

Potent anti-obesity effect of enteric-coated lactoferrin: decrease in visceral fat accumulation in Japanese men and women with abdominal obesity after 8-week administration of enteric-coated lactoferrin tablets

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Lactoferrin (LF), a multifunctional glycoprotein in mammalian milk, is reported to exert a modulatory effect on lipid metabolism. The aim of the present study was to elucidate whether enteric-coated LF (eLF) might improve visceral fat-type obesity, an underlying cause of the metabolic syndrome. Using a double-blind, placebo-controlled design, Japanese men and women (n 26; aged 22–60 years) with abdominal obesity (BMI > 25 kg/m², and visceral fat area (VFA) > 100 cm²) consumed eLF (300 mg/d as bovine LF) or placebo tablets for 8 weeks. Measurement of the total fat area, VFA and subcutaneous fat area from computed tomography images revealed a significant reduction in VFA (–14.6 cm²) in the eLF group, as compared with the placebo controls (–1.8 cm²; $P=0.009$ by ANCOVA). Decreases in body weight, BMI and hip circumference in the eLF group (–1.5 kg, –0.6 kg/m², –2.6 cm) were also found to be significantly greater than with the placebo (+1.0 kg, +0.3 kg/m², –0.2 cm; $P=0.032$, 0.013, 0.041, respectively). There was also a tendency for a reduction in waist circumference in the eLF group (–4.4 cm) as compared with the placebo group (–0.9 cm; $P=0.073$). No adverse effects of the eLF treatment were found with regard to blood lipid or biochemical parameters. From these results, eLF appears to be a promising agent for the control of visceral fat accumulation.

Lactoferrin: Enteric-coated lactoferrin tablets: Visceral fat: Metabolic syndrome

The metabolic syndrome is a combination of medical disorders that increase the risk of developing CVD and other chronic ailments. Recently, the number of affected individuals has been rapidly increasing worldwide, because of a shift towards dietary excess, lack of exercise and increasing stress, causing social problems. Visceral fat-type obesity is one underlying reason for the metabolic syndrome. Excessive visceral fat accumulation disrupts the production of adiponectin, plasminogen activator inhibitor type 1, TNF and NEFA, which induces insulin resistance linked with high blood glucose, high blood pressure and dyslipidaemia. To prevent the metabolic syndrome, it is important to improve lifestyle habits and maintain the balance of energy intake and consumption. One idea attracting increasing attention is the possibility of using specific food factors as supplements.

Lactoferrin (LF) is an Fe-binding glycoprotein which is found at highest concentrations in mammary breast milk. It is multi-functional, with anti-bacterial, antiviral,

immunostimulatory, antioxidant and cancer-preventive potential^(1–5) and because LF is a natural component of breast milk which is ingested by infants, it is considered to be highly safe. Thus it has been approved as a food additive in Japan, and is included in the ‘generally recognized as safe’ (GRAS) category in the USA. Firstly, we focused on the anti-bacterial activity of LF, and found potent anti-pathogenesis activities against periodontal disease⁽⁶⁾. In the course of conducting oral care research on LF, we also noted another highly interesting effect. Our results pointed to a new function – reducing the visceral fat that is the key cause of the metabolic syndrome – discovered through animal studies. There have been reports on the influence of LF on lipid metabolism. In the study of Takeuchi *et al.*⁽⁷⁾, bovine LF reduced plasma TAG and NEFA accompanied by decreases in hepatic cholesterol and TAG contents in rodents. Tamano *et al.* reported a significant decrease of serum TAG to 72% of the control level⁽⁸⁾. However, these reports were

Abbreviations: eLF, enteric-coated lactoferrin; JSCC, Japan Society of Clinical Chemistry; LF, lactoferrin.

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the results of animal experiments, and, to our knowledge there, has up till now been no human clinical trial aimed at determining the influence of LF on lipid metabolism. Furthermore, there has been no examination of the effects of LF on visceral fat accumulation. Therefore, we conducted the present investigation, focusing on lipid metabolism and visceral fat in a human clinical study. Since orally administered proteins are generally degraded by pepsin in the stomach, we used enteric-coated LF (eLF) tablets as the test food in the present evaluation of LF activity in a randomised double-blind placebo-controlled trial.

Experimental methods

Design and subjects

This trial was performed during the period of January 2008 to May 2008 with volunteers at the Moriyama Hospital in the Kanto District in Japan. The protocol was approved by the institutional board and the trial was conducted in accordance with the Helsinki Declaration under the supervision of clinical investigators. The subjects provided informed consent, including their permission for the findings to be published. Inclusion criteria were healthy Japanese men and women more than 20 years of age, with a BMI > 25 kg/m², and a visceral fat area > 100 cm², who were considered to be visceral fat-type obese, but had not been treated at an out-patient department and had no serious disease.

This was a randomised double-blind placebo-controlled trial, consisting of a 2-week run-in period and an 8-week treatment period. After the run-in period, the subjects were allocated to two groups designated as the eLF group (daily ingestion of three eLF tablets; 300 mg/d as bovine LF) and the control group (ingestion of three enteric-coated placebo tablets). Randomisation was stratified by age, sex, and visceral fat area measured at the time of the run-in period at hospital (control group, six men and seven women; eLF group, five men and eight women).

