0.679	0.249
Dry matter intake (kg/d)	25.0	24.5	24.9	25.5	0.93	0.803	<0.001	0.734	0.975	0.332	0.542

[1] Standard error of the mean for n=15 measurements.
[2] Probability corresponding to the effect of treatment, time, or treatment by time interaction
[3] Where 1=Control vs all other diets, 2=HyD pre-calving vs HyD post-calving, 3=D3max vs post-calving

Table 4. Effect of supplements on milk and plasma 25-hydroxyvitamin D_3 or mineral concentrations (least square means).

	Treatment					P[2]			Contrast P[3]		
Measurements	Control	HyD pre-calving	D3max	HyD post-calving	SEM[2]	Treatment	Time	Treatment × time	1	2	3
Plasma (whole study)											
25(OH) D_3 (ng/ml)	43.0	122.7	39.5	87.1	5.11	<0.0001	<0.001	<0.001	<0.001	<0.001	<0.001
Calcium (mmol/l)	2.38	2.34	2.35	2.33	0.028	0.399	<0.001	0.715	0.118	0.593	0.515
Phosphorus (mmol/l)	1.64	1.73	1.72	1.77	0.053	0.130	<0.001	0.717	0.030	0.482	0.344
Magnesium(mmol/l)	0.98	0.97	0.98	0.97	0.021	0.933	<0.001	0.379	0.733	0.875	0.875
Milk											
25(OH) D_3 (ng/kg)	869	1132	889	1001	130.7	0.193	0.004	<0.001	0.214	0.339	0.412
Calcium (mg/kg)	1105	1064	1085	1133	26.9	0.111	<0.001	0.782	0.644	0.020	0.111
Phosphorus (mg/kg)	896	923	880	930	24.4	0.215	<0.001	0.240	0.484	0.789	0.065
Magnesium (mg/kg)	95.8	96.3	93.9	98.2	2.95	0.591	<0.001	0.906	0.901	0.554	0.175

[1] Standard error of the mean for n=15 measurements.
[2] Provability corresponding to the effect of treatment, time, or treatment by time interaction
[3] Where 1=Control vs all other diets, 2=HyD pre-calving vs HyD post-calving, 3=D3max vs post-calving

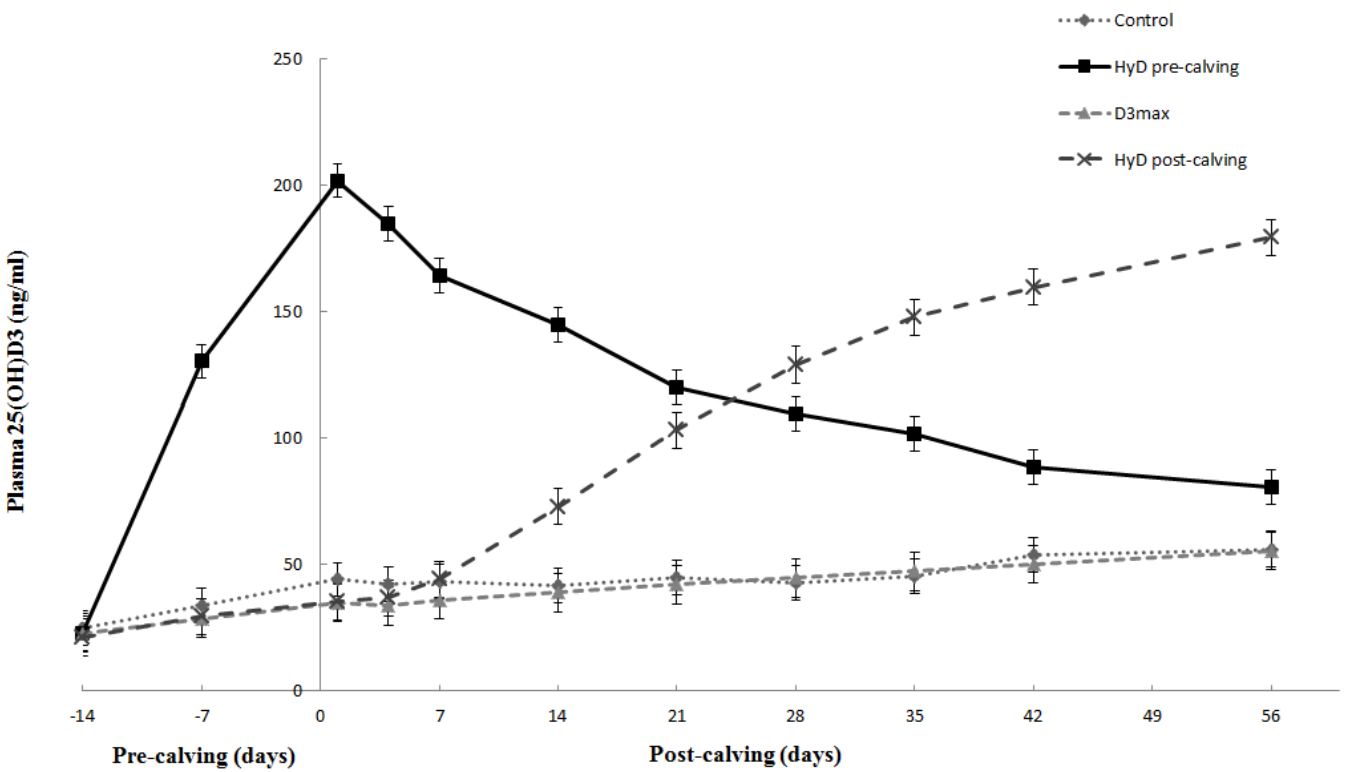

Figure 1. Effect of treatments on 25(OH) D_3 concentrations in plasma. Control for both transition and early lactation containing 0.625 mg Vitamin D_3; HyD pre-calving had same diet with Control at early lactation, but the transition diet supplemented with 6 mg 25(OH) D_3 during pre-calving in addition to Control diet; HyD post-calving had same transition diet with Control, but early lactation diet included 1.5 mg 25(OH) D_3 supplements in addition to Control; D3max had same transition diet with Control, but with supplemented 2 mg vitamin D_3 in addition to Control diet. Least squares means ± s.e.m. for 15 measurements.

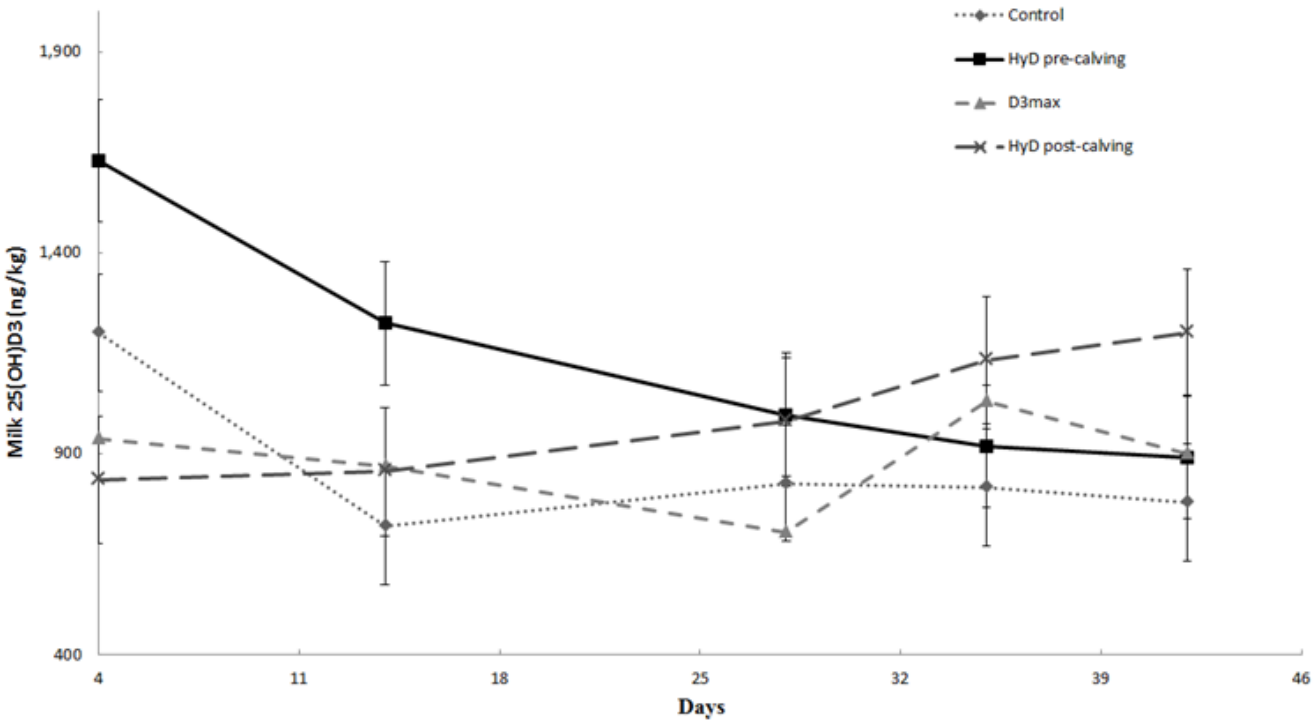

Figure 2. Effect of treatments on 25(OH) D_3 concentrations in milk. Control for both transition and early lactation containing 0.625 mg Vitamin D_3; HyD pre-calving had same diet with Control at early lactation, but the transition diet supplemented with 6 mg 25(OH) D_3 during pre-calving in addition to Control diet; HyD post-calving had same transition diet with Control, but early lactation diet included 1.5 mg 25(OH) D_3 supplements in addition to Control; D3max had same transition diet with Control, but with supplemented 2 mg vitamin D_3 in addition to Control diet. Least squares means ± s.e.m. for 15 measurements.

