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Consumption of protein-enriched milk has minor effects on inflammation in older adults - a 12-week double-blind randomized controlled trial

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Highlights

- The consumption of a low-fat, protein-enriched milk had minor effects on inflammatory related markers after 12 weeks of intervention in older subjects compared to an isocaloric intake of carbohydrate.
- Significant differences between study groups were observed in the mRNA expression levels of *NR1H3* and *INFG*.
- The mRNA level of *TNFRSF1A* was significantly reduced, while the mRNA level of *DPP4* was significantly increased in the control group, with no differences between groups.
- The serum level of sTNFRSF1A increased significantly in both groups after the intervention, while the serum level of TNFα increased significantly in the control group only. No differences were observed between groups.

Abstract

Introduction: Aging is associated with increased levels of circulating inflammatory markers and reduced muscle mass and strength.

Objective: We investigated whether intake of protein-enriched milk for 12 weeks would influence markers of inflammation among adults ≥ 70 years of age with reduced physical strength.

Methods: In a double-blind randomized controlled intervention study, subjects were randomly allocated into two groups, receiving a protein-enriched milk (2 x 20 g protein/d, n=14, mean (±SD) age 76.9 ± 4.9 yrs) or an isocaloric carbohydrate drink (n=17, age 77.7 ± 4.8 yrs) for 12 weeks. We measured serum and mRNA expression levels of inflammatory markers in PBMCs.

Results: Significant differences in the mRNA expression of *nuclear receptor subfamily, group H, member 3* (*NR1H3,* encoding the LXRα transcription factor*)* and *interferon gamma* (*INFG)* were observed between groups. The mRNA level of *TNFRSF1A* was significantly reduced, while the mRNA level of *dipeptidyl-peptidase 4* (*DPP4)* was significantly increased, in the control group. The serum level of TNFα increased significantly in the control group, while sTNFRSF1A increased significantly in both groups, but with no significant differences between groups.

Conclusion: Consumption of a low-fat, protein-enriched milk for 12 weeks had minor effects on inflammatory related markers in older adults compared to an isocaloric carbohydrate drink.

Key words: milk protein, older adults, high-protein, inflammation, peripheral blood mononuclear cells, mRNA.

Highlights

- The consumption of a low-fat, protein-enriched milk had minor effects on inflammatory related markers after 12 weeks of intervention in older subjects compared to an isocaloric intake of carbohydrate.
- Significant differences between study groups were observed in the mRNA expression levels of *NR1H3 (the gene encoding LXRα)* and *INFG*.
- The mRNA level of *TNFRSF1A* was significantly reduced, while the mRNA level of *DPP4* was significantly increased in the control group, with no differences between groups.
- The serum level of sTNFRSF1A increased significantly in both groups after the intervention, while the serum level of TNFα increased significantly in the control group only. No differences were observed between groups.

Abbreviations list

B2M, beta-2-microglobulin; BMI, body mass index; CCL, chemokine (C-C motif) ligand; cDNA, complimentary deoxyribonucleic acid; CXCL16, chemokine (C-X-C motif) ligand 16; DPP4, dipeptidyl-peptidase 4; DXA, dual energy X-ray absorptiometry; g, gravity; Gadd45, growth arrest and DNA-damage-inducible, alpha; hs-CRP, high sensitive C-reactive protein; IFNG, interferon gamma; IGF1, insulin-like growth factor 1; IL, interleukin; IL1RN/IL1Ra, interleukin 1 receptor antagonist; liver X receptor alpha, LXRα; mRNA, messenger RNA; NF-kB, nuclear factor kappa-light-chain-enhancer of activated B cells; NR1H3, nuclear receptor subfamily, group H, member 3; PBMC, peripheral blood mononuclear cells; PDK4, pyruvate dehydrogenase kinase, isozyme 4; qPCR, quantitative polymerase chain reaction; rpm, rounds per minute; Runx2, runt-related transcription factor 2; sTNFRSF1A, soluble tumor necrosis factor receptor 1; TBP, TATA box binding protein; TLDA, TaqMan Low-Density array; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha; TNFSF11, tumor necrosis factor ligand superfamily member 11; TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TNFRSF11A, tumor necrosis factor receptor superfamily, member 11A

Introduction

The risk of developing chronic diseases is increased in older adults (1-5), and an elevated level of circulating inflammatory markers are often observed (3, 6). Growing evidence indicates that inflammatory markers, such as high-sensitive C-reactive protein (hs-CRP), interleukin 6 (IL6), and tumor necrosis factor alpha (TNFα), are associated with loss of muscle mass and muscle strength (7-15), possibly contributing to the development of sarcopenia (13, 16, 17).

Dietary strategies to prevent the onset of chronic, low-grade inflammation are therefore suggested to improve several health outcomes and to prolong longevity among elderly (3, 6, 18, 19). Foods, including fruits and vegetables, fish, whole grains and some vitamins, are shown to exert anti-inflammatory effects (2). The effects of dairy products on inflammatory markers are less clear (20). Some epidemiological evidence indicates that low-fat dairy products are inversely associated with the level of inflammatory markers among healthy adults (21-23), but the data are inconclusive (24). In randomized controlled trials a reduction in the circulating levels of TNFα, IL6 and chemokine (C-C motif) ligand 2 (CCL2) have been shown after consuming dairy products in obese subjects (25, 26), but the data are not conclusive (27, 28). A reduction of the same inflammatory markers have been observed in subjects with the metabolic syndrome (29), but not in healthy adults (30, 31), nor in adult subjects with an elevated level of hs-CRP (32) after consuming dairy products.

High protein diets have increased in popularity, and these are widely used in combination with weight reduction (33) and in sports nutrition (34) to preserve muscle mass and promote muscle strength. Few have examined the possible effects of high protein diets on risk factors for chronic diseases (24, 35-37), among them level of inflammatory markers (38, 39). No negative effects on inflammatory markers are observed (38, 39), but long-term clinical studies are scarce (38-40).

In a double-blind randomized controlled intervention study, we investigated whether an increased daily intake of a low-fat, protein-enriched milk could alter markers of inflammation in peripheral mononuclear blood cells (PBMCs) and in serum among community dwelling elderly men and women above the age of 70 years with reduced muscle strength and functional performance.

Materials and methods

Study population and study design

The present study is part of a research project were men and women (≥ 70 years) living at home were recruited to a 12 week double-blind, randomized controlled intervention trial, conducted from August 2014 to September 2015 at Oslo and Akershus University College of Applied Sciences, Norway. The primary aim of this study was to investigate the effect of increased intake of protein-enriched milk on muscle mass and physical strength. A detailed description of participant recruitment, enrollment, selection criteria, and compliance are given elsewhere (41). In brief, 2820 subjects were invited to participate in the study. 438 subjects met to screening of which 388 were excluded. Thus, 50 older subjects, with either reduced hand grip strength (< 20 kg in women and < 30 kg in men), gait speed < 1 m/s, timed step stair test ≥ 8.4 s or timed five times sit to stand test > 12.5 s, and otherwise weight stable and apparently healthy, were randomized. Among the exclusion criteria were a Mini-Mental State Examination score < 24, a Mini Nutritional Assessment score < 17 and high intakes of dairy products (≥ 4 dl/day of milk, cultured milk and/or yoghurt). In total, 36 subjects completed the study. The intervention group received a protein-enriched milk (n= 17, 2 x 0.4 L/d; 2 x 20 g protein/d), whereas the control group received an isocaloric carbohydrate drink (n=19, 2 x 0.4 L/d), for 12 weeks. The subjects consumed the test drinks together with breakfast and the evening meal, and they were encouraged to maintain their habitual diet and physical activity level throughout the study period.