The test tablets that we used in the present study were eLF tablets, containing LF 100 mg/tablet, and control enteric-coated tablets, containing lactose instead of LF. Other constituents of each tablet were crystalline cellulose, carboxymethylcellulose-Ca, sucrose ester, silicon dioxide, shellac, sorbitol, arginine, dextrin and long pepper powder. In this formulation, LF molecules are protected from proteolytic digestion in the stomach, since the tablets are coated with an acid-resistant material, shellac, which dissolves easily under the neutral pH conditions in the intestine. The enteric-coated properties of this formulation were checked by the standard disintegration test to satisfy the criterion for the Japanese Pharmacopoeia.

The subjects consumed three tablets of the test material per d for 8 weeks. The time for ingestion of the test tablets was not limited, but it was recommended that the subjects take three test tablets after their evening meal/before sleep, to maintain compliance.

Energy and fat intake was not limited throughout the trial period, but supplemental food products or medications known to influence lipid or carbohydrate metabolism were prohibited. The subjects were instructed to maintain their usual dietary intake and physical activity.

The subjects visited the medical institution at 4-week intervals after the run-in period. Eating and drinking, except for water, were prohibited from 21.00 hours on the day before the visit until various measurements were completed.

Anthropometry, measurements of circulatory parameters, fasting blood sampling for biochemical and haematological parameters, and interviews were performed at -2 (before treatment), 0, 4 and 8 weeks. Computed tomography (CT) was performed at 0 and 8 weeks to measure the abdominal fat area.

Anthropometric and vital sign measurements

Height (only at -2 weeks), body weight, waist circumference and hip circumference were measured at each visit. BMI was calculated from the height and body weight. Waist and hip circumference, at the umbilical level and at the level of the greatest posterior protuberance (maximal gluteal circumference), respectively, were measured using a non-elastic anthropometric tape measure. Systolic blood pressure, diastolic blood pressure and pulse rate were assessed using an Hg manometer with subjects in a seated position after resting quietly for 10 min.

Evaluation of the abdominal fat level

The abdominal fat level, including total fat area, visceral fat area and subcutaneous fat area, were measured from CT images by Pronto-Xi/Si (Hitachi, Tokyo, Japan), using Fat Pointer software (version 2; Hitachi Medico Co., Tokyo, Japan), under X-ray conditions of a tube voltage of 120 kVp (peak voltage) and 100 mA⁽⁹⁾. Several CT images around the umbilicus were obtained and single scan images at the precise point of the umbilicus were used for analysis.

Blood biochemical examination

The serum total cholesterol (cholesterol oxidase method⁽¹⁰⁾), HDL-cholesterol (selective inhibition method⁽¹¹⁾), LDL-cholesterol (enzymic method⁽¹²⁾), TAG (enzymic method after eliminating endogenous free glycerol⁽¹³⁾), total lipid (sulfo-phospho-vanillin method⁽¹⁴⁾), NEFA (enzymic method⁽¹⁵⁾), total protein (biuret method⁽¹⁶⁾), albumin (bromocresol green (BCG) method⁽¹⁷⁾), glutamic oxaloacetic transaminase (standard methods established by the Japan Society of Clinical Chemistry (JSCC)⁽¹⁸⁾), glutamic pyruvate transaminase (standard methods established by the JSCC⁽¹⁸⁾), lactate dehydrogenase (standard methods established by the JSCC⁽¹⁸⁾), alkaline phosphatase (standard methods established by the JSCC⁽¹⁸⁾), γ -glutamyl transferase (standard methods established by the JSCC⁽¹⁸⁾), total bilirubin (enzymic method⁽¹⁹⁾), direct bilirubin (enzymic method⁽¹⁹⁾), creatinine (enzymic method⁽²⁰⁾), blood urea N (enzymic method⁽²¹⁾), uric acid (uricase-peroxidase method⁽²²⁾), creatine kinase (standard methods established by the JSCC⁽¹⁸⁾), C-reactive protein (immunonephelometry⁽²³⁾), blood glucose (glucose oxidase-peroxidase method⁽²⁴⁾), glycosylated HbA1c (latex particle agglutination method⁽²⁵⁾) and insulin (enzyme immunoassay (EIA) method⁽²⁶⁾) were measured in fasting blood samples. Non-HDL-cholesterol was calculated from the

value of total cholesterol and HDL-cholesterol. The albumin:globulin ratio was calculated from the value of total protein and albumin.

Dietary diary and daily living records

The subjects recorded the content of their meals in a dietary diary for 3 d before the visits at 0, 4 and 8 weeks. Based on the information in the diaries, dietitians analysed the daily energy intake, fat intake and fat:energy ratio, using *Standard Tables of Food Composition in Japan*, the 5th revised and enlarged edition⁽²⁷⁾, and mean values for the 3 d were calculated. In addition, the subjects recorded their compliance of the test tablet intake, and daily activities, including eating habits and exercise, measured by a passometer (Spalding cumulative passometer PS453; Tokyo Compass Mfg. Co. Ltd, Tokyo, Japan) every day from 0 to 8 weeks, in a daily living record using a simple checklist. The clinical investigators provided feedback of the daily living record to the subjects to encourage a constant level of daily activity. Physical conditions and adverse effects were examined by a physician in the interview at each visit.

