

## Effects of low-fat dairy consumption on markers of low-grade systemic inflammation and endothelial function in overweight and obese subjects: an intervention study

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Although increased concentrations of plasma inflammatory markers are not one of the criteria to diagnose the metabolic syndrome, low-grade systemic inflammation is receiving large attention as a metabolic syndrome component and cardiovascular risk factor. As several epidemiological studies have suggested a negative relationship between low-fat dairy consumption and the metabolic syndrome, we decided to investigate the effects of low-fat dairy consumption on inflammatory markers and adhesion molecules in overweight and obese subjects in an intervention study. Thirty-five healthy subjects ( $BMI > 27 \text{ kg/m}^2$ ) consumed, in a random order, low-fat dairy products (500 ml low-fat milk and 150 g low-fat yogurt) or carbohydrate-rich control products (600 ml fruit juice and three fruit biscuits) daily for 8 weeks. Plasma concentrations of  $\text{TNF-}\alpha$  were decreased by 0.16 (SD 0.50) pg/ml ( $P=0.070$ ), and soluble  $\text{TNF-}\alpha$  receptor-1 (s-TNFR-1) was increased by 110.0 (SD 338.4) pg/ml ( $P=0.062$ ) after the low-fat dairy period than after the control period. s-TNFR-2 was increased by 227.0 (SD 449.0) pg/ml ( $P=0.020$ ) by the dairy intervention. As a result, the  $\text{TNF-}\alpha$  index, defined as the  $\text{TNF-}\alpha$ :s-TNFR-2 ratio, was decreased by 0.000053 (SD 0.00012) ( $P=0.015$ ) after the dairy diet consumption. Low-fat dairy consumption had no effect on IL-6, monocyte chemoattractant protein-1, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 concentrations. The present results indicate that in overweight and obese subjects, low-fat dairy consumption for 8 weeks may increase concentrations of s-TNFR compared with carbohydrate-rich product consumption, but that it has no effects on other markers of chronic inflammation and endothelial function.

### Low-fat dairy consumption: Inflammatory markers: Adhesion molecules

The metabolic syndrome is a metabolic disorder that strongly enhances the risk of developing CVD and type 2 diabetes mellitus. Abdominal obesity, atherogenic dyslipidaemia, hypertension, insulin resistance, a pro-thrombotic state and a low-grade pro-inflammatory state have now been identified as components of the metabolic syndrome that are related to CVD risk. Although inflammatory markers are currently not included in the ATP III or WHO diagnostic criteria for the metabolic syndrome<sup>(1)</sup>, low-grade systemic inflammation is receiving large attention as a metabolic syndrome component and cardiovascular risk factor. Inflammatory markers such as C-reactive protein<sup>(2)</sup>, IL-6<sup>(3)</sup>,  $\text{TNF-}\alpha$ <sup>(4)</sup> and fibrinogen<sup>(5)</sup>, among others, have been linked to the metabolic syndrome.

The consumption of dairy products has been inversely associated with the prevalence or incidence of the metabolic syndrome in a number of epidemiological studies<sup>(6–12)</sup>. In the Coronary Artery Risk Development in Young Adults Study<sup>(11)</sup>, for example, the intake of dairy products was negatively correlated with the development of obesity, dyslipidaemia, glucose intolerance and hypertension over the next 10 years in overweight subjects. However, the relationship

between dairy consumption and the chronic inflammatory state linked to the metabolic syndrome has not yet been studied in depth. Recently, Zemel & Sun<sup>(13)</sup> reported positive effects of dairy and Ca intakes on inflammatory markers, including  $\text{TNF-}\alpha$ , IL-6 and adiponectin, in mice. Moreover, they observed reduced plasma concentrations of C-reactive protein and increased concentrations of plasma adiponectin in obese human subjects after the consumption of a euenergetic or hypoenergetic high-dairy diet<sup>(13)</sup>. Therefore, in the present intervention study, we investigated the effects of low-fat milk and yogurt consumption on a broad range of inflammatory markers and adhesion molecules in overweight and obese human subjects.