All subjects provided written informed consent, and we conducted the study according to the Declaration of Helsinki. We received approval for all procedures involving human subjects by the Regional Committees for Medical and Health Research Ethics, Health Region South East, Norway. The study was registered at Clinicaltrials.gov (ID no. NCT02218333).

Study products

TINE SA (Oslo, Norway) produced and provided the protein-enriched milk and the isocaloric control drink. The protein-enriched milk contained on average 5.0% protein, 4.6% carbohydrates, < 0.1% fat and provided approximately 167 kJ (39 kcal)/100 g. The control drink was prepared from carbohydrates (sugar, xantan gum and Maltosweet$^{TM®}$). To give the control drink a milky appearance the producer added titandioksid (E171). Both drinks contained approximately 178 mg/100 g of calcium.

Blood sampling and sample preparation

Venous blood samples were collected after an overnight fast (≥ 12 h) in BD Vacutainer® CPT™ cell preparation tubes with sodium heparin (Becton Dickenson, NJ, USA) and in silica gel tubes (Becton Dickenson Vacutainer Systems, Plymouth, UK) at baseline and after 12 weeks. Within two hours of blood collection PBMCs were collected by density gradient centrifugation of the blood samples (1636 *g*) for 25 min at room temperature (RT). The cells were washed twice (300 *g*, 10 min at RT) in phosphate-buffered saline (PBS) without calcium chloride and magnesium chloride. After the last washing step, excess PBS was discarded. The pellet was dissolved in the remaining liquid and transferred to an Eppendorf tube, centrifuged (13000 *g*, 3 min at 4°C) and frozen at -80°C until further analysis. Serum samples were centrifuged (1500 *g*, 15 min at RT) after being left on the bench top for at least 30 min. Serum sample for the determination of cytokines were frozen at -80°C until further analysis. Serum samples for the determination of hs-CRP, and EDTA-blood for the differential blood count, were sent to an accredited laboratory (Fürst Laboratories, Oslo, Norway) for further analysis.

Isolation of RNA

mRNA was isolated from thawed PBMCs using QiaCube from QIAGEN GmbH (Germany) in accordance with the protocol RNeasy Mini Kit with qiashredder and DNase digest (QIAGEN). Thirty µl high-quality mRNA was eluted in RNase free water and frozen at -80 °C until further analysis.

RNA quantity was measured using NanoDrop-1000 (NanoDrop Technologies, Inc., Delaware, USA), while RNA quality was checked with Aglient 2100 Bioanalyzer (Agilent Technologies, Inc., California, USA). All PBMC samples included in further analysis had a RIN-value above 9. One participant was excluded from further analysis due to low mRNA content in one of the PBMC samples.

Synthesis of cDNA

Complementary DNA (500 ng) was made using a RNA to cDNA kit from Applied Biosystems (Applied Biosystems, UK) in accordance with the manufacturer's protocol. Samples were stored at -20 °C for further analysis.

RNA analysis/Real-time qPCR

We analyzed mRNA levels of 48 genes using TaqMan Low-Density array (TLDA) cards from Applied Biosystems, UK (see supplementary table 1 for an overview of all genes analyzed). TLDA cards were run on a 7900 HT Applied Biosystems RT-qPCR machine (Applied Biosystems, UK). The cycle threshold values (Ct-values) were determined using SDS 2.4 (Applied Biosystems) and ExpressionSuite Sofware v1.0.3 (Applied Biosystems, UK). We normalized the Ct-values to *TATA box binding protein* (*TBP*) and *beta-2-microglobulin* (*B2M*) mRNA transcripts, and manually calculated relative changes in mRNA expression levels at baseline and at the end of the intervention ($2^{-\Delta Ct}$). Fold changes in mRNA transcripts from baseline to end of intervention were calculated by dividing $2^{-\Delta Ctend}$ with $2^{-\Delta Ctbaseline}$, using the $2^{-\Delta\Delta Ct}$-method (42).

CRP and cytokine measurements

The serum levels of interleukin 10 (IL10), interferon gamma (IFNG) and TNFA were measured using a Magnetic Luminex Performance Assay (R&D Systems Inc., Minneapolis, USA) in accordance with the protocol provided. IL6 and sTNFRSF1A were analyzed using Quantikine ELISA (R&D Systems Inc., Minneapolis, USA) in accordance with the protocols provided. The levels of IL10 and IFNG were below the detection limit in the multiplex analysis. The inter-individual variation (CV) for IL6, TNFα and sTNFRSF1A were 6.5, 10.2 and 6.7%, respectively. All samples were measured in duplicates.

Statistics

Subjects were stratified by gender and smoking prior to a 1:1 block-randomization. Power calculations were made on the primary outcome of the study (muscle mass), as described elsewhere (41). In all analysis, subjects with levels of hs-CRP above >10 mg/L at baseline (n=3) or at end of study (n=1) were excluded as such levels may indicate an ongoing inflammation. In total, samples from 14 participants in the protein group and 17 participants in the control group were analyzed.

We used non-parametric tests for statistical analysis of data not normally distributed. Mann-Whitney-test was used to determine possible differences between the study groups, while Wilcoxon signed-rank test was used when investigating possible differences in variables between end of study and baseline within one study group. For normally distributed data, differences between study-groups at baseline were performed by the independent samples t-test. The Spearman correlation test was used to reveal possible correlations between the

change in muscle mass and physical strength (chest press) with TNFA and sTNFRSF1A. Due to an explorative study design, correction for multiple testing was not performed. We considered a p-value of < 0.05 statistically significant. SPSS statistical software, version 22 from Microsoft (SPSS, Inc., USA), was used for statistical calculations.

Results

Table 1 shows baseline characteristics of the participants. There were no significant differences in gender, age, muscle mass, fat mass or BMI at baseline between the two study groups. As previously reported by Ottestad and colleagues (41), increased protein intake daily for 12 weeks did not significantly improve muscle mass, muscle strength or functional performance in healthy older and weight stable adults.

Effects of protein-enriched milk on mRNA expression in PBMCs

In the present study, we analyzed mRNA levels of inflammatory markers in PBMCs in order to test if an increased intake of a low-fat, protein-enriched milk daily would alter these markers in adults > 70 years, when compared to an isocaloric intake of carbohydrates. We found that the change in mRNA levels of *nuclear receptor subfamily, group H, member 3* (*NR1H3),* the gene that encodes for liver X receptor alpha (LXRα), and *IFNG* were significantly different between the two study groups after 12 weeks, whereas we observed no within-group changes for these genes in either of the two study groups (figure 1, panels A and B). In contrast, the mRNA level of *TNFRSF1A* decreased significantly (figure 2, panel A), whereas the mRNA level of *dipeptidyl-peptidase 4 (DPP4)* increased significantly (figure 2, panel B) in the control group after 12 weeks.

The mRNA levels of inflammatory genes, such as *IL6, IL1β, IL18, TNFα, IL10* and *interleukin 1 receptor antagonist (IL1RN)* did not change significantly neither between, nor within the two study groups (supplementary tables 2 and 3).

In addition to inflammatory related genes, we investigated possible changes in genes involved in the regulation of muscle mass. We observed no significant changes in mRNA levels of *runt-related transcription factor 2 (Runx2), growth arrest and DNA-damage-inducible alpha (Gadd45), tumor necrosis factor receptor superfamily member 11A (TNFRSF11A), tumor necrosis factor ligand superfamily member 11 (TNFSF11) or insulin-like growth factor 1 (IGF1)* neither between, nor within the two study groups (supplementary tables 2 and 3).

Finally, we investigated possible changes in the distribution of cell types within the PBMC samples at baseline and end of study to be able to control for possible changes in the distribution of cell types. However, no differences were found in the distribution of cell types after the intervention period (supplementary table 4).