Statistical analysis

Data presented for all test parameters are mean values and standard deviations. Results are expressed either in actual values or changes from 0 to 4 weeks (Δ value at week 4) or 0 to 8 weeks (Δ value at week 8). To compare the week 0 values for the two groups, an unpaired *t* test (two-sided) was employed. An intergroup comparison by repeated-measures ANOVA was performed using actual values from week 0 to week 8. *Post hoc* analysis was conducted by ANCOVA, the Dunnett test, or the paired *t* test. The statistically significant level was set at $P < 0.05$. The data were analysed using JMP (version 5.0.1a; SAS Institute Inc., Cary, NC, USA).

Results

A total of thirty subjects volunteered to participate in the study. Of the subjects, two withdrew agreement and were excluded from the original thirty subjects enrolled before the release of the double-blinding. In addition, two subjects (one in the control group and one in the eLF group) were discontinued because of job relocation or pressure of work. Data were analysed using the per-protocol samples of twenty-six subjects (control group, six men and seven women; eLF group, five men and eight women). The flow of participants in the trial is shown in Fig. 1. The baseline characteristics of the study subjects did not differ significantly between the groups (Table 1). Compliance of test tablet intake in the eLF group and the control group was 98.0 and 96.7%, respectively.

Table 2 shows the daily energy, protein, carbohydrate and fat intakes. No significant differences were found between the two groups. Daily living records indicated that exercise levels were maintained at a constant level during the study.

Table 3 shows changes in anthropometric parameters and circulatory parameters. Body weight ($P < 0.05$ at week 4, $P < 0.01$ at week 8), BMI ($P < 0.05$ at week 4, $P < 0.01$ at week 8), waist circumference ($P < 0.01$ at week 8) and hip circumference ($P < 0.05$ at week 8) decreased significantly in the eLF group by the Dunnett test as compared with week 0. Body weight ($P = 0.037$ at week 4, $P = 0.013$ at week 8), BMI ($P = 0.041$ at week 8) and hip circumference ($P = 0.032$ at week 8) were statistically different between the eLF and control groups as analysed by ANCOVA. There was also a tendency for a greater reduction in waist circumference ($P = 0.073$ at week 8) in the eLF group than with the placebo. No significant differences in circulatory parameters were found between the groups.

Table 4 shows changes in abdominal fat areas. Visceral fat area and total fat area decreased significantly over time ($P < 0.01$ at week 8; paired *t* test) in the eLF group. The decreases in visceral fat area, subcutaneous fat area and total fat area at week 8 from baseline were -14.6 , -13.4 and -28.0 cm in the eLF group, and -1.8 , -9.9 and -11.7 cm

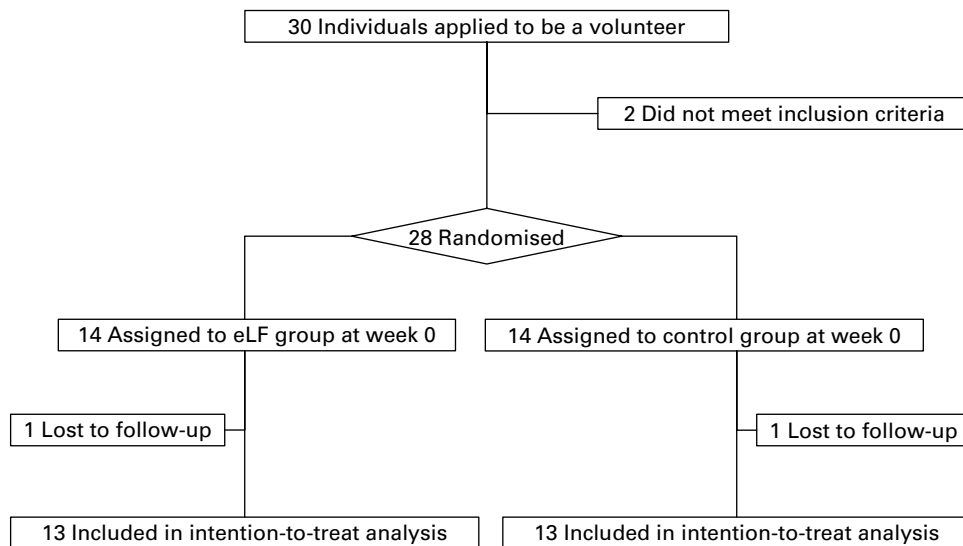


Fig. 1. Flow of participants in a study investigating the effect of enteric-coated lactoferrin (eLF) on body weight and abdominal fat area level in Japanese men and women.