### Subjects and methods

#### Subjects

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Medical Ethics Committee of Maastricht University. Written informed

**Abbreviations:** 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; En%, % energy; MCP-1, monocyte chemoattractant protein-1; s-TNFR, soluble  $\text{TNF-}\alpha$  receptor.

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consent was obtained from all the subjects. The study protocol has been reported in detail<sup>(14)</sup> previously. Briefly, forty male and female subjects were recruited in Maastricht and surroundings areas through advertisements in the local newspapers and in the University Hospital newsletter, and through posters in university and hospital buildings. During the screening visits, weight, height, waist circumference and blood pressure were measured. Two fasting blood samples, separated by a 3 d period, were taken for the determination of serum lipid and lipoprotein concentrations. Subjects were enrolled into the study when they met the following criteria: 18–70 years of age; BMI > 27 kg/m<sup>2</sup> or waist circumference > 88 cm (women) or > 102 cm (men); no active CVD, familial hypercholesterolaemia or other conditions that might interfere with the study outcomes; no pregnancy or breast-feeding; no abuse of alcohol or drugs; stable body weight during the past 3 months; and dairy (milk, yogurt and cheese (products)) < 500 g/d, as asked during the screening visits. Ten male and thirty female subjects were selected. Four subjects withdrew for personal reasons, and one subject was excluded from the analyses due to non-adherence to the protocol. Thirty-five subjects (ten males and twenty-five females, of which twelve were pre-menopausal and thirteen were post-menopausal) were used for the analyses. Subjects were asked not to change their dietary habits, level of physical exercise, alcohol intake, smoking habits or use of oral contraceptives during the study period.

#### Study design and intervention

The present study consisted of two intervention periods of 8 weeks, in a crossover design, separated by a washout period of at least 2 weeks. Subjects were randomly allocated to one of two treatment groups. The first group (*n* 17) consumed low-fat dairy products as a dietary supplement during the first intervention period, and carbohydrate-rich control products during the second intervention period, and for the second group of subjects (*n* 18), it was vice versa. The subjects maintained their habitual diet during the entire study. The dairy products consisted of 500 ml low-fat

(1.5 %, w/w) milk and 150 g low-fat (1.5 %, w/w) yogurt (Campina, Woerden, The Netherlands) per day. The control products consisted of 600 ml fruit juice (Refresco, Dordrecht, The Netherlands) and 43 g (three pieces) fruit biscuits (Verkade, Zaandam, The Netherlands) per day. The subjects received the products in daily packages, which they had to consume throughout the day. Total energy contents of the dairy and control products were similar (Table 1). At the end of each treatment period, energy and nutrient intakes during the previous 4 weeks were estimated using a validated FFQ<sup>(15)</sup>. Subjects had to record all signs of illness, use of medication or deviations from the study protocol in a diary.

#### Blood sampling and analyses

At the start of each treatment period, and after 4, 7 and 8 weeks, blood samples were taken after an overnight fast. Subjects were not allowed to consume alcohol during the previous day or to smoke on the morning before blood sampling. Venous blood was drawn into EDTA tubes using a Vacutainer system (Becton Dickinson, Franklin Lakes, NJ, USA). After sampling, the tubes were kept on ice and centrifuged within 1 h of venepuncture at 2500 *g* for 30 min at 4°C, and plasma samples were snap-frozen in liquid N<sub>2</sub> and stored at –80°C.

Samples collected at weeks 7 and 8 were pooled before the analysis. Plasma concentrations of TNF- $\alpha$  and IL-6 were determined using ELISA kits (R&D Systems, Abingdon, UK). ELISA kits were also used for the measurement of plasma concentrations of monocyte chemoattractant protein-1 (MCP-1) (Human MCP-1 Ultra-Sensitive Kit), soluble TNF- $\alpha$  receptors (s-TNFR) 1 and 2 (Human TNFR1 and TNFR2 Ultra-Sensitive Kit), intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 (Human Vascular Injury II Kit; Meso Scale Discovery, Gaithersburg, MD, USA). TNF- $\alpha$  index was calculated as (TNF- $\alpha$ )/(s-TNFR-2)<sup>(16)</sup>. To test subjects' compliance, plasma concentrations of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>) were determined by ELISA (Immunodiagnostic Systems, Boldon, UK). 1,25-(OH)<sub>2</sub>D<sub>3</sub> concentrations are expected to decrease when dietary