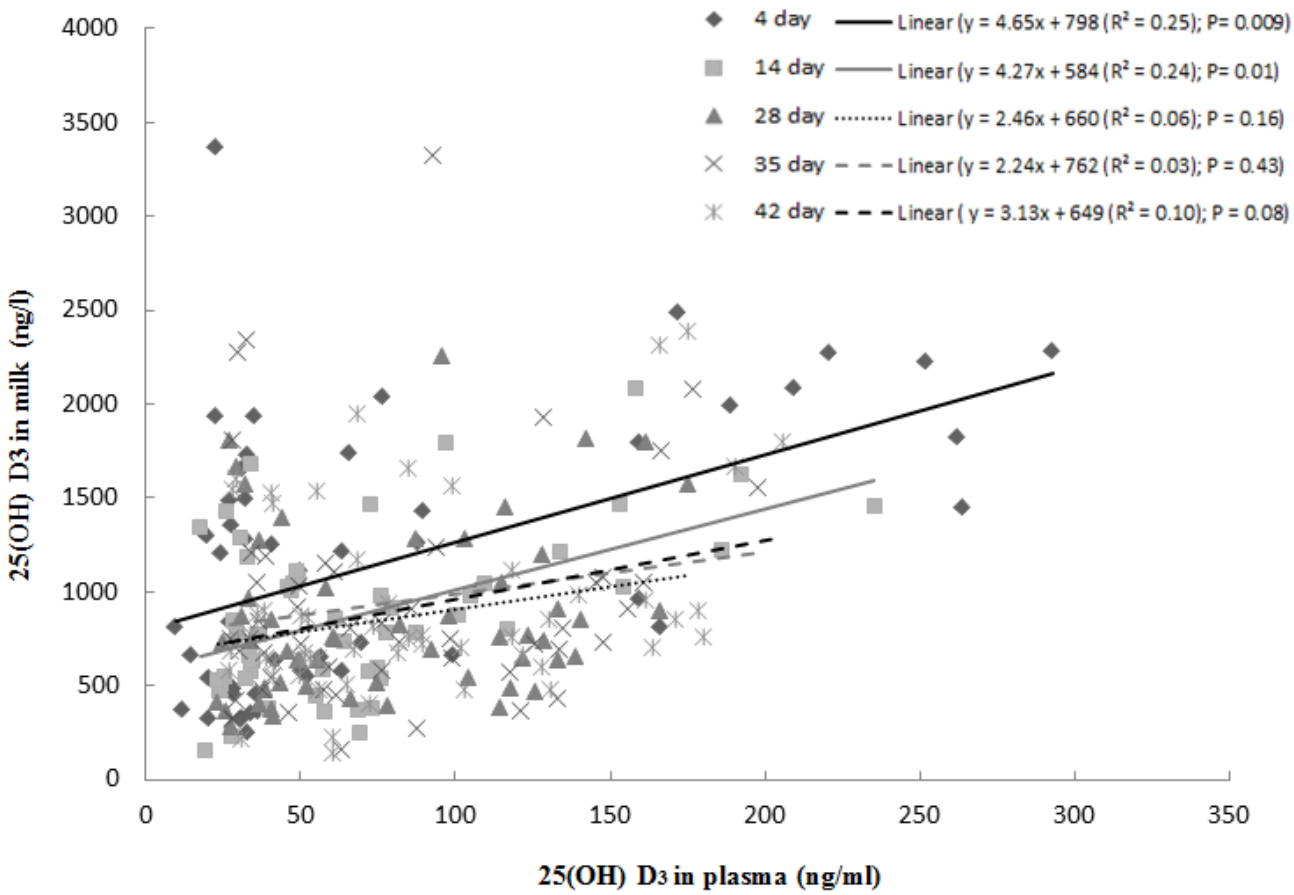

Figure 3. Corrections between 25-hydroxyvitamin D_3 in plasma and in milk.

Chapter 8 - Differential effect of 25-hydroxyvitamin D_3 and vitamin D_3 fortified dairy drinks on postprandial markers of vitamin D status and cardiovascular disease risk markers in men with sub-optimal vitamin D status.

The present chapter aims to compare the acute effect of a dairy drink enriched with vitamin D_3 or 25(OH) D_3 on vitamin D status and markers of CVD risk in humans.

JAL, DIG, KGJ and JG designed the study; JG conducted the research. JG analysed the data, JG wrote the manuscript.

Differential effect of 25-hydroxyvitamin D_3 and vitamin D_3 fortified dairy drinks on postprandial markers of vitamin D status and cardiometabolic risk markers in men with sub-optimal vitamin D status: results from the VITD randomized controlled trial

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[3] Supported by the Barham Benevolent Foundation.

[4] Abbreviations used: apolipoprotein B (apoB); area under curve (AUC); augmentation index (AI); blood pressure (BP); C-reactive protein (CRP); diastolic blood pressure (DBP); digital volume pulse (DVP); incremental area under curve (iAUC); maximal change of the variables (imaxC); non-esterified fatty acids (NEFA); maximum concentration (maxC); pulse pressure (PP); systolic blood pressure (SBP); triacylglycerol (TAG); 25-hydroxyvitamin D_3 (25(OH) D_3).

[5] Running title: Dairy drink fortification with vitamin D isoforms

[6] Author names for indexing: Guo, Jackson, Che Taha, Li, Givens, Lovegrove.

Abstract

Background: One strategy for improving vitamin D status in the population is the consumption of vitamin D fortified foods. However, the effects of dairy products fortified with different vitamin D isoforms on vitamin D status and metabolic outcomes have not been addressed.

Objective: We investigated whether a dairy drink fortified with either 25-hydroxyvitamin D_3 (25(OH) D_3) or vitamin D_3 had differential effects on 24 h circulating plasma 25(OH) D_3 concentrations (marker of vitamin D status) and cardiometabolic risk markers.

Design: A randomised, controlled, cross-over, double-blind postprandial study was conducted in 17 men of sub-optimal vitamin D status. They were randomised to three different test meals which contained either a non-fortified dairy drink (control), 20 µg 25(OH) D_3 fortified or 20 µg vitamin D_3 fortified dairy drinks on separate occasions, separated by 2 weeks. Plasma 25(OH) D_3 and cardiometabolic risk markers (including vascular function) were measured frequently up to 8 h postprandially, and at 24 h after the dairy drink was consumed.

Results: Plasma 25(OH) D_3 was significantly higher following 25(OH) D_3 compared with vitamin D_3 fortified dairy drink and control (P=0.019), reflected in the 1.5-fold and 1.8-fold greater incremental area under the curve for the 0-8 h response, respectively. Change in plasma 25(OH) D_3 from baseline to 24 h for the 25(OH) D_3 fortified dairy drink was also significantly higher than the vitamin D_3 fortified and control (P<0.0001) dairy drinks. There was no significant effect of the test meals on the cardiometabolic risk markers.

Conclusion: A 25(OH) D_3 fortified dairy drink was more effective at raising plasma 25 (OH) D_3 concentrations postprandially than the vitamin D_3 fortified drink. The long-term effect of 25(OH) D_3 dairy drink consumption on vitamin D status and cardiometabolic risk markers should be investigated.

Key words: vitamin D_3, 25(OH) D_3, dairy drink, milk, butter, vascular function, augmentation index, vitamin D status.

Introduction

Vitamin D deficiency has been reported to be associated with an increased risk of many common and chronic diseases, including cardiovascular disease, some cancers and diabetes (1). Circulating 25-hydroxyvitamin D (25(OH) D) concentration is commonly used as the measure of vitamin D status (2). The Institute of Medicine reported circulating concentrations of 50 nmol/L or above as adequate for sustaining musculoskeletal health outcomes (3). Hypovitaminosis D is now prevalent in the general European population (4) with 23% of UK adults presenting with a vitamin D status below 25 nmol/L (5). Due to diet and lifestyle changes and the frequent use of sunscreen, many individuals do not endogenously synthesise sufficient vitamin D from sunlight exposure (6). Therefore, vitamin D from dietary sources has become more important for maintenance of adequate vitamin D status. However there are only a few foods naturally rich in vitamin D such as egg yolk and oily fish (7). Thus, one strategy used in some countries, including USA and Canada, to improve population vitamin D status is fortification of milk with vitamin D, which has resulted in milk being the major contributor to vitamin D intake in these countries (8).

The relative efficacy of 25(OH) D_3 and vitamin D_3 for improving vitamin D status is inconsistent between studies (9-14), yet it is generally found that 25(OH) D_3 supplementation can increase vitamin D status more effectively than vitamin D_3 after chronic supplementation. To our knowledge, there are no human studies which have compared the efficacy of foods fortified with these two forms of vitamin D_3 to increase postprandial circulating 25(OH) D_3 concentrations, or their differential effects on chronic disease risk markers in the short term. Therefore, our study aimed to address this knowledge gap by comparing the acute effect of consuming test meals containing dairy drinks which have been fortified with either 20 µg vitamin D_3 or 20 µg 25(OH) D_3 on changes in postprandial plasma vitamin D_3 and 25(OH) D_3, cardiometabolic risk markers including vascular reactivity, blood pressure (BP), lipid profile, indexes of insulin resistance, inflammatory and vascular biomarkers. In addition,

whole blood culture cytokine production was examined as a real-time measure of inflammatory status.

Methods

Subjects

The study was conducted according to the Declaration of Helsinki and approved by the University of Reading Research Ethics Committee (approval no. 15/15), and was registered at www.clinicaltrials.gov (NCT02535910). Non-smoking men (n=18) aged 30-65 years with a body mass index (BMI) between 20-35 kg/m^2 with sub-optimal vitamin D status (plasma 25(OH) D $\leq$ 50 nmol/l) were recruited from the population in Reading, UK and the surrounding areas, from May to October 2015 by email, internet, poster or newspaper advertisements. Subjects who expressed an interest in the study were asked to complete a medical, lifestyle and ethnicity questionnaire. The key exclusion criteria included: women, cardiovascular, renal, gastrointestinal, respiratory and endocrine diseases, diabetes or cancer; hypertension; use of nutritional supplements; on long-term medication; milk allergy/intolerance or lactose intolerance; outdoor workers and those who used tanning beds; overseas holidays two months before or during the study period; vigorous exercise (>3 times of 30 min aerobic exercise/week) and excessive alcohol intake (>14 units/week). Those who complied with the inclusion criteria were invited to attend a screening visit following a 12-hour overnight fast consuming nothing but water during this time. All subjects provided written informed consent. Blood samples were taken by venipuncture for determination of the full blood count at the Royal Berkshire Hospital (Reading UK), men who had anemia (haemoglobin < 125 g/L) were excluded. Blood samples were also collected for measuring vitamin D status (performed at the Royal Berkshire Hospital) and fasting serum glucose, total cholesterol, triacylglycerol (TAG), markers of liver and kidney function using an automated clinical chemistry analyser (ILAB 600, Werfen UK Limited). Furthermore, static BP was

measured during the screening visit to exclude subjects with abnormal blood pressure. Normal blood pressure was considered to be a systolic blood pressure (SBP) of 90-120 mmHg and a diastolic blood pressure (DBP) between 60-80 mmHg.

Study design

This study was an acute, randomised, controlled, 3-way-crossover, double-blinded study conducted between October 2015 and February 2016. After participants were accepted onto the study, they were invited to the clinical unit of the Hugh Sinclair Unit of Human Nutrition at the University of Reading for a familiarisation visit to be acquainted with the clinical facilities and vascular function study measurements. Before the first study visit, the participants were asked to complete a 4-day diet diary (including 3 weekdays and 1 weekend day within the same week) and Dietplan 6.6 software was used to assess habitual dietary intake including dietary vitamin D. The first study day was performed 2-weeks after the familiarisation visit and there was a 2-week washout period between the 3 study visits (see **Supplemental Figure 1)**. The participants were randomly assigned to the study interventions by web-based random letter sequence generator (https://www.randomizer.org/). A double-blinded protocol was maintained throughout the study until all of the statistical analysis was completed. Throughout the study, participants were asked to maintain their normal diet and lifestyle, to avoid taking any dietary supplements and to minimize sun exposure.

Participants were asked to avoid alcohol, caffeine or any vigorous physical activity for 24 h before each visit and to consume a standard low-fat evening meal provided by the researchers. In addition, no foods that were fortified or high in vitamin D were permitted for the 24 h study period and low-nitrate water (The Buxton Mineral Water Company Ltd) was provided to the subjects to consume the day before the study visit and throughout the postprandial day until the 24 h time point.