Table 1. The baseline (week 0) characteristics of the study subjects (Mean values and standard deviations)

Parameter	Group				P*
	eLF (n 13)		Control (n 13)		
	Mean	SD	Mean	SD	
Sex (n)					0.50†
Men		5		6	
Women		8		7	
Age (years)	42.8	10.1	46.8	9.2	0.30
Height (cm)	161.1	8.4	161.0	11.3	0.99
Weight (kg)	77.7	12.3	72.9	17.0	0.41
BMI (kg/m ²)	30.0	4.8	27.7	2.9	0.15
Waist circumference (cm)	99.6	10.1	93.2	12.8	0.17
Hip circumference (cm)	105.7	7.4	101.9	10.1	0.28
Visceral fat area (cm ²)	118.1	32.2	116.6	49.1	0.93
Subcutaneous fat area (cm ²)	284.6	126.4	241.7	107.7	0.36
Total fat area (cm ²)	402.7	128.5	358.3	133.7	0.40
Systolic blood pressure (mmHg)	128.2	11.3	132.0	8.7	0.35
Diastolic blood pressure (mmHg)	79.5	11.9	78.6	14.1	0.87
Pulse rate (beats/min)	77.6	11.8	80.3	11.6	0.56
Total cholesterol (mmol/l)	5.73	0.87	5.63	0.55	0.72
HDL-cholesterol (mmol/l)	1.54	0.37	1.51	0.44	0.87
LDL-cholesterol (mmol/l)	3.69	0.64	3.42	0.51	0.26
TAG (mmol/l)	1.47	0.87	1.95	1.50	0.33
Total lipid (g/l)	6.97	1.30	7.34	1.62	0.52
NEFA (mmol/l)	0.76	0.36	0.61	0.21	0.21
Non-HDL-cholesterol (mmol/l)	4.20	0.69	4.12	0.58	0.76

eLF, enteric-coated lactoferrin.

* Except for sex, † test between groups.

† Square test between groups.

in the control group, respectively. A significant difference between the eLF and the control visceral fat area was found at week 8 ($P=0.0089$), as analysed by ANCOVA.

Tables 5 and 6 show changes in blood lipid parameters and biochemical parameters. No significant differences were found between the two groups. No adverse effects of eLF were apparent.

Discussion

The present investigation of effects of eLF tablets (as 300 mg LF/d for 8 weeks) on visceral fat accumulation in Japanese men and women with abdominal obesity demonstrated a significant benefit as compared with the placebo regarding

the visceral fat area and a tendency for greater improvement in anthropometric data.

Major sources of exogenous LF in our daily diet are dairy products from bovine milk. LF contents in bovine colostrum and normal milk are 1000 and 20–350 µg/ml, respectively. During the milk pasteurisation process, LF is inactivated by heating, but unpasteurised dairy products, such as natural cheese, contain approximately 300 mg LF per 100 g. Therefore, during the treatment period, the daily intake of LF for the eLF group was almost the same as approximately 100 g of natural cheese per d⁽²⁸⁾, although LF in natural cheese naturally will be degraded in the stomach. Under our conditions, 8 weeks of oral administration of eLF tablets significantly reduced the accumulation of visceral fat, compared

Table 2. Daily energy, protein, carbohydrate and fat intakes (Mean values and standard deviations)

Parameter	Group	Week 0		Week 4		Week 8		P*		
		Mean	SD	Mean	SD	Mean	SD	Time	Group	Time × group
Energy intake (kJ/d)	Control	8552	1703	7719	1774	7778	1523	0.84	0.70	0.07
	eLF	8004	2038	8577	2531	8326	2494			
Protein intake (g/d)	Control	82.8	17.6	71.1	15.5	75.3	13.6	0.66	0.15	0.46
	eLF	70.5	24.6	78.3	19.9	76.6	25.7			
Fat intake (g/d)	Control	72.6	11.7	60.8	19.2	60.2	16.1	0.61	0.86	0.13
	eLF	62.2	16.4	70.2	14.1	66.3	25.0			
Carbohydrate intake (g/d)	Control	255.0	63.2	240.7	59.8	243.6	52.2	0.57	0.84	0.21
	eLF	251.1	77.0	252.6	86.3	257.4	81.1			

eLF, enteric-coated lactoferrin.

* Repeated-measures ANOVA.

Table 3. Changes in anthropometric and circulatory parameters after taking enteric-coated lactoferrin (eLF) or control tablets for 8 weeks (Mean values and standard deviations for thirteen subjects per group)