**Table 1.** Composition of dairy and control products (Mean values and standard deviations)

	Dairy			Control		
	Product content	Daily intake (%)		Product content	Daily intake (%)	
		Mean	SD		Mean	SD
Energy (MJ)	1.5	16.3	3.7	1.7	18.9	3.4
Protein (g)	24.4	24.0	5.0	2.8	3.3	0.7
Fat total (g)	9.8	12.8	3.6	3.8	5.5	1.4
SFA (g)	6.3	21.2	5.9	1.6	6.6	2.1
MUFA (g)	2.4	10.4	3.7	0	–	–
PUFA (g)	0.3	2.0	0.5	0.6	3.9	1.0
TFA (g)	0.4	23.6	14.5	0	–	–
Carbohydrates (g)	39.7	15.7	4.3	85.0	30.6	6.0
Mono- and disaccharides (g)	39.2	30.4	9.9	70.2	45.1	11.0
Fibre (g)	0	–	–	2.7	12.1	3.5
Cholesterol (mg)	27.5	13.7	3.7	0	–	–
Ca (mg)	800	53.2	9.2	75	9.0	3.1

TFA, *trans*-fatty acids.

Ca intake is increased<sup>(17)</sup>. Samples from one subject were analysed within the same run. All intra- and inter-assay variations were < 15 %.

### Statistics

The statistical power to detect a true difference of 10 % was more than 80 % for all parameters, except for IL-6. All statistical analyses were performed using SPSS 16.0 for Macintosh OS X (SPSS, Inc., Chicago, IL, USA). Differences in endpoints between dairy and control periods, which were normally distributed as indicated by the Shapiro–Wilk test, were examined by paired *t* test analysis. Values are presented as means and standard deviations and as absolute changes (95 % CI for absolute change). A *P* value < 0.05 (two-sided) was considered as statistically significant. The presence of time and sequence effects was tested as described<sup>(18)</sup>. The differences between men and women were also tested by an unpaired *t* test.

## Results

### Subjects, dietary intakes and compliance

Subjects were 49.5 (SD 13.2) years old, and their BMI was 32.0 (SD 3.8) kg/m<sup>2</sup>. Mean body weight at the end of the intervention periods was not different between the dairy diet (91.1 (SD 13.1) kg) and the control diet (91.3 (SD 13.5) kg; *P*=0.561).

The mean dietary intakes in the dairy and control periods were estimated from an FFQ. The exchange of low-fat dairy products for the carbohydrate-rich control products was reflected in the changes in the intakes of protein (19.9 (SD 3.2) v. 16.0 (SD 2.4) % energy (En%)), total fat (33.1 (SD 4.7) v. 29.9 (SD 4.9) En%), SFA (12.8 (SD 2.1) v. 10.7 (SD 2.1) En%), MUFA (10.3 (SD 1.9) v. 9.2 (SD 1.9) En%), carbohydrates (45.9 (SD 6.1) v. 52.5 (SD 5.8) En%), fibre (2.3 (SD 0.6) v. 2.6 (SD 0.7) g/MJ), cholesterol (23.3 (SD 5.6) v. 19.7 (SD 4.5) mg/MJ) and Ca (1550 (SD 281) v. 931 (SD 291) mg) (all *P*<0.05). Total energy intake was not different between the dairy and control interventions.

Plasma concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub> were significantly lower at the end of the dairy period (119 (SD 30) pmol/l) than at the end of the control period (128 (SD 37) pmol/l; *P*=0.034).