For each study visit, participants arrived at the clinical unit of the Hugh Sinclair Unit of Human Nutrition at approximately 8.00 am after a 12 h overnight fast. Height, weight, waist and hip circumferences were measured before a cannula was inserted into the antecubital vein of the dominant arm. BP and vascular reactivity measurements were performed after a 30 min rest in a temperature controlled (23±1 °C) clinical room before a fasting blood sample was taken. After the baseline measurements were completed, the test meal was provided and consumed within 15 minutes. Ten postprandial blood samples, four BP and four vascular reactivity measurements were performed up to 8 h after the test meal (see Supplemental Figure 1**)**. Subjects remained in the clinical unit for the duration of the 8 h study visit and no additional food was consumed during the postprandial study period. A standard controlled evening meal (Marks and Spencer Ltd) was consumed at the end of study visit (no vitamin D enriched or fortified foods), after which the participants fasted overnight. The following morning, they returned to the clinical unit for their 24 h assessment in which a fasting blood sample was collected, and BP and vascular reactivity were measured.

Acute test meals

Vitamin D_3 and 25(OH) D_3 supplements (Dishman Netherlands B.V.) were dissolved in refined olive oil (Sainsbury's Supermarkets Ltd) to achieve a concentration of 1 µg/100 µl vitamin D_3 or 25(OH) D_3 stock fortified oil. Aliquots of vitamin D_3 test oil (containing 20 µg vitamin D_3), 25(OH) D_3 test oil (containing 20 µg 25(OH) D_3), and control (olive oil) were assigned a random code and store at -20 °C.

On the morning of each study visit, the dairy drink was prepared from 300 ml full fat milk (Co-operative Limited), 32 g unsalted butter (Co-operative Ltd) and 25 g Askeys Treat Strawberry sauce (The Silver Spoon Ltd). Milk and strawberry sauce were warmed and mixed with melted butter using a hand blender (Sainsbury's Supermarkets Ltd), before 2 ml of the defrosted test/control oil was added into the warm dairy drink and homogenised well.

Subjects were given a test breakfast which included the dairy drink, 3 slices (120 g) of white toast (Hovis Ltd) with 40 g strawberry jam (Sainsbury's Supermarkets Ltd) and 15 g unsalted butter (Co-operative Ltd). Each of the test meals contained 51 g fat, 125 g carbohydrate, 23 g protein and 4.54 MJ. The nutrient compositions of the foods were obtained from the product labels.

Assessment of vascular function, blood pressure and anthropometric measures

Height and weight was measured using a wall-mounted stadiometer and Tanita BC-418 digital scale (Tanita Europe BV) respectively. BP was measured on the upper left arm using a BP monitor (TM-2430; A&D Ltd) in triplicate after a minimum of 10 min rest in a supine position at baseline (0 min) and at 1.5, 3, 6 and 8 h after breakfast and also at the 24 h visit. An Endo-PAT 2000 device (Itamar Medical Ltd) was used to assess the peripheral artery tonometry at baseline (before breakfast) and at the 24 h visit as described elsewhere (15). In brief, after the subjects had rested in a supine position for 20 min, the occlusion cuff was placed on the non-dominant upper arm, and fingertip probes were secured to the index finger of both hands. Measurements were taken for 5 min baseline, 5 min occlusion (cuff was inflated to 60 mm Hg above the subjects' SBP (between 200 and 300 mmHg)) and 5 min post-occlusion after deflation of the cuff. Pulse wave amplitude (PWA) was recorded automatically by the EndoPAT software (EndoPAT^{TM} 2000). From the PWA recordings, reactive hyperemia index (RHI), Framingham reactive hyperemia index (F-RHI), augmentation index (AI), and AI adjusted for a heart rate of 75 beats/min (AI@75) were automatically calculated (16). In addition, digital volume pulse (DVP) photoplethysmography (Pulse Trace; Micro Medical) was measured at baseline (0 min) and 1.5, 3, 6 and 8 h after breakfast and also at the 24 h visit to determine arterial stiffness index (SI), reflection index (RI), peak-to-peak time (PPT) and heart rate (HR) (17).

Plasma collection and analysis

Blood samples collected from the cannula were placed into serum separating tubes (for the analysis of blood lipids, apolipoprotein B (apoB), C-reactive protein (CRP), glucose and insulin); lithium heparin tubes (for the analysis of total nitrates and nitrites (NOx)); and K_3EDTA-coated tubes (for the analysis of tumor necrosis factor alpha (TNF-α), Interleukin 6 (IL-6), vitamin D_3 and 25(OH)D_3). After blood collection, the serum separating tubes were stored at room temperature for 15 min, whereas those containing anticoagulant were stored on ice. All blood samples were centrifuged within 30 min at 1700 × *g* for 15 min at room temperature (serum) or 4 °C (plasma). After centrifugation, the serum or plasma were aliquoted and stored at -20 °C until analysis.

Analysis of plasma vitamin D_3 and 25(OH) D_3 (as sum of 25(OH) D_3 and 3-epi-25(OH) D_3) was conducted by DSM Nutritional Products Ltd using a method validated according to Food and Drug Administration (18) and European Medicines Agency (19) bioanalytical guidelines. In brief, after addition of a deuterated internal standard solution, a protein precipitation was performed with a mixture of tetrahydrofuran, acetonitrile and methanol. After centrifugation, the supernatant was evaporated and the residue was reconstituted with acetonitrile-methanol solution. An aliquot was then injected into a LC-MS/MS system (Agilent 1290, C^{18} column) with APPI source (ABSciex 4000) and the detection of the specific fragment ions was performed using multiple reactions monitoring mode. To assess the daily and long-term laboratory performance of the method, dedicated standard and quality control samples were analyzed daily with the unknown samples to ensure the accuracy and precision of the method. Data acquisition of extracted ion chromatograms, integration and quantification were performed using Analyst® software from ABSciex.

Serum total cholesterol, HDL-cholesterol, non-esterified fatty acids (NEFA), TAG, apoB and CRP were determined using the ILAB 600 autoanalyser with standard kits and appropriate quality controls (reagents and analyser: Werfen (UK) Ltd; NEFA reagent: Alpha

Laboratories; apoB reagent: Randox Laboratories). The fasting LDL-cholesterol concentration was calculated from total cholesterol, HDL-cholesterol and TAG by using the Friedewald formula (20). ELISA kits were used to detect TNF-α (R&D Systems Europe Ltd), IL-6 (R&D Systems Europe Ltd) and insulin (Dako Ltd). Insulin resistance markers: QUICKI, Revised QUICKI (rQUICKI) and HOMA IR were calculated by using standard equations (21). Plasma samples were analysed for nitrite and nitrate using Eicom NOx Analyzer (ENO-30) as described elsewhere (22).

Blood was collected into K_2EDTA tubes (Greiner BioOne Limited) at baseline, 8 and 24 h after the consumption of the test meal for whole blood culture and cytokine analysis as previously described (23). Cytokines of TNF-α and IL-6 were measured in whole blood culture supernatants using ELISA kits (R&D Systems Europe Ltd). The data were normalized for monocyte number and only samples stimulated for 24 h with lipopolysaccharide (0.5 µg/ml) were used in the final analysis.

Study power

According to earlier research by Jetter et al. (24), the expected difference between the treatments (i.e. single dose of 20 µg vitamin D_3 or 20 µg 25(OH) D_3) for plasma 25(OH) D_3 is 3.7 ng/ml (peak concentration of the first day) with a standard deviation (SD) of 13.2 ng/ml. Thus, it was estimated that 15 subjects were required to detect a significant change in this primary outcome measurement with a power of 80% and 5% significance level. A total of 18 subjects were recruited to allow for a drop-out rate of 15%.

Statistical analysis

All data analyses were conducted using STATA (version 13.0; STATA Corporation, 2014). Results are expressed as means ± standard errors (SEMs). Data were checked for

normality and natural logarithm transformation was calculated if needed. The primary analysis of the time courses from baseline to 8 h for outcome variables were analysed by two-factor repeated measures ANOVA to assess the effect of treatment, time, and treatment by time interactions with Bonferroni correction to control for multiple comparisons.

For secondary data analysis, postprandial summary measures were calculated which included area under curve (AUC), incremental AUC (iAUC), maximum concentration (maxC), increment from baseline to maximal concentration imaxC (imaxC=maxC-fasting value) and time to reach maximum concentration (Tmax). These measures were analysed by one-way ANOVA and subsequently Bonferroni correction was applied if post-hoc multiple pairwise comparisons were performed. Kruskal-Wallis equality-of-populations rank test was applied to data which could not be normalised.

For NEFAs, the postprandial summary measures AUC, iAUC, max C, imaxC, Tmax were calculated from the average minimum concentration (approximately 2 h) to 8 h (25).

Results

Of the 18 men who completed the study, the data for one subject whose baseline vitamin D status on the study visit was higher than 50 nmol/L was excluded from the statistical analysis. Therefore, 17 men were included in the current study dataset (**Table 1**) with mean (± SEM) sub-optimal vitamin D status of 31.7 (± 3.4) nmol/L and low dietary vitamin D intake of 4.4 (± 1.5) µg/d.

There were no differences in fasting (0 min) vitamin D status, lipid, indices of insulin resistance and glycaemia, vascular biomarkers, SBP or vascular function measurements between study visits. However, the fasting DBP and pulse pressure (PP) were significantly different between study visits. Thus, only iAUC was calculated to determine the effects of the fortified and control dairy drinks on DBP and PP.

Postprandial response of plasma vitamin D_3 and 25(OH) D_3

Following the test dairy drinks, there was a significant time by treatment interaction for the postprandial plasma 25(OH) vitamin D_3 concentrations (P<0.0001) (**Figure 1**). After the 25(OH) D_3 fortified dairy drink consumption, imaxC (0-8h) (P=0.0001) was 1.2-fold higher than control and 1.7-fold higher than vitamin D_3 fortified dairy drink (**Table 2**). Furthermore, the iAUC (0-8 h) for the 25(OH) D_3 fortified dairy drink was 1.5-fold higher than vitamin D_3 fortified dairy drink and 1.8-fold higher than control (P=0.019), whereas the iAUC (0-8 h) for the vitamin D_3 fortified dairy drink was not different from the control. The change in plasma 25(OH) D_3 concentration calculated from baseline to 24 h after the 25(OH) D_3 fortified dairy drink was also significantly higher than following the vitamin D_3 fortified and control dairy drinks (P<0.0001)(Table 2).

Statistical analysis of the plasma vitamin D_3 responses was not conducted as only 42/648 plasma samples had vitamin D_3 concentrations above the limit of detection of the LC-MS/MS technique (2.5 nmol/L).

Vascular function and postprandial blood pressure

Treatment effects on vascular function and postprandial BP are presented in **Table 3**. There was no difference in the change from baseline to 24 h for the vascular function measurements by EndoPAT and DVP devices. There were no significant effects of treatments on postprandial blood pressure (SBP and DBP) or PP.

Blood lipid profile and indices of insulin resistance and glycaemia

There were no treatment effects on postprandial blood lipids or indices of insulin resistance and glycaemia determined over the 8 h (**Supplemental Table 1**). In addition, there was no difference in the change from baseline to 24 h for any of these measures.

Postprandial responses of vascular and inflammatory biomarkers

No significant effect of treatments on serum CRP, plasma NOx and IL-6 were observed (**Supplemental Table 2**). Statistical analysis of TNF-α was not conduced as 37% of the samples had concentrations below the lower level of detection of the ELISA kit (0.11 pg/ml).

Ex vivo *Cytokine production*

There was no effect of the fortified or control dairy drinks on *ex vivo* production of IL-6 or TNF-α after stimulation of whole blood cultures with LPS, measured using blood samples collected at baseline, 8 or 24 h, or calculated as change from baseline to 8 or 24 h (**Supplemental Table 3**).