Effects of protein-enriched milk on serum levels of inflammatory markers

Based on the results from the mRNA analysis, we analyzed the effects of protein-enriched milk consumption on serum levels of sTNFRSF1A, TNFA and IL6. In contrast to the mRNA data, we found that the serum level of sTNFRSF1A increased significantly in both study groups (p=0.022 in the protein group, p=0.009 in the control group), whereas the serum level of TNFα increased in the control group (p=0.03), but with no statistically significant differences between the two study groups. The serum levels of IL6, hs-CRP and the ratio TNFα/sTNFRSF1A were not significantly different, neither within nor between the two study groups (table 2).

We have previously reported that combining the two study groups, a significant improvement in muscle mass and chest press was observed from baseline to end of study (41). In the present study, we observed a significant increase in serum levels of TNFA (p=0.001) and sTNFRSF1A (p=0.049) from baseline to end of study when combining the two study groups (n=31). However, we observed no relationship between the serum level of TNFA and muscle mass, r=-0.12 (p=0.54), or chest press, r=-0.13 (p=0.49) in the groups combined (n=31). Nor did we observed any relationship between changes in the serum level of sTNFRSF1A and muscle mass, r= 0.16 (p=0.40) (n=) or chest press, r=0.15 (p=0.43) in the groups combined (n=31).

Discussion

In the present study, we explored the impact of adding a daily intake of low-fat, high-protein milk to the regular diet of community-dwelling subjects > 70 years of age with reduced muscle strength and/or physical function, for 12 weeks, on inflammatory markers. Our data indicate that the intake of this milk had minor effects on mRNA expression and circulating inflammatory markers, compared to an isocaloric control drink containing carbohydrates only.

In the majority of studies where possible effects of consuming dairy products (25, 27, 28, 43), or high-protein diets (38, 39, 44-46) on circulating inflammatory markers were investigated, no significant effects on circulating markers, such as hs-CRP, IL6 or TNFα were observed, supporting the results of the present study. We did observe a significant increase in the serum level of TNFα, but only in the control group receiving carbohydrates, indicating an increased TNFα activity in the control group. The serum level of sTNFRSF1A increased significantly in both groups after the intervention with no statistically significant changes between the two groups. The increased level of sTNFRSF1A in the protein group is in accordance with Van Meijl and colleagues, who also found an increased level of sTNFRSF1A after providing overweight subjects (n = 35, mean age 49.5 yrs) with low-fat dairy products (500 ml milk and 150 g yoghurt, equals ~ 24 g protein, 1.5 MJ/day) daily for eight weeks. In contrast, they did not observe this effect in the control group who received 600 ml fruit juice and three fruit biscuits daily (1.7 MJ/day) (25). Van Meijl and colleagues suggested that these effects may be related to the content of calcium, as calcium has been shown to enhance the TNFα-signaling in human embryonic kidney cells (47). In the present study, the calcium content was similar in the two drinks, suggesting that this effect may be independent of calcium.

Elevated circulating levels of hs-CRP, TNFα, IL6 and sTNFRSF1A have been associated with a decline in muscle mass (8-10, 12) and increased mortality in older adults (48, 49), but few randomized controlled trials have been performed investigating possible effects of consuming protein with the level of inflammatory markers in combination with muscle mass and muscle strength (13, 50). Lower rates of muscle protein synthesis have also been associated with an upregulation of the NF-κB pathway, a key regulator of inflammation (13). Both TNFA and IL6 may activate the NF-κB pathway (2, 51). Bartali and colleagues found an association between a low protein intake, high levels of TNFα, IL6 and CRP and muscle strength in older subjects (13). We have previously reported that the additional intake of 40 g protein or an isocaloric carbohydrate drink daily increased chest press after 12 weeks of intervention, in the

groups combined (41). We therefore investigated possible correlations between changes in muscle mass and chest press with changes in circulating levels of TNFA and sTNFRSF1A. However, we observed no correlations between these parameters. In contrast to the subjects included in the study of Bartali and colleagues (13), subjects included in the present study already had a protein intake within the recommended levels (52) at baseline (1.0 ± 0.3 g) (41). This may be one explanation for observing no associations between changes in muscle mass, chest press and circulating levels of TNFα or sTNFRSF1A when additional protein was provided throughout the day.

After the intervention period, we observed a statistically significant difference in mRNA expression levels between the two study groups for two out of the 44 genes analyzed; *NR1H3* and *INFG*. The mRNA levels of both *NR1H3* and *INFG* increased slightly in the protein group, with the opposite effect in the control group (figure 1, panel A and B). LXRα plays a central role in the transcriptional regulation of metabolism and inflammation (53), and LXRα activation has been shown to reduce inflammation by decreasing the level of Th-1 cytokine release, among them the pro-inflammatory cytokine INFG, in stimulated T-cells (54). Alternations in the endogenous regulatory system for LXRα in immune cells may potentially lead to immune related diseases (55). LXRα also have an important role in promoting the elimination of excessive cholesterol from the cell (53, 55). In HepG2 cells, an upregulation of the mRNA expression level of *NR1H3* has been observed after supplementation with branched chained amino acids (BCAA), suggesting that BCAA may improve glucose metabolism (56). INFG coordinates a diverse array of cellular programs within the immune system, and it is believed to induce a Th-1 cytokine release (57). In the present study, expression of *NR1H3* does not seem to suppress the expression of *INFG* as previously shown in stimulated T-cells (54) as the mRNA levels of *INFG* increased in the protein group, but decreased in the control group. Based on the present results and lack of human studies, it is difficult to interpret the physiological meaning of the observed differences in the present study. To our knowledge, few, if any, have previously investigated the effects of long-term dairy protein or high-protein diets on the mRNA expression levels of *NR1H3* or *INFG* in healthy, elderly humans.

In the protein group, we found no statistically significant changes in mRNA levels of any of the genes analyzed. These results are in accordance with data presented by Labonté and colleagues, who found no differences in mRNA expression levels of *CCL2, IL18, IL6, IL1β* or *TNFA* in whole blood after consuming three servings of dairy products daily, compared to an

isocaloric amount of fruit- and vegetable juices, cashews and cookies (32). Fifty-three subjects with low-grade systemic inflammation were included in this randomized controlled trial (32). Van Loan and colleagues (27) also performed a randomized controlled trial, investigating possible effects of a high vs a low intake of dairy products daily (4 vs 0.5 serving/day). Seventy-one obese subjects were included in that study where they observed no changes in mRNA levels in adipose tissue of *TNFα, IL6, CCL2, IL1β, IL10* or *IL15* after the intervention period, supporting our mRNA data. Low-fat milk, yoghurt and full-fat cheese were provided in these studies (27, 32).

In the control group, we observed a ~0.6 fold downregulation of *TNFRSF1A* mRNA expression (figure 2, panel A) and a ~1.3 fold upregulation of *DDP4* mRNA expression (figure 2, panel B), with no significant differences between the two groups. The upregulation of *DDP4* may be related to the increased intake of carbohydrate in the control group. Simple carbohydrates may increase the activation of the NF-κB pathway (2, 51), and both TNFRSF1A and DDP4 are able to induce the transcription of inflammatory genes through this pathway (49). However, we observed no changes in target genes of the NF-κB pathway (58), such as *IL6, TNFα, IL8, CCL2* and *chemokine (C-C motif) ligand 3* (*CCL3),* in the control group. The down-regulation of *TNFRSF1A* gene expression was in contrast to the increased serum level of sTNFRSF1A. Many other tissues (e.g. endothelial cell, adipose tissue and tissue macrophages) than PBMCs, may also contribute to the circulating levels of inflammatory markers, making the comparison between serum and PBMC levels difficult.