Parameter	Group	Week 0		Week 4		ΔValue at week 4†		Week 8		ΔValue at week 8‡		P§			P																																																																																																																																																																			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Time	Group	Time × group	Week 4	Week 8																																																																																																																																																																		
Weight (kg)	Control	72.9	17.0	72.5	17.5	-0.3	1.1	73.9	20.1	+1.0	4.0	0.0047	0.55	0.045	0.037	0.013																																																																																																																																																																		
	eLF	77.7	12.3	76.4*	12.0	-1.3	1.2	76.2**	11.7	-1.5	1.4						BMI (kg/m ²)	Control	27.7	2.9	27.6	3.0	-0.2	0.4	28.0	3.7	+0.3	1.2	0.0040	0.23	0.053	0.12	0.041	eLF	30.0	4.8	29.5*	4.6	-0.5	0.5	29.4**	4.37	-0.6	0.6	Waist circumference (cm)	Control	93.2	12.9	93.2	13.4	0.0	6.8	92.3	12.0	-0.9	3.3	0.0049	0.30	0.083	0.45	0.073	eLF	99.6	10.1	97.4	9.1	-2.2	2.1	95.2**	8.9	-4.4	4.0	Hip circumference (cm)	Control	101.9	10.1	100.0	8.9	-1.9	4.5	101.7	10.0	-0.2	1.8	0.0036	0.35	0.053	0.54	0.032	eLF	105.7	7.4	104.1	7.7	-1.6	2.1	103.1*	6.5	-2.6	2.9	Systolic blood pressure (mmHg)	Control	132.0	8.7	132.7	9.6	+0.7	10.4	126.8	11.9	-5.2	9.4	0.07	0.18	0.62			eLF	128.2	11.3	125.8	9.8	-2.5	8.1	124.9	6.8	-3.3	8.5	Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62			eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0	Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4
BMI (kg/m ²)	Control	27.7	2.9	27.6	3.0	-0.2	0.4	28.0	3.7	+0.3	1.2	0.0040	0.23	0.053	0.12	0.041																																																																																																																																																																		
	eLF	30.0	4.8	29.5*	4.6	-0.5	0.5	29.4**	4.37	-0.6	0.6						Waist circumference (cm)	Control	93.2	12.9	93.2	13.4	0.0	6.8	92.3	12.0	-0.9	3.3	0.0049	0.30	0.083	0.45	0.073	eLF	99.6	10.1	97.4	9.1	-2.2	2.1	95.2**	8.9	-4.4	4.0	Hip circumference (cm)	Control	101.9	10.1	100.0	8.9	-1.9	4.5	101.7	10.0	-0.2	1.8	0.0036	0.35	0.053	0.54	0.032	eLF	105.7	7.4	104.1	7.7	-1.6	2.1	103.1*	6.5	-2.6	2.9	Systolic blood pressure (mmHg)	Control	132.0	8.7	132.7	9.6	+0.7	10.4	126.8	11.9	-5.2	9.4	0.07	0.18	0.62			eLF	128.2	11.3	125.8	9.8	-2.5	8.1	124.9	6.8	-3.3	8.5	Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62			eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0	Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4	+2.2	8.9	72.8	10.0	-4.8	9.1																						
Waist circumference (cm)	Control	93.2	12.9	93.2	13.4	0.0	6.8	92.3	12.0	-0.9	3.3	0.0049	0.30	0.083	0.45	0.073																																																																																																																																																																		
	eLF	99.6	10.1	97.4	9.1	-2.2	2.1	95.2**	8.9	-4.4	4.0						Hip circumference (cm)	Control	101.9	10.1	100.0	8.9	-1.9	4.5	101.7	10.0	-0.2	1.8	0.0036	0.35	0.053	0.54	0.032	eLF	105.7	7.4	104.1	7.7	-1.6	2.1	103.1*	6.5	-2.6	2.9	Systolic blood pressure (mmHg)	Control	132.0	8.7	132.7	9.6	+0.7	10.4	126.8	11.9	-5.2	9.4	0.07	0.18	0.62			eLF	128.2	11.3	125.8	9.8	-2.5	8.1	124.9	6.8	-3.3	8.5	Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62			eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0	Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4	+2.2	8.9	72.8	10.0	-4.8	9.1																																																		
Hip circumference (cm)	Control	101.9	10.1	100.0	8.9	-1.9	4.5	101.7	10.0	-0.2	1.8	0.0036	0.35	0.053	0.54	0.032																																																																																																																																																																		
	eLF	105.7	7.4	104.1	7.7	-1.6	2.1	103.1*	6.5	-2.6	2.9						Systolic blood pressure (mmHg)	Control	132.0	8.7	132.7	9.6	+0.7	10.4	126.8	11.9	-5.2	9.4	0.07	0.18	0.62			eLF	128.2	11.3	125.8	9.8	-2.5	8.1	124.9	6.8	-3.3	8.5	Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62			eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0	Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4	+2.2	8.9	72.8	10.0	-4.8	9.1																																																																														
Systolic blood pressure (mmHg)	Control	132.0	8.7	132.7	9.6	+0.7	10.4	126.8	11.9	-5.2	9.4	0.07	0.18	0.62																																																																																																																																																																				
	eLF	128.2	11.3	125.8	9.8	-2.5	8.1	124.9	6.8	-3.3	8.5				Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62			eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0	Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4	+2.2	8.9	72.8	10.0	-4.8	9.1																																																																																																												
Diastolic blood pressure (mmHg)	Control	78.6	14.1	83.6	9.7	+5.0	15.3	78.8	11.0	+0.2	12.6	0.30	0.92	0.62																																																																																																																																																																				
	eLF	79.5	11.9	80.8	9.6	+1.3	7.8	79.7	9.4	+0.2	10.0				Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47			eLF	77.6	11.8	79.8	8.4	+2.2	8.9	72.8	10.0	-4.8	9.1																																																																																																																																								
Pulse rate (beats/min)	Control	80.3	11.6	81.8	9.6	+1.5	10.1	79.1	10.2	-1.2	12.9	0.033	0.29	0.47																																																																																																																																																																				
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Mean value was significantly different from that at baseline (week 0): * $P < 0.05$, ** $P < 0.01$ (Dunnett's test).

† The value is the change from week 0 to week 4.

‡ The value is the change from week 0 to week 8.

§ Repeated-measures ANOVA.

|| ANCOVA.