Discussion

This study is the first to compare the postprandial responses to vitamin D_3 and 25(OH) D_3 fortified dairy drinks on plasma 25(OH) D_3 concentrations in addition to markers of cardiometabolic risk. It was observed that a 25(OH) D_3 fortified dairy drink (20 µg) resulted in higher and more sustained plasma 25(OH) D_3 concentrations over 8 h and at the 24 h time point compared with the control and vitamin D_3 fortified dairy drinks (20 µg). However, we did not detect changes in vascular function measurements or cardiometabolic risk markers after consumption of the test meals containing the dairy drinks.

To date, no randomized controlled trials (RCTs) have evaluated the effects of fortified 25(OH) D_3 dairy products on vitamin D status. However, Jetter et al. (24) compared the effect of capsules containing 20 µg of 25(OH) D_3 and vitamin D_3 on plasma 25(OH) D_3 in healthy postmenopausal women who had similar baseline plasma 25(OH) D_3 concentrations (30.7 ± 10.2 (SD) nmol/L) to the participants in the current study. A tendency for a 28% higher plasma 25(OH) D_3 AUC (0 to 24 h) after the 25(OH) D_3 supplementation compared with the

vitamin D_3 supplement was reported, although this did not reach statistical significance. This direction of effect was in line with the current study where a 25(OH) D_3 fortified dairy drink resulted in a 1.5-fold higher plasma 25(OH) D_3 iAUC compared with the vitamin D_3 fortified dairy drink which was evident within 8 h of ingestion, although in the current study the iAUC between treatments reached statistical significance. The differences between studies may be due in part to the characteristics of the study participants. The current study was conducted in men aged 30-54 y, while Jetter et al. (24) studied postmenopausal women aged 50-70 y, although there has been no evidence from any study reported of an age or sex effect on the absorption of vitamin D supplements. In addition, the form of the 25(OH) D_3 may have influenced absorption, with a preferential absorption with a fat containing meal rather than from capsules taken with water. This speculative explanation would require further confirmation.

We were unable to quantify plasma vitamin D_3 concentrations since plasma levels were below the detection limit of the LC MS/MS assay. One explanation may relate to the findings of Barger et al. (9). Their study investigated the dose response to supplemental vitamin D_3 (25, 250, 1250 µg/d) and 25(OH) D_3 (10, 20, 50 µg/d) for 8 and 4 weeks, respectively. It was observed that both serum vitamin D_3 and 25(OH) D_3 increased after vitamin D_3 supplementations, whereas only serum 25(OH) D_3 increased after 25(OH) D_3 supplementations. The lack of detection of changes in plasma vitamin D_3 after either fortified drink suggests that higher dose of vitamin D_3 may be required over a longer period of time to change plasma vitamin D_3 concentrations.

A study by Stamp (26) investigated the acute effect of a single dose of supplemental 25(OH) D_3 at 10 µg per kg body weight in healthy subjects over 24 h. The peak concentration of circulating 25 (OH) D_3 was reached between 4 and 8 h. In contrast, Jetter et al. (24) reported the time to reach peak plasma 25(OH) D_3 concentrations for a supplemental dose of 20 µg of 25(OH) D_3 and vitamin D_3 to be 10.8 and 22.2 h respectively. In the current study

the peak circulating concentration of 25(OH) D_3 could not be identified precisely as blood samples were not collected between 8 and 24 h, although 24 h concentrations were still above baseline concentrations. Thus, it could be speculated that the peak concentration was reached earlier, after ingestion of 25(OH) D_3 fortified dairy drink, compared with vitamin D_3 fortified dairy drink, although this would need to be confirmed in a study with frequent blood sampling over 8-36 h.

Effective dietary strategies to increase population vitamin D status are required to address the high incidence of sub-optimal vitamin D status within the population (5). The Scientific Advisory Committee for Nutrition published new dietary guidance in 2016 (1), recommending a daily vitamin D intake of 10 µg/day for adults, which is challenging to achieve through diet unless fortified foods are consumed. The average daily intake of vitamin D for adults is only 3.1 µg for men and 2.6 µg for women, respectively (5). Therefore, vitamin D fortified foods are one strategy that would increase vitamin D dietary intake. Milk and dairy are ideal foods for fortification as they are consumed by the majority of the population within Europe and USA (5, 27). The current study verified that dairy products were suitable vehicles for fortification with 25(OH) D_3 resulting in a more rapid increase in markers of vitamin D status than using vitamin D_3. The mechanism for the more rapid absorption of 25(OH) D_3 is unclear, but it might be because hepatic metabolism of vitamin D_3 to 25(OH) D_3 is circumvented (6), and so the bioactive form of vitamin D, 1,25$(OH)_2$ D_3, can be more rapidly synthesised by the kidney, whereas vitamin D_3 needs to be transported in chylomicron particles from the gut to the liver for further metabolism (3).

No treatment effects on postprandial arterial stiffness in men with sub-optimal vitamin D status were observed, which is in line with a previously study (28) which also reported no changes in arterial stiffness after consumption of a single dose of 7500 µg or 1875 µg vitamin D_3. In addition, a recent systematic review and meta-analysis (29) has summarised 28 RCTs on vitamin D_3 supplementation and concluded there was no effect of vitamin D

supplementation (doses ranged from 25 µg/day to 3000 µg/month) on arterial stiffness after administration periods ranging from 2 to 12 months.

In contrast with our study of no effect on BP, Bischoff-Ferrari et al. (10) reported a 5.7-mmHg decrease (P=0.0002) in SBP after daily 20 µg 25(OH) D_3 supplementation compared with 20 µg vitamin D_3 consumption over 4 months in subjects who had normal BP. Note that, in our study, the effect of the test meal containing the dairy drink was followed up for 24 h only as opposed to the study of Bischoff-Ferrari et al. (10) which was a 4 months intervention, which suggests that a chronic intervention period may have been required for significant changes in BP.

Our findings for a lack of effect of the fortified dairy drinks on the postprandial lipid profiles (TAG and NEFA) are in line with a previously study (28), which also reported there were no effects of a single higher dose of vitamin D_3 of 7500 µg or 1875 µg on postprandial lipid profiles (TAG, total-/HDL-/LDL-cholesterol) up to 8 h in overweight vitamin D deficient women (vitamin D level of 27.1 (SD=13.8) nmol/L). Furthermore, the current study is the first to investigate the effects of vitamin D fortified dairy drinks on the production of the inflammatory cytokines, IL-6 and TNF-α, in whole blood culture following stimulation with lipopolysaccharide. No differences between the dairy drinks were observed, suggesting that longer supplementation periods or higher doses may be required to determine the chronic effect on inflammation.

This study has some potential limitations. It was powered to detect a significant difference in the primary outcome of postprandial plasma 25(OH) D_3, however it may not have been suitably powered to detect changes in the secondary outcomes. In addition, blood samples were not collected between 8 and 24 h, which restricted estimation of the peak 25(OH) D_3 concentration. Furthermore, the participants were men with sub-optimal vitamin D levels and the results may not be representative of responses in women or those individuals with adequate vitamin D levels.

In conclusion, the current study confirmed that a 25(OH) D_3 fortified dairy drink was able to increase a marker of vitamin D status more efficiently postprandially than a vitamin D_3 fortified dairy drink. For future studies it is important to investigate the impact of daily 25(OH) D_3 fortified dairy drink consumption on vitamin D status and cardiometabolic risk markers over a longer period in both men and women.

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The author's responsibilities were as follows: JAL, DIG, JG, KGJ designed the study; JG conducted the research analysed the data, and wrote the manuscript; CSBCT: assisting the whole study; YL: substantially contributed to the conduct of research on whole blood culture; KGJ, DIG and JAL modified the writing of the manuscript; all of authors read and approved the final manuscript. None of the authors reported a conflict of interest relating to this study.

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Table 1. Baseline characteristics of participants[1].

	All (n=17)
Age, y	49 ± 3
BMI, kg/m^2	26.4 ± 0.61
Blood pressure (mmHg)	
Systolic	122 ± 2
Diastolic	64 ± 2
Total-cholesterol, mmol/L	5.04 ± 0.21
LDL-cholesterol, mmol/L	3.61 ± 0.09
HDL-cholesterol, mmol/L	1.21 ± 0.03
Triacylglycerol, mmol/L	1.48 ± 0.21
Glucose, mmol/L	5.42 ± 0.14
Insulin, pmol/L	47.7 ± 3.2
Vitamin D dietary intake[2], µg/d	4.40 ± 1.51
Vitamin D status, nmol/L	31.7 ± 3.4

[1]Values are means ± SEM of three visits. BMI: body mass index;
[2]Derived from 4-day diet diary.

Table 2. Baseline and postprandial changes of plasma 25(OH) D_3 concentrations from baseline after consumption of 25(OH) D_3 fortified dairy drink (25(OH) D_3), vitamin D_3 fortified dairy drink (Vitamin D_3) or unfortified dairy drink (Control)[1].

	Meal			
Measures of 25(OH) D_3	Control	Vitamin D_3	25(OH) D_3	P^2
Baseline, nmol/L	28.5 ± 2.8	31.0 ± 3.4	30.4 ± 3.3	0.847
maxC (0-8h), nmol/L	32.9 ± 3.6	34.6 ± 3.8	40.2 ± 3.9	0.368
imaxC (0-8h), nmol/L	4.4 ± 1.1[b]	3.6 ± 0.7[b]	9.8 ± 1.2[a]	0.0001
AUC (0-8h), nmol/L×8h	238.2 ± 24.9	259.6 ± 29.1	272.3 ± 28.4	0.677
IAUC (0-8h), nmol/L×8h	10.3 ± 5.8[b]	11.7 ± 4.2[b]	29.2 ± 5.2[a]	0.019
Change from baseline to 24h	1.6 ± 1.1[b]	4.5 ± 0.8[b]	8.7 ± 0.9[a]	<0.0001
Change from 8 h to 24h	-0.2 ± 1.2	3.1 ± 0.9	1.6 ± 1.0	0.101

[1]Values are means ± SEMs. Different superscript letters within a row identify intervention groups significantly different from one another ($P \leq 0.05$).
[2]One-factor ANOVA was applied to the outcome variable, Bonferroni multiple pairwise comparison *post hoc* tests was used to identify the significant differences between the treatments.

Table 3. Baseline and change from baseline to 24 h for the vascular measurements and postprandial blood pressure after consumption of 25(OH) D_3 fortified dairy drink (25(OH) D_3), vitamin D_3 fortified dairy drink (Vitamin D_3) or unfortified dairy drink (Control)[1].