There are several methodological differences among the studies where intakes of dairy products/high-protein diets and potential implications on health are investigated, possibly explaining some of the conflicting results. The above-mentioned studies were performed in different groups (subjects with low-grade inflammation, the metabolic syndrome or obese subjects), different products were provided in the control groups, different levels and sources of protein were given and the metabolic status of the subjects included differed (39, 59-61). In addition, dairy products are a heterogeneous group of foods that may exert different health effects. Most dairy products contain high levels of calcium, which is a component suggested to be able to suppress inflammation by inhibiting the production of inflammatory substances from adipose tissue through the inhibition of calcitriol (27, 29, 62). In the present study, we supplied an equal amount of calcium to both groups, in an attempt to nullifying such an effect.

This may be a possible explanation for the lack of differences observed between the two study groups.

The major limitations of the present study were the few subjects included in the final analysis (n=31), that only a selection of markers known to be involved in chronic-low grade inflammation were analyzed, and that we measured the selected markers at mRNA level only. Moreover, the present study did not include measurement of postprandial gene expression levels, which could have provided important insight into the acute response to the consumption of dairy protein compared to carbohydrates. Major strengths were the double-blind randomized controlled design, the strictly controlled methodology, the isocaloric composition of the test products and the very high compliance to the experimental procedure of the subjects completing the study.

Conclusion

In the present study, we investigated possible effects of consuming a low-fat, protein-enriched milk, compared to an isocaloric carbohydrate drink, in older adults with reduced physical strength and/or functional performance, on mRNA levels in PBMCs and serum levels of selected inflammatory markers. Altogether, the results from the present study indicate that consuming low fat, protein-enriched milk for 12 weeks had minor effects on these markers, compared to an isocaloric control drink.

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References

1. Chung HY, Lee EK, Choi YJ, Kim JM, Kim DH, Zou Y, et al. Molecular inflammation as an underlying mechanism of the aging process and age-related diseases. J Dent Res. 2011;90(7):830-40.
2. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, et al. Dietary factors and low-grade inflammation in relation to overweight and obesity. Br J Nutr. 2011;106 Suppl 3:S5-78.
3. Singh T, Newman AB. Inflammatory markers in population studies of aging. Ageing research reviews. 2011;10(3):319-29.
4. Simon AK, Hollander GA, McMichael A. Evolution of the immune system in humans from infancy to old age. Proc Biol Sci. 2015;282(1821):20143085.
5. Guarner V, Rubio-Ruiz ME. Low-grade systemic inflammation connects aging, metabolic syndrome and cardiovascular disease. Interdisciplinary topics in gerontology. 2015;40:99-106.
6. Franceschi C. Inflammaging as a major characteristic of old people: can it be prevented or cured? Nutr Rev. 2007;65(12 Pt 2):S173-6.
7. Legrand D, Adriaensen W, Vaes B, Mathei C, Wallemacq P, Degryse J. The relationship between grip strength and muscle mass (MM), inflammatory biomarkers and physical performance in community-dwelling very old persons. Arch Gerontol Geriatr. 2013;57(3):345-51.
8. Schaap LA, Pluijm SM, Deeg DJ, Harris TB, Kritchevsky SB, Newman AB, et al. Higher inflammatory marker levels in older persons: associations with 5-year change in muscle mass and muscle strength. J Gerontol A Biol Sci Med Sci. 2009;64(11):1183-9.
9. Visser M, Pahor M, Taaffe DR, Goodpaster BH, Simonsick EM, Newman AB, et al. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. J Gerontol A Biol Sci Med Sci. 2002;57(5):M326-32.
10. Cesari M, Penninx BW, Pahor M, Lauretani F, Corsi AM, Rhys Williams G, et al. Inflammatory markers and physical performance in older persons: the InCHIANTI study. J Gerontol A Biol Sci Med Sci. 2004;59(3):242-8.
11. Marzetti E, Landi F, Marini F, Cesari M, Buford TW, Manini TM, et al. Patterns of circulating inflammatory biomarkers in older persons with varying levels of physical performance: a partial least squares-discriminant analysis approach. Frontiers in medicine. 2014;1:27.
12. Aleman H, Esparza J, Ramirez FA, Astiazaran H, Payette H. Longitudinal evidence on the association between interleukin-6 and C-reactive protein with the loss of total appendicular skeletal muscle in free-living older men and women. Age Ageing. 2011;40(4):469-75.
13. Bartali B, Frongillo EA, Stipanuk MH, Bandinelli S, Salvini S, Palli D, et al. Protein intake and muscle strength in older persons: does inflammation matter? J Am Geriatr Soc. 2012;60(3):480-4.
14. Taaffe DR, Harris TB, Ferrucci L, Rowe J, Seeman TE. Cross-sectional and prospective relationships of interleukin-6 and C-reactive protein with physical performance in elderly persons: MacArthur studies of successful aging. J Gerontol A Biol Sci Med Sci. 2000;55(12):M709-15.
15. Vina J, Tarazona-Santabalbina FJ, Perez-Ros P, Martinez-Arnau FM, Borras C, Olaso-Gonzalez G, et al. Biology of frailty: Modulation of ageing genes and its importance to prevent age-associated loss of function. Mol Aspects Med. 2016.

16. Beyer I, Mets T, Bautmans I. Chronic low-grade inflammation and age-related sarcopenia. Curr Opin Clin Nutr Metab Care. 2012;15(1):12-22.
17. Laviano A, Gori C, Rianda S. Sarcopenia and nutrition. Adv Food Nutr Res. 2014;71:101-36.
18. Kritchevsky SB, Cesari M, Pahor M. Inflammatory markers and cardiovascular health in older adults. Cardiovasc Res. 2005;66(2):265-75.
19. Panickar KS, Jewell DE. The beneficial role of anti-inflammatory dietary ingredients in attenuating markers of chronic low-grade inflammation in aging. Horm Mol Biol Clin Investig. 2015;23(2):59-70.
20. Draganidis D, Karagounis LG, Athanailidis I, Chatzinikolaou A, Jamurtas AZ, Fatouros IG. Inflammaging and Skeletal Muscle: Can Protein Intake Make a Difference? J Nutr. 2016.
21. Panagiotakos DB, Pitsavos CH, Zampelas AD, Chrysohoou CA, Stefanadis CI. Dairy products consumption is associated with decreased levels of inflammatory markers related to cardiovascular disease in apparently healthy adults: the ATTICA study. J Am Coll Nutr. 2010;29(4):357-64.
22. Azadbakht L, Mirmiran P, Esmaillzadeh A, Azizi F. Dairy consumption is inversely associated with the prevalence of the metabolic syndrome in Tehranian adults. Am J Clin Nutr. 2005;82(3):523-30.
23. Esmaillzadeh A, Azadbakht L. Dairy consumption and circulating levels of inflammatory markers among Iranian women. Public Health Nutr. 2010;13(9):1395-402.
24. Rashidi Pour Fard N, Karimi M, Baghaei MH, Haghighatdoost F, Rouhani MH, Esmaillzadeh A, et al. Dairy consumption, cardiovascular risk factors and inflammation in elderly subjects. ARYA atherosclerosis. 2015;11(6):323-31.
25. van Meijl LE, Mensink RP. Effects of low-fat dairy consumption on markers of low-grade systemic inflammation and endothelial function in overweight and obese subjects: an intervention study. Br J Nutr. 2010;104(10):1523-7.
26. Zemel MB, Sun X, Sobhani T, Wilson B. Effects of dairy compared with soy on oxidative and inflammatory stress in overweight and obese subjects. Am J Clin Nutr. 2010;91(1):16-22.
27. Van Loan MD, Keim NL, Adams SH, Souza E, Woodhouse LR, Thomas A, et al. Dairy Foods in a Moderate Energy Restricted Diet Do Not Enhance Central Fat, Weight, and Intra-Abdominal Adipose Tissue Losses nor Reduce Adipocyte Size or Inflammatory Markers in Overweight and Obese Adults: A Controlled Feeding Study. J Obes. 2011;2011:989657.
28. Wennersberg MH, Smedman A, Turpeinen AM, Retterstol K, Tengblad S, Lipre E, et al. Dairy products and metabolic effects in overweight men and women: results from a 6-mo intervention study. Am J Clin Nutr. 2009;90(4):960-8.
29. Stancliffe RA, Thorpe T, Zemel MB. Dairy attentuates oxidative and inflammatory stress in metabolic syndrome. Am J Clin Nutr. 2011;94(2):422-30.
30. Drouin-Chartier JP, Gagnon J, Labonte ME, Desroches S, Charest A, Grenier G, et al. Impact of milk consumption on cardiometabolic risk in postmenopausal women with abdominal obesity. Nutrition journal. 2015;14:12.
31. Tricon S, Burdge GC, Jones EL, Russell JJ, El-Khazen S, Moretti E, et al. Effects of dairy products naturally enriched with cis-9,trans-11 conjugated linoleic acid on the blood lipid profile in healthy middle-aged men. Am J Clin Nutr. 2006;83(4):744-53.
32. Labonte ME, Cyr A, Abdullah MM, Lepine MC, Vohl MC, Jones P, et al. Dairy product consumption has no impact on biomarkers of inflammation among men and women with low-grade systemic inflammation. J Nutr. 2014;144(11):1760-7.
33. Hu FB. Protein, body weight, and cardiovascular health. Am J Clin Nutr. 2005;82(1 Suppl):242s-7s.