### Inflammatory markers and adhesion molecules

Concentrations of plasma IL-6 were not different between the dairy and control periods (Table 2), while concentrations of TNF-α tended to be lower after dairy diet consumption (*P*=0.070). Concentrations of s-TNFR-1 tended to be higher after dairy diet consumption (*P*=0.062), and concentrations of s-TNFR-2 were significantly higher after the dairy diet consumption than after the control diet consumption (*P*=0.020). Although the change in s-TNFR-1 was not statistically significant, it was correlated with the change in s-TNFR-2 (Pearson *r* 0.692, *P*<0.001). Calculated TNF-α index was lower after dairy consumption than after control consumption (*P*=0.015). Dairy consumption had no effect on plasma concentrations of MCP-1, intracellular adhesion molecule-1 and vascular cell adhesion molecule-1.

No time or sequence effects were present, and responses did not differ between men and women.

## Discussion

Data from the present study indicate that low-fat dairy consumption for 8 weeks may affect markers reflecting low-grade systemic inflammation in overweight and obese subjects. We found a significant increase in plasma s-TNFR-2 concentrations after low-fat dairy consumption, while there was a trend towards higher s-TNFR-1 and lower TNF-α concentrations. Subjects' compliance was confirmed by the expected decrease in plasma concentrations of 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

Elevated concentrations of TNF-α have been found to be related to obesity, insulin resistance and the metabolic syndrome<sup>(19,20)</sup>. An enlarged adipose tissue mass increases the production of TNF-α, which may in turn cause insulin resistance by affecting signalling pathways in different organs. Although animal studies have established TNF-α as a link between obesity and insulin resistance<sup>(21,22)</sup>, evidence from human studies is less conclusive. Reduced insulin-induced glucose uptake after TNF-α infusion has been shown in healthy subjects<sup>(23)</sup>. Furthermore, the use of anti-TNF-α drugs in inflammatory conditions induced a concomitant improvement in insulin sensitivity in several human trials<sup>(24–26)</sup>, whereas no beneficial effects of TNF-α neutralisation on insulin sensitivity were found in other studies<sup>(27–29)</sup>. Furthermore, the function of s-TNFR (1 and 2) is not yet fully

**Table 2.** Effects of dairy consumption on inflammatory markers and adhesion molecules

(Mean values, standard deviations and 95 % confidence intervals)

	Dairy		Control		Change		95% CI	<i>P</i>
	Mean	SD	Mean	SD	Mean	SD		
s-TNFR-1 (pg/ml)	3729	853	3619	769	110.4	338.4	–5.77, 226.7	0.062
s-TNFR-2 (pg/ml)	4367	1359	4140	1122	227.0	549.0	38.4, 415.6	0.020
TNF-α (pg/ml)	2.32	0.64	2.48	0.76	–0.16	0.50	–0.33, 0.01	0.070
TNF-α index	0.00057	0.00021	0.00062	0.00020	–0.000053	0.00012	–0.000096, –0.000011	0.015
IL-6 (pg/ml)	3.01	3.47	3.05	3.24	–0.04	1.46	–0.54, 0.46	0.875
MCP-1 (pg/ml)	291.1	68.5	289.7	66.5	1.45	29.4	–8.64, 11.5	0.772
ICAM-1 (ng/ml)	280.5	78.6	286.9	86.6	–6.43	27.7	–15.9, 3.07	0.178
VCAM-1 (ng/ml)	447.7	110.0	436.1	103.1	11.6	76.4	–14.7, 37.8	0.377

s-TNFR, soluble TNF-α receptor; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intracellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