Measures	Meal			P[3]
	Control	Vitamin D_3	25(OH) D_3	
EndoPAT device[2]				
RHI				
Baseline	1.94 ± 0.18	2.04 ± 0.15	1.85 ± 0.12	0.683
Change from baseline to 24h	0.15 ± 0.18	0.05 ± 0.14	0.13 ± 0.12	0.895
F-RHI				
Baseline	0.29 ± 0.11	0.41 ± 0.09	0.31 ± 0.08	0.625
Change from baseline to 24h[6]	0.20 ± 0.11	0.04 ± 0.10	0.08 ± 0.08	0.599
AI				
Baseline	3.85 ± 3.24	6.33 ± 3.37	4.66 ± 3.74	0.872
Change from baseline to 24h	-0.51 ± 1.66	-3.21 ± 1.74	-2.99 ± 1.56	0.447
AI@75				
Baseline	3.85 ± 3.24	6.33 ± 3.37	4.66 ± 3.74	0.872
Change from baseline to 24h	-0.51 ± 1.66	-3.21 ± 1.74	-2.99 ± 1.56	0.447
DVP device				
Heart Rate (HR)				
Interaction of treatment × time[5]				0.545
Baseline, (beats/min)	57.9 ± 1.8	58.1 ± 1.9	57.5 ± 1.7	0.967
AUC (0-8h), beats/min×8h	475.9 ± 13.8	465.9 ± 14.3	476.3 ± 13.8	0.838
iAUC (0-8h), beats/min×8h	12.3 ± 7.3	0.9 ± 7.8	16.3 ± 7.4	0.331
Change from baseline to 24h, (beats/min)[6]	3.0 ± 1.5	1.6 ± 1.3	3.2 ± 0.9	0.505
Stiffness index (SI)				
Interaction of treatment × time				0.084
Baseline, m/s	8.6 ± 0.6	9.0 ± 0.6	8.4 ± 0.7	0.804

AUC (0-8h), beats/min×8h	60.9 ± 3.8	59.6 ± 3.7	62.9 ± 4.4	0.845
iAUC (0-8h), beats/min×8h[6]	-7.9 ± 8.3	-12.3 ± 15.6	-4.4 ± 12.7	0.393
Change from baseline to 24h, m/s[6]	-0.04 ± 0.41	-0.73 ± 0.63	0.04 ± 0.33	0.631
Reflection index (RI)				
Interaction of treatment × time				0.307
baseline,%	73.7 ± 2.7	75.2 ± 2.7	71.1 ± 3.3	0.615
AUC (0-8h), beats/min×8h	552.3 ± 19.8	554.1 ± 16.1	552.4 ± 22.6	0.997
iAUC (0-8h), beats/min×8h	-37.0 ± 12.6	-47.2 ± 17.7	-16.7 ± 17.9	0.410
Change from baseline to 24h,%	-3.49 ± 2.61	-5.73 ± 3.16	-2.24 ± 2.80	0.686
Peak-to-peak time				
Interaction of treatment × time				0.172
Baseline, m/s	220.7 ± 14.9	212.5 ± 15.3	233.9 ± 18.2	0.643
AUC (0-8h), beats/min×8h	1999.0 ± 465.0	2014.4 ± 456.4	1967.2 ± 516.1	0.958
iAUC (0-8h), beats/min×8h	233.6 ± 228.5	314.1 ± 355.6	95.7 ± 324.6	0.123
Change from baseline to 24h, m/s[6]	5.91 ± 7.59	20.07 ± 13.80	0.55 ± 7.76	0.530
Systolic blood pressure (SBP)				
Interaction of treatment × time[5]				0.574
Baseline, mm Hg	119 ± 2	123 ± 3	121 ± 3	0.672
AUC (0-8h), mm Hg×8h	953 ± 15	961 ± 14	968 ± 17	0.802
IAUC (0-8h), mm Hg×8h	-2 ± 11	-19 ± 10	-1 ± 9	0.376
Change from baseline to 24h, mm Hg	-1 ± 2	-2 ± 2	-4 ± 1	0.551
Diastolic blood pressure (DBP)				
Interaction of treatment × time				0.924
Baseline, mm Hg	67 ± 2	64 ± 2	73 ± 2	0.0007
IAUC (0-8h), mm Hg×8h	11 ± 12	-3 ± 15	-17 ± 12	0.334
Change from baseline to 24h, mm Hg	-1 ± 2	2 ± 2	2 ± 2	0.584
Pulse pressure (PP)[4]				
Interaction of treatment × time				0.873
Baseline, mm Hg	53 ± 2[ab]	58 ± 3[b]	48 ± 3[a]	0.041

IAUC (0-8h), mm Hg×8h	-13 ± 14	-17 ± 19	16 ± 15	0.317
Change from baseline to 24h, mm Hg	-1 ± 2	-5 ± 3	-6 ± 3	0.366

[1]Values are means ± SEMs. Different superscript letters within a row identify intervention groups significantly different from one another.

[2]RHI: reactive hyperemia index; F-RHI: Framingham reactive hyperemia index; AI: augmentation index; AI@75: augmentation index adjusted for a heart rate of 75 beats/min.

[3]Two-factor repeated measure ANOVA was applied to assess the treatment by time interactions; One-factor ANOVA was applied to the outcome variable to compare overall between-group diet, Bonferroni multiple pairwise comparison *post hoc* tests was used to identify the significant differences between treatments.

[4]Calculated by subtraction of DBP from SBP.

[5]Original data were transformed to natural logarithms for the analysis.

[6]Kruskal-Wallis equality-of-populations rank test for non-normally distributed data.

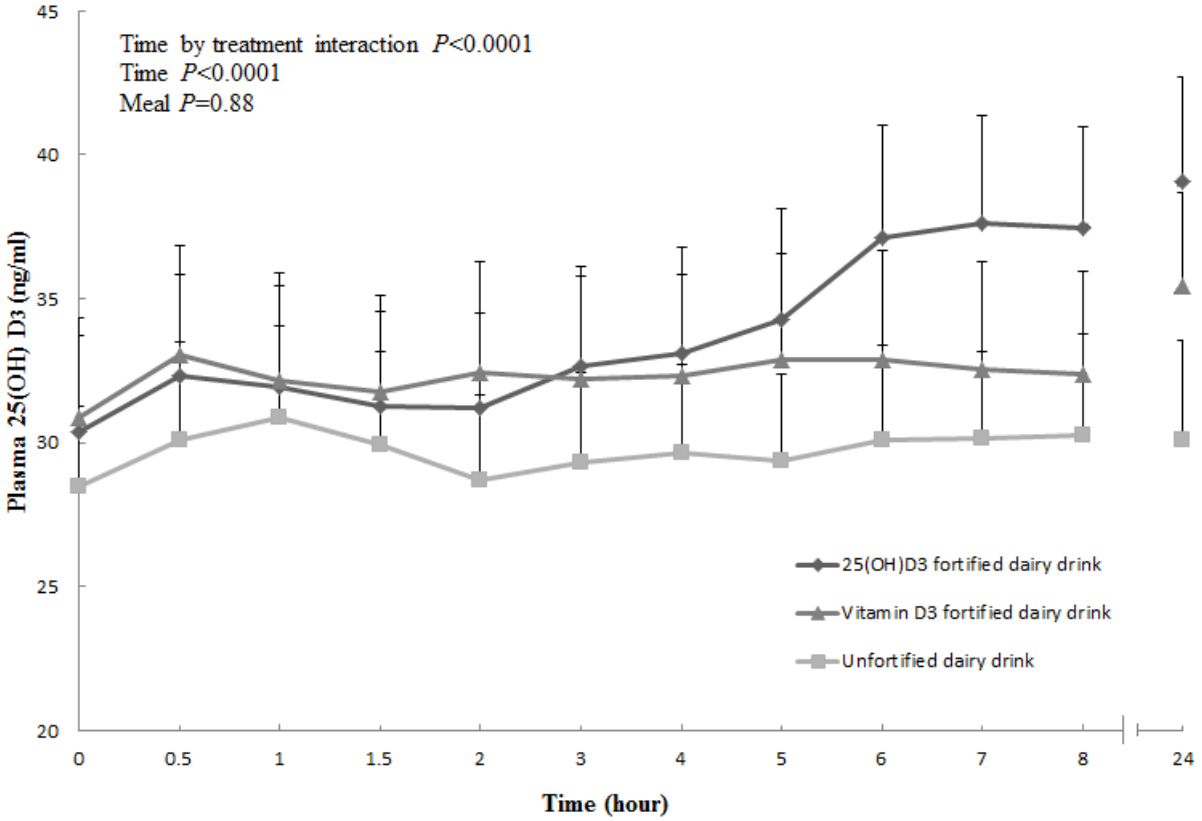
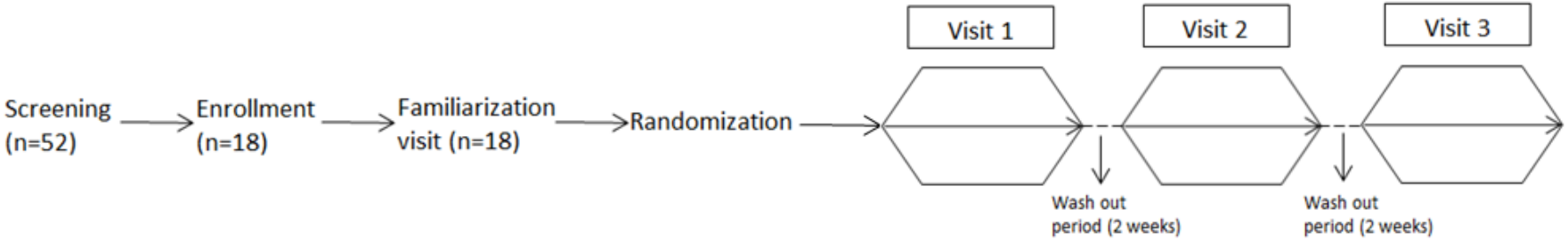
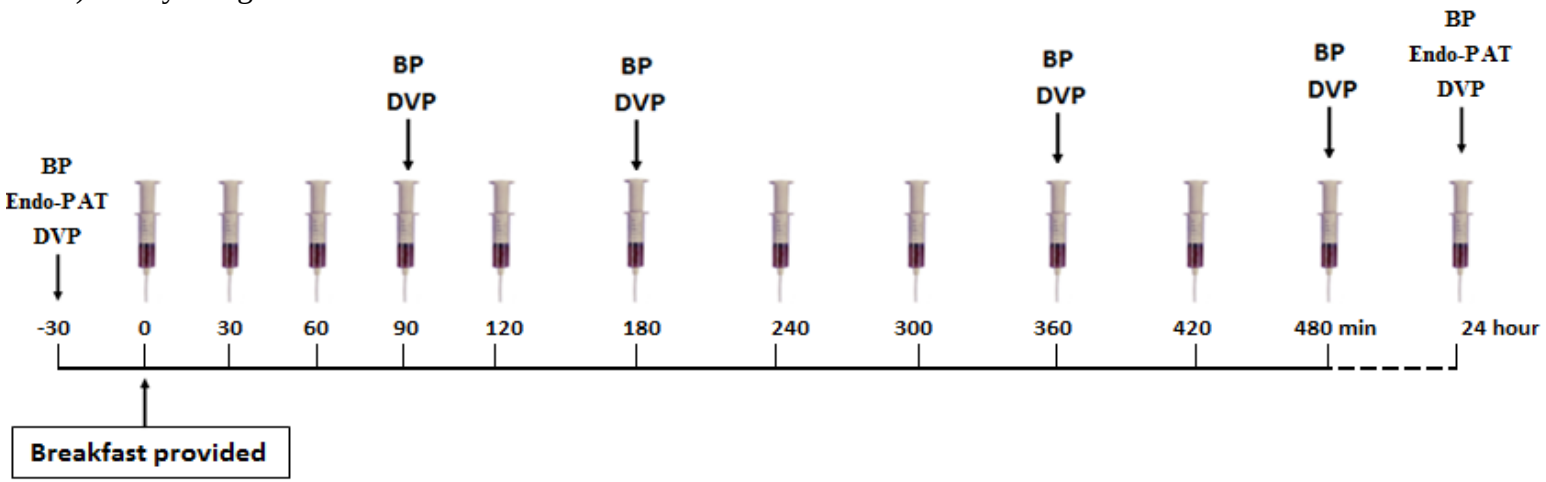


Figure 1. Postprandial responses of mean plasma 25(OH) D_3 concentrations after consumption of 25(OH) D_3 fortified dairy drink, vitamin D_3 dairy drink and unfortified dairy drink. Values are means ± SEM, n=17 for each treatment. Two-factor repeated measure ANOVA was applied to assess the effect of treatment, time, and treatment by time interactions.