34. Goisser S, Kemmler W, Porzel S, Volkert D, Sieber CC, Bollheimer LC, et al. Sarcopenic obesity and complex interventions with nutrition and exercise in community-dwelling older persons--a narrative review. Clin Interv Aging. 2015;10:1267-82.
35. Wolfe BM. Potential role of raising dietary protein intake for reducing risk of atherosclerosis. Can J Cardiol. 1995;11 Suppl G:127g-31g.
36. Clifton PM, Keogh J. Metabolic effects of high-protein diets. Current atherosclerosis reports. 2007;9(6):472-8.
37. Schwingshackl L, Hoffmann G. Long-term effects of low-fat diets either low or high in protein on cardiovascular and metabolic risk factors: a systematic review and meta-analysis. Nutrition journal. 2013;12:48.
38. Santesso N, Akl EA, Bianchi M, Mente A, Mustafa R, Heels-Ansdell D, et al. Effects of higher- versus lower-protein diets on health outcomes: a systematic review and meta-analysis. Eur J Clin Nutr. 2012;66(7):780-8.
39. Teunissen-Beekman KF, Dopheide J, Geleijnse JM, Bakker SJ, Brink EJ, de Leeuw PW, et al. Dietary proteins improve endothelial function under fasting conditions but not in the postprandial state, with no effects on markers of low-grade inflammation. Br J Nutr. 2015;114(11):1819-28.
40. Ziegler CC, Sidani MA. Diets for successful aging. Clin Geriatr Med. 2011;27(4):577-89.
41. Inger Ottestad ATL, Gyrd Omholt Gjevestad, Håvard Hamarsland, Jūratė Šaltytė Benth, Lene Frost Andersen, Asta Bye, Anne Sofie Biong, Kjetil Retterstøl, Per Ole Iversen, Truls Raastad, Stine M Ulven, Kirsten B Holven. Intake of a protein-enriched milk and effects on muscle mass and strength. A 12-week randomized placebo controlled trial among community-dwelling older adults. J Nutr Health Aging. In press.
42. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
43. Bohl M, Bjornshave A, Gregersen S, Hermansen K. Whey and Casein Proteins and Medium-Chain Saturated Fatty Acids from Milk Do Not Increase Low-Grade Inflammation in Abdominally Obese Adults. The review of diabetic studies : RDS. 2016.
44. Pal S, Ellis V. The chronic effects of whey proteins on blood pressure, vascular function, and inflammatory markers in overweight individuals. Obesity (Silver Spring, Md). 2010;18(7):1354-9.
45. Ballard KD, Bruno RS, Seip RL, Quann EE, Volk BM, Freidenreich DJ, et al. Acute ingestion of a novel whey-derived peptide improves vascular endothelial responses in healthy individuals: a randomized, placebo controlled trial. Nutrition journal. 2009;8:34.
46. Rebholz CM, Reynolds K, Wofford MR, Chen J, Kelly TN, Mei H, et al. Effect of soybean protein on novel cardiovascular disease risk factors: a randomized controlled trial. Eur J Clin Nutr. 2013;67(1):58-63.
47. Tomsig JL, Sohma H, Creutz CE. Calcium-dependent regulation of tumour necrosis factor-alpha receptor signalling by copine. Biochem J. 2004;378(Pt 3):1089-94.
48. Varadhan R, Yao W, Matteini A, Beamer BA, Xue QL, Yang H, et al. Simple biologically informed inflammatory index of two serum cytokines predicts 10 year all-cause mortality in older adults. J Gerontol A Biol Sci Med Sci. 2014;69(2):165-73.
49. Wertz IE. TNFR1-activated NF-kappaB signal transduction: regulation by the ubiquitin/proteasome system. Curr Opin Chem Biol. 2014;23:71-7.
50. Dawson BM, Axford S. Nutrition As a Part of Healthy Aging and Reducing Cardiovascular Risk: Improving Functionality in Later Life Using Quality Protein, with Optimized Timing and Distribution. Semin Thromb Hemost. 2014.

51. Minihane AM, Vinoy S, Russell WR, Baka A, Roche HM, Tuohy KM, et al. Low-grade inflammation, diet composition and health: current research evidence and its translation. Br J Nutr. 2015;114(7):999-1012.
52. Council TN. Nordic nutrition recommendations: NNR 2004 : integrating nutrition and physical activity. ISBN 92-893-1062-6. 2004.
53. Zelcer N, Tontonoz P. Liver X receptors as integrators of metabolic and inflammatory signaling. J Clin Invest. 2006;116(3):607-14.
54. Walcher D, Kummel A, Kehrle B, Bach H, Grub M, Durst R, et al. LXR activation reduces proinflammatory cytokine expression in human CD4-positive lymphocytes. Arterioscler Thromb Vasc Biol. 2006;26(5):1022-8.
55. Spann NJ, Glass CK. Sterols and oxysterols in immune cell function. Nat Immunol. 2013;14(9):893-900.
56. Higuchi N, Kato M, Miyazaki M, Tanaka M, Kohjima M, Ito T, et al. Potential role of branched-chain amino acids in glucose metabolism through the accelerated induction of the glucose-sensing apparatus in the liver. J Cell Biochem. 2011;112(1):30-8.
57. Schoenborn JR, Wilson CB. Regulation of interferon-gamma during innate and adaptive immune responses. Adv Immunol. 2007;96:41-101.
58. Bonizzi G, Karin M. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 2004;25(6):280-8.
59. Labonte ME, Couture P, Richard C, Desroches S, Lamarche B. Impact of dairy products on biomarkers of inflammation: a systematic review of randomized controlled nutritional intervention studies in overweight and obese adults. Am J Clin Nutr. 2013;97(4):706-17.
60. Da Silva MS, Rudkowska I. Dairy products on metabolic health: current research and clinical implications. Maturitas. 2014;77(3):221-8.
61. Lopez-Legarrea P, de la Iglesia R, Abete I, Navas-Carretero S, Martinez JA, Zulet MA. The protein type within a hypocaloric diet affects obesity-related inflammation: the RESMENA project. Nutrition. 2014;30(4):424-9.
62. Zemel MB. Proposed role of calcium and dairy food components in weight management and metabolic health. The Physician and sportsmedicine. 2009;37(2):29-39.