Table 4. Abdominal fat area after taking enteric-coated lactoferrin (eLF) or control tablets for 8 weeks (Mean values and standard deviations for thirteen subjects per group)

Parameter	Group	Week 0		Week 8		ΔValue at week 8†		P‡			P§
		Mean	SD	Mean	SD	Mean	SD	Time	Group	Time × group	
Visceral fat area (cm ²)	Control	116.6	49.1	114.8	46.7	-1.8	11.7	0.0013	0.76	0.0087	0.0089
	eLF	118.1	32.2	103.4**	32.9	-14.6	11.2				
Subcutaneous fat area (cm ²)	Control	241.7	107.7	231.7	113.0	-9.9	33.6	0.064	0.36	0.77	0.96
	eLF	284.6	126.4	271.3	114.0	-13.4	27.4				
Total fat area (cm ²)	Control	358.3	133.7	346.6	132.1	-11.7	36.6	0.0051	0.47	0.22	0.34
	eLF	402.7	128.5	374.7**	111.7	-28.0	28.6				

** Mean value was significantly different from that at baseline (week 0) ($P < 0.01$; paired *t* test).

† The value is the change from week 0 to week 8.

‡ Repeated-measures ANOVA.

§ ANCOVA.

with control, and total fat area, hip circumference, body weight and BMI were also significantly decreased by eLF. No adverse events were observed with regard to safety parameters. In addition to its known anti-bacterial, anti-virus, immunostimulatory, antioxidant and cancer-preventive properties^(1–5), the present results thus point to a novel functional significance of eLF in reducing visceral fat.

Recently, Moreno-Navarrete *et al.*^(29,30) reported the circulating LF concentration to be inversely associated with BMI, the waist:hip ratio and the fasting TAG and glucose concentrations, and positively with insulin sensitivity. They speculated that the preservation of LF production leads to decreased free lipopolysaccharide concentration and maintenance of an adequate lipid profile^(29,30). Metabolic endotoxaemia initiates obesity and insulin resistance⁽³¹⁾, and LF is known to bind to and inactivate lipopolysaccharide⁽³²⁾. Although, in the present study, we administered bovine LF extrinsically, there is a possibility that circulating LF could rise with oral administration of eLF, and neutralise the action of lipopolysaccharide. Takeuchi *et al.* described LF to be transported into the blood circulation from the intestine via the lymphatic pathway in adult rats⁽³³⁾ and Fischer *et al.* detected immunoreactive LF in the serum, liver, kidneys,

gall bladder, spleen and brain of mice after administration by gastric intubation⁽³⁴⁾. These reports support our hypothesis described above.

With regard to another possible mechanism underlying the effects of eLF on visceral fat, the anti-adipogenic action of LF should be considered. Two studies have demonstrated such activity by LF on pre-adipocytes. Yagi *et al.*⁽³⁵⁾ and Moreno-Navarrete *et al.*⁽³⁶⁾ reported that LF inhibits adipogenic differentiation of the MC3T3-G2/PA6 cell lines and the 3T3-L1 cell line^(35,36). We also confirmed those actions of LF against pre-adipocytes isolated from rat mesenteric fat using a primary culture system. Moreover, we proved that trypsin-degraded LF retained this activity, whereas pepsin-degraded LF did not, suggesting an important role for enteric coating in enabling the LF to bypass the stomach's digestive action in order to exert its anti-adipogenic action (T Ono, S Morishita, C Fujisaki, M Ohdera, M Murakoshi, N Iida, H Kato, K Miyashita, M Iigo, T Yoshida, K Sugiyama and H Nishino, unpublished results).

LRP1, a known LF receptor, may be a key factor for the anti-adipogenic action of LF. Dietary lipids are carried in chylomicron remnants which are taken up into the liver mainly via LRP1. Crawford & Borensztajn reported that LF

Table 5. Changes in serum lipid parameters after taking enteric-coated lactoferrin (eLF) or control tablets for 8 weeks (Mean values and standard deviations for thirteen subjects per group)

Parameter	Group	Week 0		Week 4		Week 8		P*		
		Mean	SD	Mean	SD	Mean	SD	Time	Group	Time × group
Total cholesterol (mmol/l)	Control	5.63	0.55	5.68	0.52	5.93	0.66	0.59	0.79	0.35
	eLF	5.73	0.87	5.66	0.77	5.66	0.61			
HDL-cholesterol (mmol/l)	Control	1.51	0.44	1.52	0.44	1.56	0.48	0.10	0.75	0.78
	eLF	1.54	0.37	1.61	0.50	1.61	0.41			
LDL-cholesterol (mmol/l)	Control	3.42	0.51	3.50	0.39	3.68	0.56	0.43	0.69	0.34
	eLF	3.69	0.64	3.54	0.66	3.61	0.66			
TAG (mmol/l)	Control	1.95	1.50	2.02	1.33	1.93	0.83	0.38	0.19	0.94
	eLF	1.47	0.87	1.54	0.83	1.35	0.61			
Total lipid (g/l)	Control	7.34	1.62	7.50	1.36	7.56	0.85	0.62	0.23	0.74
	eLF	6.97	1.30	7.04	1.32	6.81	0.81			
NEFA (mmol/l)	Control	0.61	0.21	0.61	0.22	0.61	0.26	0.35	0.33	0.37
	eLF	0.76	0.36	0.71	0.23	0.58	0.19			
Non-HDL-cholesterol (mmol/l)	Control	4.12	0.59	4.16	0.55	4.37	0.43	0.61	0.55	0.32
	eLF	4.20	0.69	4.05	0.62	4.05	0.57			

* Repeated-measures ANOVA.