A) Participant flowchart



B) Study design



Supplemental Figure 1. Participant flowchart and study design (BP: blood pressure measurement; vascular function measurements: DVP or Endo-PAT.

Supplemental Table 1. Postprandial responses of blood lipid profile, indexes of insulin resistance after consumption of 25(OH) D_3 fortified dairy drink (25(OH) D_3), vitamin D_3 fortified dairy drink (Vitamin D_3) or unfortified dairy drink (Control)[1].

Measures	Meal			P[2]
	Control	Vitamin D_3	25(OH) D_3	
Glucose				
Interaction of meal × time for the postprandial timecourse				1.000
Baseline, mmol/L[3]	5.39 ± 0.17	5.50 ± 0.13	5.49 ± 0.15	0.788
maxC (0-8h), mmol/L[3]	7.63 ± 0.49	7.93 ± 0.50	7.93 ± 0.51	0.872
imaxC (0-8h), mmol/L[3]	2.24 ± 0.43	2.43 ± 0.45	2.44 ± 0.46	0.922
Tmax (0-8h), min[3]	81 ± 13	67 ± 12	62 ± 15	0.259
AUC (0-8h), mmol/L×8h[3]	44.6 ± 1.4	44.3 ± 1.4	43.7 ± 1.4	0.881
IAUC (0-8h), mmol/L×8h	1.5 ± 1.2	0.3 ± 1.2	-0.2 ± 0.8	0.526
Change from baseline to 24h, mmol/L	0.12 ± 0.06	0.08 ± 0.08	0.02 ± 0.06	0.583
Insulin				
Interaction of meal × time for the postprandial timecourse[3]				0.875
Baseline, pmol/L	43.7 ± 5.4	49.3 ± 5.6	50.0 ± 5.7	0.685
maxC (0-8h) pmol/L	411.10 ± 41.77	478.23 ± 46.99	459.57 ± 46.25	0.558
imaxC (0-8h), pmol/L[3]	367.39 ± 38.96	428.94 ± 44.23	409.61 ± 42.73	0.575
Tmax (0-8h), min	72 ± 9	56 ± 8	58 ± 7	0.320
AUC (0-8h), nmol/L×8h	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	0.810
IAUC (0-8h), nmol/L×8h	0.9 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.837
Change from baseline to 24h, pmol/L	18.6 ± 3.7	5.4 ± 4.0	10.0 ± 3.8	0.055
HOMA-IR				
Baseline	1.76 ± 0.23	2.03 ± 0.24	2.06 ± 0.26	0.634
Change from baseline to 24h	0.80 ± 0.17	0.25 ± 0.18	0.40 ± 0.16	0.072
QUICKI				

Baseline[3]	0.16 ± 0.00	0.15 ± 0.00	0.15 ± 0.00	0.672
Change from baseline to 24h[4]	-0.01 ± 0.00	-0.00 ± 0.00	-0.01 ± 0.00	0.150
Revised QUICKI				
Baseline[4]	0.08 ± 0.00	0.08 ± 0.00	0.08 ± 0.00	0.825
Change from baseline to 24h[4]	-0.00 ± 0.00	-0.00 ± 0.00	-0.00 ± 0.00	0.690
Total cholesterol				
Baseline, mmol/L	5.11 ± 0.20	5.12 ± 0.22	5.14 ± 0.20	0.995
Change from baseline to 24h, mmol/L	0.36 ± 0.08	0.40 ± 0.09	0.36 ± 0.07	0.900
HDL-cholesterol				
Baseline, mmol/L	1.22 ± 0.06	1.19 ± 0.06	1.23 ± 0.06	0.881
Change from baseline to 24h, mmol/L	0.06 ± 0.01	0.04 ± 0.02	0.05 ± 0.01	0.632
LDL-cholesterol				
Baseline, mmol/L	3.58 ± 0.15	3.63 ± 0.17	3.61 ± 0.16	0.974
Change from baseline to 24h, mmol/L	0.31 ± 0.07	0.34 ± 0.07	0.30 ± 0.06	0.914
Triacylgycerol				
Interaction of meal × time for the postprandial timecourse[3]				0.977
Baseline, mmol/L[3]	1.54 ± 0.17	1.50 ± 0.20	1.49 ± 0.16	0.978
maxC (0-8h), mmol/L	2.88 ± 0.27	3.01 ± 0.33	2.96 ± 0.29	0.953
imaxC (0-8h), mmol/L	1.34 ± 0.14	1.50 ± 0.20	1.47 ± 0.18	0.793
Tmax (0-8h), min	275 ± 19	221 ± 17	277 ± 21	0.073
AUC (0-8h), mmol/L×480min	1033 ± 104	1042 ± 121	1041 ± 102	0.998
IAUC (0-8h), mmol/L×480min	296 ± 37	319 ± 49	325 ± 44	0.879
Change from baseline to 24h, mmol/L	-0.04 ± 0.07	0.16 ± 0.06	0.05 ± 0.04	0.079
Non-esterified fatty acids (NEFA)				
Interaction of meal × time for the postprandial timecourse[3]				0.999
Baseline, µmol/L[3]	475.0 ± 38.6	484.0 ± 51.4	489.0 ± 30.0	0.824
minC (0-8h), µmol/L[3]	146.5 ± 11.3	138.6 ± 12.7	143.3 ± 11.9	0.827

Suppression (0-2h) %[4]	-62.0 ± 4.5	-61.3 ± 5.3	-67.8 ± 3.0	0.749
maxC (2-8h), µmol/L[3]	654.8 ± 54.0	651.0 ± 56.6	720.7 ± 61.7	0.702
imaxC (2-8h), µmol/L[3]	492.6 ± 55.1	489.4 ± 59.8	569.3 ± 59.4	0.569
Tmax (2-8h), min	431 ± 14	441 ± 13	441 ± 11	0.792
AUC (2-8h), mmol/L×6h	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0.2	0.909
IAUC (2-8h), mmol/L×6h	-0.1 ± 0.2	-0.1 ± 0.3	-0.1 ± 0.2	0.983
Change from baseline to 24h, µmol/L	-12.7 ± 51.6	-12.7 ± 51.0	-24.4 ± 27.3	0.977
Apolipoprotein B				
Interaction of meal × time for the postprandial timecourse				0.570
Baseline, µg/mL	1039± 48	1043 ± 49.0	1043 ± 49.0	1.000
maxC (0-8h), µg/mL	1089 ± 48	1099 ± 13	1091 ± 53	0.989
imaxC (0-8h), µg/mL	49.6± 9.2	55.8 ± 8.0	52.0 ± 8.3	0.875
Tmax (0-8h), min[4]	247 ± 50	251 ± 48	173 ± 47	0.607
AUC (0-8h), µg/mL×8h	8293 ± 352	8302 ± 351	8272 ± 401	0.998
IAUC (0-8h), µg/mL×8h	-22.9 ± 71.4	-42.5 ± 73.3	-37.7 ± 44.0	0.975
Change from baseline to 24h, µg/mL	89.8 ± 10.5	78.3 ± 15.8	87.2 ± 12.7	0.811

[1]Values are means ± SEMs. Different superscript letters within a row identify different from one another

[2]Two-factor repeated measure ANOVA was applied to assess the treatment by time interactions; One-factor ANOVA was applied to the outcome variable to compare overall between-group diet, Bonferroni multiple pairwise comparison *post hoc* tests was used to identify the significant differences between the treatments.

[3]Original data were transformed to natural logarithms for the analysis.

[4]Kruskal-Wallis equality-of-populations rank test for non-normally distributed data.

Supplemental Table 2. Postprandial responses of the inflammatory and vascular biomarkers after consumption of 25(OH) D_3 fortified dairy drink (HyD_3), vitamin D_3 fortified dairy drink (25(OH) D_3) or unfortified dairy drink (Control)[1].

Measures	Meal			P[2]
	Control	Vitamin D_3	25(OH) D_3	
C-reactive protein				
Interaction of meal × time for the postprandial timecourse[3]				0.669
Baseline, µg/ mL[3]	1.37 ± 0.43	1.14 ± 0.49	0.71 ± 0.17	0.690
maxC (0-8h), µg/ mL[3]	1.50 ± 0.43	1.23 ± 0.48	0.83 ± 0.18	0.553
imaxC (0-8h), µg/ mL[3]	0.13 ± 0.03	0.10 ± 0.01	0.12 ± 0.01	0.578
Tmax (0-8h), min[4]	124 ± 25	106 ± 12	111 ± 16	0.945
AUC (0-8h), µg/ml×8h[3]	11.0 ± 3.3	8.8 ± 3.3	6.1 ± 1.4	0.623
IAUC (0-8h), µg/ml×8h[4]	0.0 ± 0.3	-0.3 ± 0.6	0.4 ± 0.1	0.342
Change from baseline to 24h, mg/mL[4]	-0.15 ± 0.17	-0.21 ± 0.25	0.06 ± 0.04	0.579
Nitric oxide				
Interaction of meal × time for the postprandial timecourse				0.755
Baseline, µmol/L	13.0 ± 1.3	11.6 ± 1.3	11.6 ± 1.5	0.730
maxC (0-8h), µmol/L[3]	14.4 ± 0.9	13.8 ± 0.7	13.4 ± 0.7	0.635
imaxC (0-8h), µmol/L[3]	1.5 ± 1.3	2.2 ± 1.0	1.7 ± 0.8	0.634
Tmax (0-8h), min	81 ± 36	141 ± 46	184 ± 44	0.238
AUC (0-8h), µmol/L×8h[3]	86.6 ± 11.1	77.2 ± 8.9	76.1 ± 8.3	0.808
IAUC (0-8h), µmol/L×8h	-17.1 ± 6.3	-15.9 ± 4.5	-10.5 ± 4.3	0.630
Change from baseline to 24h, µmol/L	-3.3 ± 1.0	-0.2 ± 2.0	-2.3 ± 1.3	0.318
Interleukin 6				
Interaction of meal × time for the postprandial timecourse[3]				0.667
Baseline, pg/mL	1.16 ± 0.14	1.00 ± 0.12	1.03 ± 0.11	0.624
maxC (0-8h), pg/mL[3]	2.28 ± 0.11	2.36 ± 0.13	2.25 ± 1.12	0.995
imaxC (0-8h), pg/mL[3]	1.12 ± 0.23	1.36 ± 0.32	1.22 ± 0.28	0.933
Tmax (0-8h), min	388 ± 28	388 ± 38	356 ± 43	0.781

AUC (0-8h), pg/mL×8h[3]	12.0 ± 1.3	11.1 ± 1.2	11.6 ± 1.3	0.858
IAUC (0-8h), pg/mL×8h	2.8 ± 0.9	3.1 ± 1.3	3.4 ± 1.2	0.926
Change of 24 h from baseline, pg/mL	0.09 ± 0.17	-0.04 ± 0.11	-0.06 ± 0.09	0.664

[1]Values are means ± SEMs. Different superscript letters within a row identify intervention groups significantly different from one another

[2]Two-factor repeated measure ANOVA was applied to assess the treatment by time interactions; One-factor ANOVA was applied to the outcome variable to compare overall between-group diet, Bonferroni multiple pairwise comparison *post hoc* tests was used to identify the significant differences between treatments.