Figure legends

Figure 1. mRNA levels in PBMCs of *NR1H3* [A] and *IFNG* [B] expressed as fold change from baseline after 12 weeks of supplementaion with proteins (n= 14) or carbohydrates (control, n=17 [A] and n= 16 [B]). Data are shown at an individual level, with one symbol (triangle, circle, sauare) representing one individual. The horizontal lines represent the median with interquartile range.

Figure 2. mRNA levels in PBMCs of *TNFRSF1A* [A] and *DDP4* [B] expressed as fold change from baseline after 12 weeks of supplementation with proteins (n=14) or carbohydrates (control, n=17). Data are shown at an individual level, with one symbol (triangle, circle, sauare) representing one individual. The horizontal lines represent the median with interquartile range.

Figure 1

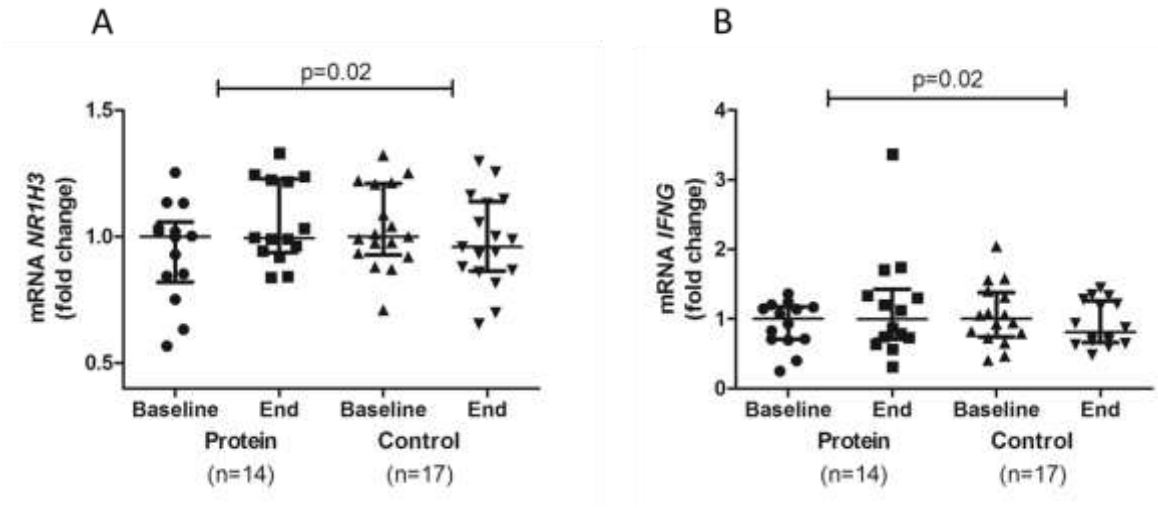

Figure 2

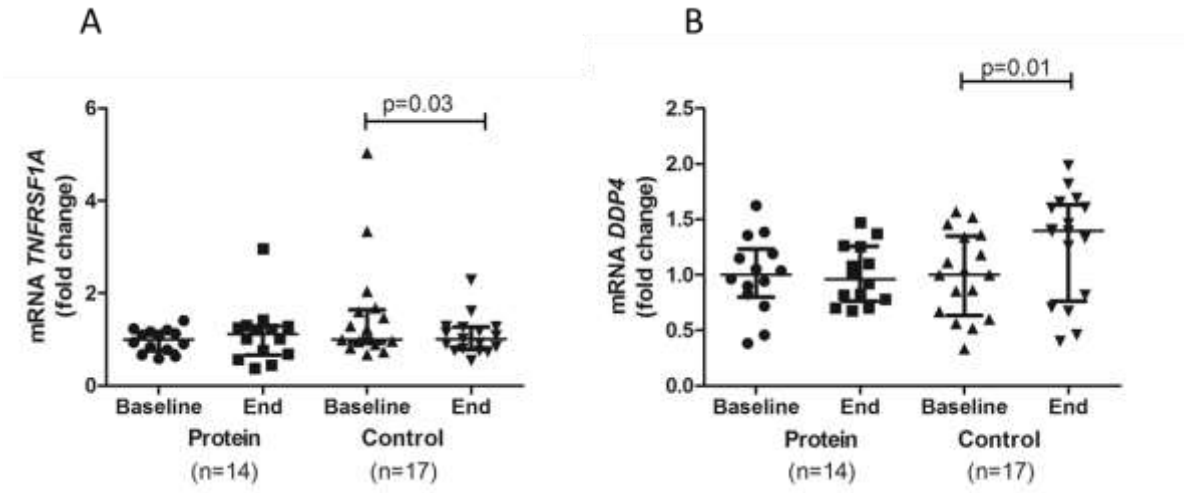

Table 1 Baseline characteristics.

	Protein group (n=14)	Control group (n=17)
Gender (n men/n women)	4/10	5/12
Age (yrs)	76.9 ± 4.9	77.7 ± 4.8
Muscle mass (kg)	43.7 ± 0.8	44.1 ± 0.9
Fat mass (kg)	28.8 ± 0.9	29.9 ± 0.9
BMI (kg/m^2)	27.1 ± 3.8	26.4 ± 4.9

Data are presented as mean ± SD. No significant differences were observed between the two groups at baseline.

Table 2 Effects of protein-enriched milk and isocaloric control drink on circulating inflammatory markers and body composition.

	Protein group (n=14)				Control group (n=17)				
	Median (min-max) baseline	Median (min.max) end of study	Δ median (min-max)	p-value[1]	Median (min-max) baseline	Median (min.max) end of study	Δ median (min-max)	p-value[1]	p-value[2]
hs-CRP (mg/L)	1.5 (0.4-6.0)	1.7 (0.9-9.0)	0.5 (-5.1-7.6))	0.10	1.1 (0.3-5.0)	1.5 (0.2-4.7)	0.1 (-1.3-1.3)	0.67	0.07
IL6 (pg/ml)	1.7 (1.0-4.5)	1.9 (1.0-4.8)	-0.1 (-1.7-1.6)	0.40	1.7 (0.6-10.7)	2.0 (0.5-7.0)	0.02 (-2.8-3.7)	0.91	0.81
TNFA(pg/ml)	3.5 (1.2-6.4)	3.5 (1.4-5.4)	1.0 (0.6 – 1.3)	0.46	3.6 (0.6-18.2)	4.1 (1.1-12.3)	1.1 (0.7 – 1.8)	0.03	0.53
TNFRSF1A (pg/ml)	992.5 (689.3-1511.1)	1087.4 (825.4-1004.4)	-117.7 (213.3-163.8)	0.02	1036.6 (133.3-2430.7)	1086.2 (789.3-2224.5)	-49.6 (-1057.0-206.2)	0.01	0.69
TNFα/sTNFRSF1A ratio (10^{-4})	3.6 (1.3-5.0)	2.9 (1.3-5.5)	-6.3 (-11.1-48.7)	0.27	3.2 (0.6-33.0)	3.3 (1.0-5.5)	-7.4 (-75.9-997)	0.69	0.55
Lean body mass (kg)	41.8 (34.6-56.7)	42.4 (34.7-57.5)	0.4 (-0.13-0.2)	0.12	41.5 (32.6-65.5)	42.1 (33.5-66.6)	0.6 (-0.1-0.3)	0.03	0.45
Fat mass (kg)	28.6 (15.1-45.3)	27.8 (15.0-46.0)	0.083 (-1.2-1.2)	0.64	24.6 (12.9-49.0)	25.3 (13.0-51.5)	0.74 (-3.3-2.5)	0.15	0.18

[1] changes from baseline were analyzed using Wilcoxon signed test

[2] between group effects were analyzed using Mann-Whitney U-test

Δ mean difference between baseline and end of study

Table S1 All genes analysed in the study.