Table 6. Changes in biochemical parameters after taking enteric-coated lactoferrin (eLF) or control tablets for 8 weeks (Mean values and standard deviations for thirteen subjects per group)

Parameter	Group	Week 0		Week 8		<i>P</i> *		
		Mean	SD	Mean	SD	Time	Group	Time × group
Total protein (g/l)	Control	74	4	74	4	0.45	0.83	0.66
	eLF	73	4	74	5			
Albumin (g/l)	Control	45	2	45	2	0.17	0.78	0.34
	eLF	45	3	46	3			
Albumin:globulin ratio	Control	1.59	0.09	1.59	0.14	0.26	0.39	0.41
	eLF	1.63	0.21	1.66	0.22			
Glutamic oxaloacetic transaminase (U/l)	Control	29	14	28	11	0.22	0.084	0.79
	eLF	23	7	21	5			
Glutamic pyruvate transaminase (U/l)	Control	37	21	34	19	0.036	0.44	0.64
	eLF	32	29	27	19			
Lactate dehydrogenase (U/l)	Control	214	29	211	29	0.21	0.21	0.76
	eLF	201	34	196	22			
Alkaline phosphatase (U/l)	Control	233	51	240	59	0.076	0.34	0.87
	eLF	214	42	222	39			
γ-Glutamyl transferase (U/l)	Control	47	31	46	32	0.31	0.46	0.47
	eLF	65	74	58	58			
Total bilirubin (μmol/l)	Control	9.4	3.6	10.1	4.1	0.19	0.87	0.84
	eLF	11.6	3.1	12.7	4.3			
Direct bilirubin (μmol/l)	Control	2.7	1.2	2.6	1.4	0.79	0.072	0.79
	eLF	3.4	1.0	3.4	1.2			
Creatinine (μmol/l)	Control	62	17	63	17	0.53	0.66	0.44
	eLF	60	14	59	15			
Blood urea N (mmol/l)	Control	4.7	1.1	5.0	1.4	0.16	0.3	0.64
	eLF	4.3	1.0	4.5	0.9			
Uric acid (mmol/l)	Control	0.34	0.12	0.36	0.12	0.59	0.36	0.063
	eLF	0.32	0.07	0.31	0.06			
Creatine kinase (U/l)	Control	120	41	124	60	0.79	0.12	0.82
	eLF	97	43	97	25			
C-reactive protein (mg/l)	Control	1.7	1.2	4.7	11.9	0.54	0.51	0.23
	eLF	2.5	2.6	1.5	1.5			
Blood glucose (mmol/l)	Control	3.5	0.9	3.5	0.5	0.74	0.6	0.78
	eLF	3.7	1.2	3.6	0.7			
Glycosylated HbA1c (%)	Control	5.6	0.6	5.3	0.5	<0.0001	0.79	0.59
	eLF	5.7	1.2	5.4	1.0			
Insulin (μg/l)	Control	0.40	0.28	0.45	0.24	0.35	0.75	0.12
	eLF	0.58	0.94	0.41	0.57			

* Repeated-measures ANOVA.

inhibits the plasma clearance of chylomicrons in the mouse⁽³⁷⁾. Moreover, Hofmann *et al.* reported that LRP1 is expressed in visceral fat and modulates postprandial lipid transport and glucose homeostasis in mice⁽³⁸⁾. These findings suggest that LF may bind LRP1 to block incorporation of lipid in the visceral fat. Further experimentation should be conducted to validate these hypotheses, as well as to determine the LF distribution after administration of eLF.

In summary, this trial clarified that the ingestion of eLF for an 8-week period can reduce visceral fat in men and women without the need for any lifestyle change. Additional analysis, with larger sample sizes, of this potential to prevent obesity and decrease risk of the metabolic syndrome is clearly warranted.

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advice for the design; T. O. and N. S. contributed to interpretation of the data and statistical analysis; M. M. helped T. O. to write the manuscript. All authors read and approved the final version of the manuscript.

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References

1. Tomita M, Bellamy W, Takase M, *et al.* (1991) Potent antibacterial peptides generated by pepsin digestion of bovine lactoferrin. *J Dairy Sci* **74**, 4137–4142.
2. Harmsen MC, Swart PJ, de Béthune MP, *et al.* (1995) Antiviral effects of plasma and milk proteins: lactoferrin shows potent activity against both human immunodeficiency virus and human cytomegalovirus replication *in vitro*. *J Infect Dis* **172**, 380–388.
3. Zimecki M, Własczyk A, Cheneau P, *et al.* (1998) Immunoregulatory effects of a nutritional preparation containing bovine lactoferrin taken orally by healthy individuals. *Arch Immunol Ther Exp* **46**, 231–240.
4. Shoji H, Oguchi S, Shinohara K, *et al.* (2007) Effects of iron-unsaturated human lactoferrin on hydrogen peroxide-induced