[3]Original data were transformed to natural logarithms for the analysis.

[4]Kruskal-Wallis equality-of-populations rank test for non-normally distributed data.

Supplemental Table 3.Ex vivo lipopolysaccharide - stimulated interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) production in whole blood cultures after consumption of 25(OH) D_3 fortified dairy drink (25(OH) D_3), vitamin D_3 fortified dairy drink (Vitamin D_3) or unfortified dairy drink (Control)[1].

Measures	Meal			P^2
	Control	Vitamin D_3	25(OH) D_3	
IL-6				
Interaction of meal × time for the postprandial time course [3]				0.872
Baseline, pg/mL[3]	3693 ± 629	3827 ± 498	4689 ± 690	0.496
Change from baseline to 8h, pg/mL[3]	-420 ± 327	-145 ± 323	-611 ± 450	0.528
Change from baseline to 24h, pg/mL[4]	741 ± 437	594 ± 502	460 ± 423	0.910
TNF-α				
Interaction of meal × time for the postprandial time course [3]				0.255
Baseline, pg/mL	465 ± 99	421 ± 58	400 ± 41	0.996
Change from baseline to 8h, pg/mL[4]	-101 ± 89	-12.1 ± 54.7	-15.1 ± 29.6	0.281
Change from baseline to 24h, pg/mL[4]	-34 ± 88	52 ± 52	110 ± 52	0.257

[1]Values are means ± SEMs, data were corrected for the number of monocytes.
[2]Two-factor repeated measures ANOVA was applied to the outcome variable
[3]Original data were transformed to natural logarithms for the analysis.
[4]Kruskal-Wallis equality-of-populations rank test for non-normally distributed data.

Chapter 9 – General discussions and conclusions.

General discussion

Hypovitaminosis D is prevalent through EU, due to diet and lifestyle changes (1, 2). In the UK, 40% and 8% adults (19-64y) whose plasma 25(OH) D concentration < 25 nmol/L in the winter and summer, respectively (3). To date, there is growing evidence for the association between low vitamin D status and increased risk of non-skeletal health outcomes, such as cardiovascular disease (CVD), diabetes and certain cancers (4). The fact that CVD and diabetes are responsible for over 18 million mortalities globally (5). Estimates from study of Grant et al. (2009) suggest a reduction of economic burden of disease is €187,000 million/year if serum 25(OH) D level to 100 nmol/L(6).

Evidence from Caerphilly Prospective Study (CAPS) in Chapter 3 is the first to show higher vitamin D dietary intake was associated with a lower plasma triacylglycerol level cross-sectionally and also at the 5-years examination, an independent risk factor for CVD and a characteristic of type 2 diabetes (T2D) (7). The findings agrees with the results of a randomized controlled trial (RCT) which showed a daily 100 µg vitamin D_3 for 6 months resulted in a decreased triacylglycerol level, however, the study was conducted in the post-menopausal women with T2D. Furthermore, evidence in Chapter 3 also suggest higher vitamin D intake was modest positive associated with diastolic blood pressure, but there were no associations between vitamin D intake and CVD after over 20 years follow-up (Chapter 3). This is consistent with a recent report and meta-analysis (8) that reports the direct associations between vitamin D and CVD are not certain. The impact of vitamin D intake on CVD events and risk markers is a complex tropical area of research, which need further large cohort studies or RCT to verify.

Due to diet and lifestyle changes and the frequent use of sunscreen, many individuals do not endogenously synthesise sufficient vitamin D from sunlight exposure (9), thus, awareness of tackling inadequate vitamin D intake has been increased (10). Egg yolk, oily fish and wild mushrooms have been regarded as foods naturally enriched with vitamin D (11). Previous studies indicated vitamin D content of salmon and some mushrooms significantly varied between different production systems (12, 13). Our research in Chapter 5 is the first UK study to show that the vitamin D content of the eggs from indoor was significantly less than free range and organic. However, study of Matt et al. (2009) (14) demonstrated eggs from organic have lower vitamin D content than indoor eggs. The inconsistencies in the findings probably be explained by the variation of system management, such as the difference in the diet or pasture usage for the birds. With the limitation of the current study, the original diet of the poultry and daily activity of the birds are unknown, but current results represent what the consumer purchases and consumes. Furthermore, the current study confirmed that one egg per day contributed about 2 µg/day vitamin D, which equivalent to 20% of RNI (10 µg/day) vitamin D.

Eggs are a nutrient-dense food with high quality protein and minerals, but also enriched with cholesterol, which could increase the risk of CVD and this has become a controversial issue (15). National Diet and Nutrition Survey (NDNS) of the UK (16) reported the average daily intake of vitamin D for adults is 3.1 µg for men and 2.6 µg for women, respectively. The percentage contribution of egg is 13%, which is much less that one egg. Therefore, it is speculate the conception of 'egg limitation' is still continues to influence the public diet. If eggs are recommended as a source of vitamin D to the general population, it is important to determine whether there are any potential detrimental effects of the consumption. Some meta-analyses have reported that higher egg consumption was associated with increasing risk of coronary heart disease in diabetic patients, but the evidence are inconsistencies (17, 18).

Therefore we investigated the association between eggs and CVD events (Chapter 4) in two UK cohort studies, the CAPS and NDNS. Our findings in agreement with previous studies, there were no association between egg consumption and CVD events in the general health population over 20 years follow-up. However, our analysis is the first study to show higher egg consumption to be associated with increasing stroke and elevated fasting glucose in the sub-group of subjects with T2D and/or impaired glucose tolerance (IGT). In addition, cross-sectional analyses of CAPS and NDNS showed egg consumption was associated with higher blood glucose and HbA1c concentrations in a T2D and/or IGT sub-group. However, the potential mechanism by eggs could increase fasting plasma glucose and stroke in T2D and/or IGT subjects is unknown. With the limitation of epidemiology studies cannot prove causality and simply represent an association, it is therefore recommended that this should be explored further by performing RCTs to verify the relationship between egg consumption and CVD risk in T2D and/or IGT subjects. Nonetheless, results of current study recommend daily consumption of one egg in general population to increase vitamin D intake.

Vitamin D naturally enriched foods are few in number and in many cases not widely consumed (19), thus, vitamin D enriched foods or vitamin D fortified foods are important strategies which will help to facilitate sufficient vitamin D intake within the general population. As highlighted in the literature review in Chapter 2, there are several studies which have enriched foods with vitamin D through a food chain approach by feeding vitamin D_3 or 25(OH) D_3 supplements to poultry, which resulted in increasing vitamin D content in the eggs. However, feeding 25(OH) D_3 supplements to poultry only resulted in elevation of 25(OH) D_3 content of the egg yolk but not significant increase in the vitamin D_3 content (20), whilst feeding vitamin D_3 supplements to poultry would increase vitamin D_3 content in egg yolk much more than 25(OH) D_3 (21). Comparison of beneficial effect of vitamin D_3 and 25(OH) D_3 enriched eggs on human's serum 25(OH) D concentrations and health need further

RCT to verify. To my knowledge, only one RCT (22) has investigated the effect of vitamin D_3 and 25(OH) D_3 enriched eggs on serum 25(OH) D concentrations and showed both types of enriched eggs sustained serum 25(OH) D through the winter period compared with consumption of normal commercial eggs. However, studies on the chronic effect of vitamin D enriched eggs on vitamin D status or health outcome are lacking.

Milk and dairy products are consumed widely all around the world (23, 24) and which contributing a substantial amount and variety of nutrients (25).Thus, milk and dairy products are ideal foods for fortification, such as USA and Canada, as a strategy to address lower vitamin D status within the general population (26). However they are not available in all countries, including the UK, due to different food policies. As mentioned previously in relation to eggs, it is important to determine any potential detrimental effects of milk and dairy products on public health if they are to be used as a vehicle for vitamin D fortification. There is relatively consistent evidence that shows that dairy, particularly milk, consumption is associated with a no long-term effect on risk of CVD or mortality (27) with some studies reporting an inverse association with CVD risk (28, 29). However, a recent study (30), which included two large Swedish cohorts (61,433 women and 45,339 men) reported higher milk consumption to be associated with a doubling of all-cause mortality risk in the women and received considerable media attention. Therefore, an updated dose-response meta-analysis of all available published perspective cohort studies up to Sep 2016, including Michaelson et al. (30) was conducted (Chapter 6). No association between milk consumption and CVD was observed from the pooled data of 29 prospective cohort studies. Our results were in agreement with recent meta-analysis study of Larsson et al. (2015) (31) who also reported the neutral associations between milk and dairy intake with mortality or CVD mortality. Although the comprehensive meta-analysis, our results are limited by the observed heterogeneity of the pooled results. Therefore, RCT of investigating the effect of milk and dairy production

consumption on CVD event should be considered in the future to provide robust evidence. Nevertheless, our research results provide the evidence that milk and dairy products have neutral effect on CVD event, and which can be considered as suitable food for vitamin D fortification.

Vitamin D_3 has become the preferred form of vitamin D for fortification (32). A few previous studies (33-37), highlighted in the literature review in Chapter 2, reported that the vitamin D metabolite 25(OH) D_3, was more effective in raising vitamin D status, and was absorbed more rapidly than vitamin D_3. To address this issue further, we performed the study to investigate the effect of feeding dairy cows with 25(OH) D_3 compared with vitamin D_3 on vitamin D content of their milk (Chapter 7). The results showed bovine plasma increased significantly after feeding 25(OH) D_3 (not vitamin D_3), but vitamin D concentration in the milk is relatively low (mean 25(OH) D_3 concentration in milk was 0.88 µg/L). Consistent with the studies of Hollis et al. 1981 (38); Reeve et al. 1982 (39); McDermott et al. 1985 (40) and Weiss et al., 2015; (41) (as highlighted in Chapter 2, Table 3): vitamin D concentrations of the milk were not significantly increased by feeding cows with vitamin D supplements. Our results from this enrichment study illustrated that although it was possible to produce vitamin D enriched milk by a food chain approach, the absolute concentrations of vitamin D were insufficient to have any impact on the vitamin D status of the general population. Therefore, vitamin D fortification would seem a logical and more practical strategy to increase vitamin D content of milk or dairy products.