Gene	**Abbreviation**	**Entrez Gene Number**	**Assay ID**	**Function**
Beta-2-Microglobulin	B2M	567	Hs00187842_m1	Component of the class I major histocompatibility complex (used as housekeeping gene)
Chemokine (C-C-Motif) Ligand 2	CCL2	6347	Hs00234140_m1	Involved in chemotactic activity for monocytes and basophils, binding to CCR2 and CCR4
Chemokine (C-X-C-Motif) Ligand 16	CXCL16	58191	Hs00222859_m1	Involved in the migration of cells, a chemoattractant
Chitinase-3-Like 1	CHI3L1	1116	Hs01072228_m1	Involved in inflammation and extracellular tissue remodeling
Dipeptidyl-Peptidase 4	DPP4	1803	Hs00897391_m1	Involved in glucose metabolism
Fas Cell Surface Death Receptor	FAS	355	Hs00163653_m1	Involved in apoptosis
F-box Protein 32	FBXO32	114907	Hs01041408_m1	Involved in muscle atrophy
Forkhead Box O1	FOXO1	2308	Hs01054576_m1	Transcription factor, regulating whole body energy metabolism
Glucuronidase, Beta	GUSB	2990	Hs00939627_m1	Involved in the degradation of dermatan and keratan sulfates (included as a potential housekeeping gene)
Glyceraldehyd-3-Phosphate Dehydrogenase	GAPDH	2597	Hs02758991_g1	Involved in the break down glucose for energy and carbon molecules (included as a potential housekeeping gene)
Growth Arrest AND DNA-Damage-Inducible, Alpha	GADD45A	1647	Hs00169255_m1	Involved in neuromuscular junction denervation
Histone deacetylase 4	HDAC4	9759	Hs01041638_m1	Involved in bone development
Hypoxia Inducible Factor 1, Alpha Subunit	HIF1A	3091	Hs00153153_m1	Involved in glucose metabolism
Insulin-Like Growth Factor 1	IGF1	3479	Hs01547656_m1	Growth promoting

Interferon, Gamma	IFNG	3458	Hs00989291_m1	Involved in the regulation of immune and inflammatory response, promotes Th1 differentiation
Interleukin 1 Receptor Antagonist	IL1RN	3557	Hs00893626_m1	Inhibits the activities of IL1A/IL1B, and modulates a variety of interleukin 1 related immune and inflammatory responses
Interleukin 1, Beta	IL1B	3553	Hs00174097_m1	Proliferation and maturation of lymphocytes, involved in inflammation and acute-phase response
Interleukin 10	IL10	3586	Hs00961622_m1	Down-regulates the expression of Th1 cytokines, enhances B cell survival, proliferation, and antibody production, able to block NF-κB activity
Interleukin 12	IL12	3592	Hs01073447_m1	Involved in the differentiation of naive T cells into Th1 cells. Able to stimulates INFG and TNFα
Interleukin 13	IL13	3596	Hs00174379_m1	Anti-inflammatory properties, closely related to IL4
Interleukin 15	IL15	3600	Hs01003716_m1	Regulates T and natural killer cell activation and proliferation
Interleukin 17A	IL17A	3605	Hs00174383_m1	Pro-inflammatory cytokine, that may stimulate the expression of IL6
Interleukin 18	IL18	3606	Hs01038788_m1	Involved in bone formation and inflammation
Interleukin 2	IL2	3558	Hs00174114_m1	Involved in differentiation, immune responses and homeostasis
Interleukin 23A	IL23A	51561	Hs00900828_g1	Involved in inflammation, increases angiogenesis
Interleukin 6	IL6	3569	Hs00985639_m1	A pleiotropic cytokine that plays important roles in the acute-phase response inflammation

Myocyte-specific enhancer factor 2C	MEF2C	4208	Hs00231149_m1	Involved in bone development
Nuclear Receptor Subfamily 4, Group A, Member 2	NR4A2	4929	Hs00428691_m1	Transcription factor, involved in energy metabolism and inflammation
Nuclear Receptor Subfamily, Group H, Member 3	NR1H3	10062	Hs00172885_m1	Transcription factor, involved in lipid metabolism and inflammation
Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Alpha	PPARGC1A	10891	Hs01016719_m1	Involved in energy metabolism and inflammation
Peroxisome Proliferator-Activated Receptor Gamma, Coactivator 1 Beta	PPARGC1B	133522	Hs00991676_m1	Involved in energy metabolism and inflammation
Pyruvate Dehydrogenase Kinase, Isozyme 4	PDK4	5166	Hs01037712_m1	Involved in glucose metabolism
Runt-Related Transcription Factor 1	RUNX1	861	Hs01021971_m1	Involved in neuromuscular junction denervation
Runt-related transcription factor 2	RUNX2	860	Hs01047973_m1	Involved in bone development
Sirtuin 1	SIRT1	23411	Hs01009006_m1	Involved in energy metabolism
Stearoyl-CoA Desaturase	SCD	6319	Hs01682761_m1	Involved in fat metabolism
Superoxide Dismutase 1, Soluble	SOD1	6647	Hs00533490_m1	Involved in the anti-oxidative defense destroying free superoxide radicals in the body
TATA Box Binding Protein	TBP	6908	Hs00427620_m1	General transcription factor that functions at the core of the DNA-binding multiprotein factor TFIID (used as housekeeping gene)
Toll-like Receptor 2	TLR2	7097	Hs01872448_s1	Involved in recognition of pathogen-associated molecular patterns, mediate the production of cytokines necessary for the development of effective immunity
Toll-like Receptor 4	TLR4	7099	Hs00152939_m1	Involved in recognition of pathogen-associated molecular patterns, mediate the production of cytokines necessary for the development of effective immunity

Tumor Necrosis Factor, alpha	TNFA	7124	Hs01113624_g1	Prototypical pro-inflammatory cytokine, play a central role in inflammation, immune system development and apoptosis
Tumor Necrosis Factor Ligand Superfamily Member 11	TNFSF11	8600	Hs00243522_m1	Involved in osteoclast differentiation and activation
Tumor Necrosis Factor Receptor Superfamily, Member 11A, NFKB activator	TNFRSF11A	8792	Hs00921372_m1	Involved in osteoclast differentiation and activation
Tumor Necrosis Factor Receptor Superfamily, Member 11B	TNFRSF11B	4982	Hs00900358_m1	Involved in bone development
Tumor Necrosis Factor Receptor Superfamily, Member 1A	TNFRSF1A	7132	Hs01042313_m1	Involved in TNF-signaling
Tumor Necrosis Factor Receptor Superfamily, Member 1B	TNFRSF1B	7133	Hs00153550_m1	Involved in TNF-signaling
Uncoupling Protein 2	UCP2	7351	Hs01075227_m1	Involved in energy metabolism, oxidation
Vitamin D (1,25-dihydroxyvitamin D3) receptor	VDR	7421	Hs00172113_m1	Involved in vitamin D metabolism

Table S2. Median values from mRNA analysis in PBMCs calculated as $2^{-\Delta Ct}$ at baseline and at the end of the intervention in subjects consuming a low-fat, protein-enriched milk (protein group). The mRNA expression was analysed by quantitative real-time RT-PCR and normilized to the endogenous control genes *TBP* and *B2M*. The p-values indicate changes between end of study and baseline values. The n equals the number of mRNA samples for each gene.