understood. Elevated concentrations of s-TNFR have been associated with obesity<sup>(30,31)</sup>, and weight loss has been found to decrease TNF- $\alpha$  and increase s-TNFR concentrations<sup>(32)</sup>. The membrane-bound forms of the two TNF receptors activate different intracellular pathways upon TNF- $\alpha$  binding, facilitating its physiological effects<sup>(33)</sup>. On the contrary, circulating TNF receptors are able to compete for TNF- $\alpha$  binding with the cell surface receptors, and have been proposed to function as inhibitors of TNF- $\alpha$  action. Through the formation of high affinity complexes and subsequent reduction of the amount of active TNF- $\alpha$ , they might protect against the potentially harmful effects of TNF- $\alpha$ <sup>(34,35)</sup>. Illustratively, a dimeric recombinant form of s-TNFR-2, known as etanercept, is often used in inflammatory conditions such as rheumatoid arthritis and psoriasis, and has been shown to improve inflammatory conditions in patients with the metabolic syndrome<sup>(27)</sup>. Our data show increased concentrations of s-TNFR-2 after low-fat dairy consumption, which might imply lower biological availability of TNF- $\alpha$  protein. In fact, when we calculated the TNF- $\alpha$  index, a measure for biologically available TNF- $\alpha$ <sup>(16)</sup>, we found reduced numbers after the dairy intervention. Thus far, the effect of dairy products on the TNF- $\alpha$  pathway in human subjects has not been explored. Experiments in mice have indicated that Ca and dairy products may reduce TNF- $\alpha$  production<sup>(13,36)</sup>, but effects in human subjects have not been studied before. The present results might imply beneficial effects of low-fat dairy consumption on TNF- $\alpha$  action, but the precise consequences of these observations have to be examined further. It might be interesting for future research to study the effects of dairy intake on the activity, besides the concentration, of TNF- $\alpha$  and related parameters, since signalling from the TNF- $\alpha$  receptor has been found to be modulated by Ca-dependent proteins<sup>(37)</sup>.

Other inflammatory markers and adhesion molecules, however, were not affected by dairy consumption. Studies addressing the effects of dairy products or their constituents on inflammation or endothelial function are scarce. Wennersberg *et al.*<sup>(38)</sup> studied the effects of 6-month dairy consumption in overweight men and women, and found no differences in the markers of inflammation (IL-6, C-reactive protein and TNF- $\alpha$ ) and endothelial dysfunction (E-selectin and von Willebrand factor), except for a decrease in vascular cell adhesion molecule-1, which was only present in women. Zemel & Sun<sup>(13)</sup> reported reductions in plasma TNF- $\alpha$  and IL-6, and an increase in plasma adiponectin in mice fed a high-dairy diet. They also evaluated samples from obese men and women who followed a high-dairy euenergetic or hypoenergetic diet for 4 weeks. Compared with a low-dairy group, they observed decreased concentrations of C-reactive protein and increased concentrations of adiponectin consumption of high-dairy diets. Although the effects of an improved body composition cannot be fully excluded, they also suggested a role for the suppression of 1,25-(OH) $_2$ D $_3$ . In previous *in vitro* experiments, they showed that 1,25-(OH) $_2$ D $_3$  stimulated TNF- $\alpha$  and IL-6 expression<sup>(39,40)</sup>. On the contrary, other *in vitro* and animal studies provide evidence that 1,25-(OH) $_2$ D $_3$  has anti-inflammatory properties<sup>(41–43)</sup>. In the present study, concentrations of 1,25-(OH) $_2$ D $_3$  were measured as marker of dietary compliance and were indeed reduced by dairy consumption, but the role in the modulation of the TNF

pathway remains to be elucidated. Recently, Zemel *et al.*<sup>(44)</sup> showed that a euenergetic dairy-rich diet reduced inflammatory markers (IL-6, TNF- $\alpha$  and MCP-1) and increased adiponectin in overweight and obese subjects than a soya-rich diet, in the absence of changes in adiposity. Effects were already present after 7 d of intervention, and were even more pronounced after 28 d. The present results suggest that effects on TNF- $\alpha$ -related parameters are still present after an 8-week intervention period. However, whether these changes are present for a longer period needs further study. Furthermore, unlike Zemel *et al.*<sup>(44)</sup>, we observed no effects on IL-6 and MCP-1, for which we have no obvious explanation.

Taken together, the present results indicate that low-fat dairy consumption for 8 weeks, compared with carbohydrate-rich product consumption, may modulate TNF- $\alpha$  signalling by increasing s-TNFR-2, but that it does not affect other markers of low-grade systemic inflammation and endothelial function in overweight and obese subjects.

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