USA has fortified fluid 100 g milk with 42 IU vitamin D_3 and which has been become one of predominant food vehicles for vitamin D intake in USA and Canada, but Calvo et al. (26) indicated the amount of vitamin D added to milk may not be adequate to produce the sufficient 25(OH) D concentrations (26). As highlighted in Chapter 2, previous studies showed 25(OH) D_3 is highly effective in raising serum 25(OH) D level (42). Therefore, may 25(OH) D_3 fortified milk or dairy product is needed to increase vitamin D status. With

novelty as the first study to investigate the potential differential effects of 25(OH) D_3 and vitamin D_3 fortified dairy drink on vitamin D status and CVD risk markers, a double-blind, randomised, controlled acute human study (Chapter 8) was performed in 17 men with sub-optimal vitamin D status (mean (± SEM) plasma 25(OH) D_3: 32.8 ± 2.4 nmol/L). As expected, the 25(OH) D_3 fortified dairy drink was found to be more effective and faster at raising vitamin D status postprandially within 24 hours than the vitamin D_3 fortified dairy drink. Although novel, may the neutral results of the vitamin D fortified dairy drinks and cardiometabolic markers may due to the study is limited by the number of subjects which resulted in a lack of power to detect the significant difference of the secondary outcomes of cardiometabolic markers. In addition, the longer term effects of consuming a 25(OH) D_3 fortified dairy drink is unknown, which should be explored further.

In the UK, estimation from NDNS data suggesting 64-75% of adults consume semi-skimmed milk (16). However, due to lactose intolerance or low dairy product consumption in some individuals (e.g. vegetarian), other foods (such as bread or flour) should be considered as possible vehicles for vitamin D fortification to accommodate the food diversity. However, few studies have investigated if vitamin D is stable and bioavailable by adding into those foods.

Regarding the cost of the vitamin D supplementation, a study of Holick et al. (43) identified which strategy would be a cost-effective, however, there were no studies comparing the cost-effectiveness of vitamin D_3 fortification compared with 25(OH)D_3 fortified foods. More evidence on this is needed. Furthermore, apart from the general population, the effect of food fortification strategies for people who are at greatest risk of sub-optimal vitamin D status (e.g. dark-skinned and elderly subjects) should be explored.

Conclusions

This thesis has presented new and valuable epidemiological, animal and human studies on the role of eggs and dairy products in relation to vitamin D status and cardiovascular health. Novel findings that the vitamin D content of eggs is significantly affected by production system; association of higher egg consumption and increasing risk of stroke in subjects with T2D and/or IGT; an inverse association between vitamin D intake and plasma triacylglycerol level was demonstrated, as well as an updated dose-response meta-analysis of dairy consumption and CVD or mortality. Furthermore, the efficiency and faster effect of the vitamin D metabolic form 25(OH) D_3 in raising plasma 25(OH) D_3 in both dairy cows and humans were confirmed. Therefore to increase vitamin D dietary intake, one egg per day is recommended to a generally healthy population but not to subjects who have T2D and/or IGT. Additionally, the strategy of daily consumption of vitamin D fortified foods should be recommended to the general population, especially in winter and spring, to guarantee adequate vitamin D dietary intake.

Future research

The present studies have addressed a number of important research questions, while also highlighting some key opportunities for future research. In Chapter 3, whether vitamin D is associated with CVD events is a contentious topic with conflicting findings from the literature. In Chapter 4: with no prior longitudinal prospective evidence for higher egg consumption in increasing risk of stroke in T2D and/or IGT subjects. Both Chapter 3 and Chapter 4 are mainly based in same cohort of CAPS. As there are some limitations of the CAPS (highlighted in Chapter 3 and 4), further cohort studies with large subject numbers and both genders are needed to verify the findings in Chapters 3 and 4. In addition, updated meta-analysis of prospective studies on the association between egg consumption and CVD risk in T2D and/or IGT is needed in the future; also large RCTs are needed to verify the findings in relation to T2D and/or IGT.

A further area of research address in Chapter 6 was the meta-analysis of milk and dairy consumption and risk of cardiovascular disease and all-cause mortality. There is distinct lack of RCTs research into the impact of milk and dairy consumption on CVD events, thus, generating a considerable opportunity for future research. Intervention-based evidence has focused mainly on milk component (e.g. why protein), rather than milk as whole. Furthermore, milk and dairy products should be studies for particular effect of different dairy foods (e.g. high-/low-fat milk, yogurt, cheese) on CVD events.

The study presented in Chapter 5 was the effect of production system, supermarket and purchase date on the vitamin D content of the eggs at UK retail. Such kind of study is not possible to investigate the reasons behind the vitamin D variation of the eggs from different production system. Therefore, future research of collected eggs from different farm through the whole UK, even EU to investigate the effect of the production system on vitamin D content of the eggs, additional investigation of the effect of the hens feeding on vitamin D content of the eggs should be explored as well.

The study presented in Chapter 7 demonstrated supplemental 25(OH) D_3 is an effective means of enhancing 25(OH) D_3 concentration than vitamin D_3 supplementation to cows. However, vitamin D content of the milk was not significantly increased by either 25(OH) D_3 or vitamin D_3 supplementation, may future studies could explore the physiology reason behind this.

The beneficial effects of consuming dairy drinks with added 25(OH) D_3 or vitamin D_3 on vitamin D status is presented in Chapter 8. Further research including undertaking chronic RCT in a large, health subjects or subjects who have liver disease to compare the effect of consuming dairy drinks fortified 25(OH) D_3 with vitamin D_3 on raising vitamin D concentrations.

Furthermore, the amount of vitamin D added to milk and dairy products in the UK for public population to reach desired circulating 25(OH) D concentrations should be studied. For

people who are not milk or dairy consumers, the possible 25(OH) D_3 fortification of other staple foods (e.g. bread) could be explored with the aim of ensuring that a high proportion of the population achieved a satisfactory vitamin D status throughout the year. Finally, the stability of the vitamin D fortified foods in terms of processing and storage conditions should be explored to guarantee the vitamin D content of the fortification foods are in compliance.

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Appendix - 1

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Summer Meeting, 6–9 July 2015, The future of animal products in the human diet: health and environmental concerns

Egg consumption and cardiovascular disease events – evidence from the Caerphilly prospective cohort study

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Eggs are regarded as an economic and nutrient dense food, but they are also rich in cholesterol[(1)]. Limiting egg consumption is recommended as a strategy for LDL-cholesterol reduction, a key risk factor for cardiovascular disease (CVD). However, recent evidence suggests the effect of cholesterol in eggs to be negligible when compared with the impact of dietary saturated fatty acids[(2)]. A limited number of studies have investigated the effects of egg consumption on CVD using prospective cohort data, with inconsistent results. The present study investigated the prospective relationship between egg consumption and incidence of stroke, myocardial infarction (MI), heart failure (HF), and (any cause) mortality as well as cross-sectional relationships between egg consumption and metabolic risk markers using data from the Caerphilly Prospective Cohort Study.

Included in this cohort were 2,512 men, aged 45–59 years at baseline, who were followed up at 5 years intervals for a mean of 22·8 years. With adjustments for dietary and lifestyle variables, Cox regression and multiple linear regression analysis were used to examine the longitudinal and cross-sectional relationships, respectively. Furthermore, because earlier studies suggest that higher egg consumption will increase the risk of heart disease in diabetics but not in healthy individuals[(3)], separate analyses were completed using data from i) healthy men and ii) men with impaired glucose tolerance (IGT) and diagnosed diabetes (DM) at baseline.

	Stroke (adjusted)							
Egg intake	Healthy (n = 2036)		DM & IGT (n = 279)		DM (n = 72)		IGT(n = 254)	
(number/week)	HR	SE	HR	SE	HR	SE	HR	SE
0–1	1·00		1·00		1·00		1·00	
2	1·00	0·19	0·54	0·27	0·13	0·15	0·68	0·38
3	1·03	0·21	0·83	0·41	1·20	1·15	1·03	0·58
4–5	1·13	0·23	1·47	0·64	1·72	1·58	1·81	0·91
6–40	1·40	0·30	1·71	0·78	0·32	0·38	2·36	1·23
P for trend	0·100		0·028		0·729		0·009	

The findings from the longitudinal analysis suggest that weekly consumption of up to six eggs is unlikely to have a substantial impact on the risk of CVD or mortality (all cause) among healthy men (P for trend = 0·100). However, increased risk of stroke was associated with higher egg consumption among participants with IGT or suffering from diabetes (P for trend = 0·028). However, the cross-sectional analysis did not find any significant effect of higher egg consumption on the concentration of total cholesterol, HDL-cholesterol, LDL-cholesterol, triacylglycerol and insulin in venous blood from fasted subjects. Similarly there was no effect of egg consumption on systolic and diastolic blood pressure. The cross-sectional analysis did however indicate that consumption of up to six eggs per week may elevate fasting glucose concentration in subjects who had DM and IGT (P for trend = 0·017), but this was not found in the healthy population (P for trend = 0·664). Clearly the interaction between egg consumption and diabetes (DM and IGT) needs detailed study.

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Appendix - 2

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Effect of production system, supermarket and purchase date on the vitamin D content of eggs

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There is mounting evidence to show that vitamin D deficiency may increase the risk of many common and serious diseases, including osteoporosis, cardiovascular disease, some cancers and type 1 diabetes[(1)]. Hypovitaminosis D is prevalent in the UK general population. Due to lifestyle changes most people do not endogenously synthesise sufficient vitamin D from sunlight exposure[(2)]. Therefore, vitamin D intakes from dietary sources have become very important. Egg yolk is known to be a useful source of vitamin D yet very few studies have investigated the effect of production method on vitamin D content of UK hens' eggs. The purpose of this study was to explore the effects of production system, supermarket and time of the year on the concentration of vitamin D_3 and 25(OH)D_3 in UK hens' eggs at retail.

Eggs ($n = 259$) from free range, organic (also allows outdoor access) and caged (as identified on the label) production systems were purchased from three supermarkets (Asda, Tesco and Budgens) each month from July to November in 2012. The concentrations of vitamin D_3 ($n = 130$) and 25(OH)D_3 ($n = 129$) were analysed by HPLC/Tandem MS. Statistical analysis was undertaken using general linear model of ANOVA in Minitab 16·0 and the least square means for concentration of vitamin D_3 and 25(OH)D_3 in egg yolks according to production system (PS) and effect of supermarket (SM) and month (M) are shown in the table.

	PS				P for			
Vitamin D form (μg/kg)	Caged	Free range	Organic	SE	PS	SM	M	PS x SM
vitamin D_3	40·2[b]	57·2[a]	57·2[a]	3·10	<0·001	0·009	NS	<0·001
25(OH)D_3	13·0[b]	13·8[b]	16·1[a]	0·59	0·001	NS	<0·001	0·033

[a,b]different superscripts indicate significantly different means ($P < 0·05$); NS, not significant ($P > 0·05$).

Overall, concentrations of vitamin D_3 were similar to those of FSA[(3)] but lower than a more recent study[(4)]. The significantly higher vitamin D_3 concentrations in free range and organic eggs is presumably related to increased vitamin D synthesis by birds that have access to sunlight although why the effect on 25(OH)D_3 was only seen for organic eggs is unclear. The significant interaction effect of system and supermarket for both forms of vitamin D reflects inconsistencies in the ranking of vitamin D by production systems between supermarkets, perhaps indicating some incorrect labelling. The effect of month of purchase on 25(OH)D_3 reflected significantly higher values in July and September than in August (data not shown). Whether this is related to more sunshine hours is not known and there was no such effect on vitamin D_3.

In conclusion, these results confirm that eggs from outdoor production systems are likely to have higher vitamin D concentrations but this may not be a consistent effect in all supermarkets. Future work could expand the sampling number and research time through the whole year and match feeding schemes for the birds. Furthermore, genotype of birds that go outdoors may also be a factor.

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