Gene	**Timepoint**	**n**	**$2^{-\Delta Ct}$ value (median)**	**p-value**
CCL2	Baseline	12	0.0014	0.29
	End		0.0017	
CHI3L1	Baseline	14	0.0006	0.58
	End		0.0007	
CXCL16	Baseline	12	0.0599	0.25
	End		0.0618	
DPP4	Baseline	14	0.0704	0.74
	End		0.0677	
FAS	Baseline	14	0.0774	0.65
	End		0.0717	
FBX	Baseline	14	0.0798	0.74
	End		0.0766	
FOXO1	Baseline	14	0.0654	0.73
	End		0.0626	
GADD45A	Baseline	14	0.0069	0.25
	End		0.0068	
HDAC4	Baseline	11	0.0534	0.85
	End		0.0577	
HIF1A	Baseline	14	0.2395	0.96
	End		0.2261	
IFNG	Baseline	14	0.0141	0.12
	End		0.0140	
IGF1	Baseline	13	0.0002	0.25
	End		0.0001	
IL10	Baseline	13	0.0005	0.89
	End		0.0005	
IL12A	Baseline	13	0.0103	0.72
	End		0.0103	
IL15	Baseline	13	0.0119	0.61
	End		0.0110	
IL18	Baseline	11	0.0030	0.18
	End		0.0032	
IL1B	Baseline	14	0.0204	0.58
	End		0.0174	

IL1RN	Baseline	14	0.0559	0.83
	End		0.0557	
IL23A	Baseline	14	0.0130	0.45
	End		0.0155	
IL2	Baseline	14	0.0004	0.93
	End		0.0003	
IL6	Baseline	14	0.0010	0.63
	End		0.0011	
MEF2C	Baseline	13	0.1033	0.14
	End		0.1061	
NR1H3	Baseline	14	0.0098	0.06
	End		0.0100	
NR4A2	Baseline	14	0.0018	0.81
	End		0.0016	
PDK4	Baseline	14	0.0597	0.24
	End		0.0496	
PPARGC1A	Baseline	13	0.0018	0.15
	End		0.0011	
PPARGC1B	Baseline	14	0.0165	0.63
	End		0.0172	
RUNX1	Baseline	14	0.0971	0.80
	End		0.0968	
RUNX2	Baseline	14	0.0832	0.83
	End		0.0790	
SCD	Baseline	14	0.0086	0.80
	End		0.0090	
SIRT1	Baseline	14	0.0452	0.76
	End		0.0448	
SOD1	Baseline	14	0.2823	0.19
	End		0.2232	
TLR2	Baseline	14	0.0504	0.20
	End		0.0455	
TLR4	Baseline	14	0.0580	0.88
	End		0.0531	
TNFα	Baseline	14	0.0478	0.71
	End		0.0471	
TNFRSF11A	Baseline	13	0.0030	0.39
	End		0.0032	
TNFRSF1A	Baseline	14	0.0791	0.76
	End		0.0884	
TNFRSF1B	Baseline	14	0.2635	0.63
	End		0.3494	
TNFSF11	Baseline	13	0.0019	0.29
	End		0.0015	
UCP2	Baseline	14	1.7140	0.49

	End		2.0163	
VDR	Baseline	7	0.0093	0.41
	End		0.0080	

Table S3. Median values from mRNA analysis in PBMCs calculated as $2^{-\Delta Ct}$ at baseline and at the end of the intervention in subjects consuming an isocaloric control drink consisting of carbohydrate only. The mRNA expression were analysed using quantitative real-time RT-PCR and normilized to the endogenous control genes *TBP* and *B2M*. The p-values are indicating changes between end of study and baseline values. The n equals the number of mRNA samples for each gene.

Gene	**Timepoint**	**n**	**$2^{-\Delta Ct}$ value (median)**	**p-value**
CCL2	Baseline	16	0.0017	0.34
	End		0.0018	
CHI3L1	Baseline	16	0.0006	0.96
	End		0.0008	
CXCL16	Baseline	15	0.0692	0.81
	End		0.0728	
DPP4	Baseline	17	0.0588	0.01
	End		0.0819	
FAS	Baseline	17	0.0745	0.34
	End		0.0685	
FBX	Baseline	17	0.0741	0.17
	End		0.0787	
FOXO1	Baseline	16	0.0672	0.38
	End		0.0690	
GADD45A	Baseline	17	0.0064	0.27
	End		0.0062	
HDAC4	Baseline	17	0.0601	0.76
	End		0.0573	
HIF1A	Baseline	17	0.2549	0.38
	End		0.2283	
IFNG	Baseline	16	0.0107	0.07
	End		0.0080	
IGF1	Baseline	16	0.0002	0.85
	End		0.0002	
IL10	Baseline	15	0.0004	0.29
	End		0.0003	
IL12A	Baseline	17	0.0101	0.98
	End		0.0091	
IL15	Baseline	17	0.0131	0.09
	End		0.0105	
IL18	Baseline	16	0.0034	0.64
	End		0.0035	
IL1B	Baseline	17	0.0209	0.19
	End		0.0171	

IL1RN	Baseline	17	0.0601	0.34
	End		0.0589	
IL23A	Baseline	17	0.0117	0.31
	End		0.0142	
IL2	Baseline	17	0.0004	0.79
	End		0.0004	
IL6	Baseline	17	0.0011	0.08
	End		0.0010	
MEF2C	Baseline	15	0.1073	0.90
	End		0.1072	
NR1H3	Baseline	17	0.0089	0.18
	End		0.0085	
NR4A2	Baseline	17	0.0022	0.62
	End		0.0017	
PDK4	Baseline	17	0.0659	0.06
	End		0.0550	
PPARGC1A	Baseline	15	0.0016	0.55
	End		0.0008	
PPARGC1B	Baseline	17	0.0186	0.26
	End		0.0158	
RUNX1	Baseline	17	0.0932	0.48
	End		0.0999	
RUNX2	Baseline	17	0.0778	0.41
	End		0.0816	
SCD	Baseline	16	0.0079	0.08
	End		0.0075	
SIRT1	Baseline	17	0.0505	0.21
	End		0.0456	
SOD1	Baseline	17	0.2248	0.87
	End		0.2127	
TLR2	Baseline	16	0.0487	0.99
	End		0.0468	
TLR4	Baseline	16	0.0666	0.94
	End		0.0667	
TNFα	Baseline	17	0.0445	0.96
	End		0.0443	
TNFRSF11A	Baseline	14	0.0038	0.94
	End		0.0034	
TNFRSF1A	Baseline	17	0.0675	0.03
	End		0.0685	
TNFRSF1B	Baseline	15	0.3333	0.34
	End		0.3595	
TNFSF11	Baseline	16	0.0017	0.25
	End		0.0021	
UCP2	Baseline	15	2.1228	0.76

	End		1.8480	
VDR	Baseline	6	0.0097	0.25
	End		0.0093	

Table S4. Distribution of lymphocytes and monocytes, expressed as percent, in PMBCs in subjects receiving protein-enriched drinks (n = 14) or control (n=17). The p-values indicate changes between baseline values and end of study.

Drink	**Cell type**		**Percentiles**			**p-value**
			25	**50**	**75**	
Protein (n=14)	Lymphocytes	Baseline	14.4	16.1	19.7	0.78
		End	14.7	15.8	18.2	
	Monocytes	Baseline	3.6	4.3	4.9	0.93
		End	3.5	4.4	5.0	
Control (n=17)	Lymphocytes	Baseline	13.9	17.5	19.3	0.92
		End	14.1	16.1	19.0	
	Monocytes	Baseline	3.8	4.2	4.7	0.10
		End	4.1	4.0	5.2	