- oxidative damage in intestinal epithelial cells. *Pediatr Res* **61**, 89–92.
5. Sekine K, Ushida Y, Kuhara T, *et al.* (1997) Inhibition of initiation and early stage development of aberrant crypt foci and enhanced natural killer activity in male rats administered bovine lactoferrin concomitantly with azoxymethane. *Cancer Lett* **121**, 211–216.
 6. Suzuki N, Kigawa H, Ono T, *et al.* (2007) Potent action of bovine lactoferrin against pathogenesis factors of periodontal disease: alteration of gene expression profiles of human gingival fibroblast by lactoferrin. In *The Lactoferrin 2007*, pp. 68–72 [H Thuda, K Shimazaki and K Tanaka, editors]. Tokyo: Nihon Igakukan.
 7. Takeuchi T, Shimizu H, Ando K, *et al.* (2004) Bovine lactoferrin reduces plasma triacylglycerol and NEFA accompanied by decreased hepatic cholesterol and triacylglycerol contents in rodents. *Br J Nutr* **91**, 533–538.
 8. Tamano S, Sekine K, Takase M, *et al.* (2008) Lack of chronic oral toxicity of chemopreventive bovine lactoferrin in F344/DuCrj rats. *Asian Pac J Cancer Prev* **9**, 313–316.
 9. Tokunaga K, Matsuzawa Y, Ishikawa K, *et al.* (1983) A novel technique for the determination of body fat by computed tomography. *Int J Obes* **7**, 437–445.
 10. Richmond W (1973) Preparation and properties of a cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin Chem* **19**, 1350–1356.
 11. Sugiuchi H, Uji Y, Okabe H, *et al.* (1995) Direct measurement of high-density lipoprotein cholesterol in serum with polyethylene glycol-modified enzymes and sulfated α -cyclodextrin. *Clin Chem* **41**, 717–723.
 12. Kanno T, Sakurabayashi I, Saito Y, *et al.* (1997) Evaluation of a new assay for determination of LDL-cholesterol. *Jpn J Med Pharm Sci* **37**, 635–644.
 13. Eggstein M & Kreutz FH (1966) A new determination of the neutral fats in blood serum and tissue. I. Principles, procedure, and discussion of the method (article in German). *Klin Wochenschr* **44**, 262–267.
 14. Frings CS & Dunn RT (1970) A colorimetric method for determination of total serum lipids based on the sulfo-phospho-vanillin reaction. *Am J Clin Pathol* **53**, 89–91.
 15. Okabe H, Uji Y, Nagashima K, *et al.* (1980) Enzymic determination of free fatty acids in serum. *Clin Chem* **26**, 1540–1543.
 16. Gornall AG, Bardawill CJ & David MM (1949) Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177**, 751–766.
 17. Doumas BT, Watson WA & Biggs HG (1971) Albumin standards and the measurement of serum albumin with bromocresol green. *Clin Chim Acta* **31**, 87–96.
 18. Japan Society of Clinical Chemistry (2005) Recommendations <http://www.jscc-jp.gr.jp/eng/measurement.html> (accessed 10 February 2005).
 19. Otsuji S, Mizuno K, Ito S, *et al.* (1988) A new enzymatic approach for estimating total and direct bilirubin. *Clin Biochem* **21**, 33–38.
 20. Tanganelli E, Prencipe L, Bassi D, *et al.* (1982) Enzymic assay of creatinine in serum and urine with creatinine iminohydrolase and glutamate dehydrogenase. *Clin Chem* **28**, 1461–1464.
 21. Tabacco A, Meiattini F, Moda E, *et al.* (1979) Simplified enzymic/colorimetric serum urea nitrogen determination. *Clin Chem* **25**, 336–337.
 22. Gochman N & Schmitz JM (1971) Automated determination of uric acid, with use of a uricase-peroxidase system. *Clin Chem* **17**, 1154–1159.
 23. Férard G, Goester C, Klumpp T, *et al.* (1980) Evaluation of immunonephelometry of C-reactive protein in serum. *Clin Chem* **26**, 782–783.
 24. Sugiura M & Hirano K (1977) A new colorimetric method for determination of serum glucose. *Clin Chim Acta* **75**, 387–391.
 25. John WG (1996) Hemoglobin A1c measurement: new precise immunoassay method involving latex particle agglutination. *Clin Chem* **42**, 1874–1875.
 26. Yoshioka M, Taniguchi H, Kawaguchi A, *et al.* (1979) Evaluation of a commercial enzyme immunoassay for insulin in human serum, and its clinical application. *Clin Chem* **25**, 35–38.
 27. Ministry of Education, Culture, Sports, Science and Technology, Japan (2005) Standard Tables of Food Composition in Japan, Fifth Revised and Enlarged Edition (website in Japanese). http://www.mext.go.jp/b_menu/shingi/gijyutu/gijyutu3/toushin/05031802.htm (accessed November 2009).
 28. Shimazaki K & Yoshimoto Y (1988) Distribution of bovine lactoferrin in curd and whey (article in Japanese). *Jpn J Dairy Food Sci* **37**, A106–A108.
 29. Moreno-Navarrete JM, Ortega FJ, Bassols J, *et al.* (2008) Association of circulating lactoferrin concentration and 2 non-synonymous LTF gene polymorphisms with dyslipidemia in men depends on glucose-tolerance status. *Clin Chem* **54**, 301–309.
 30. Moreno-Navarrete JM, Ortega FJ, Bassols J, *et al.* (2009) Decreased circulating lactoferrin in insulin resistance and altered glucose tolerance as a possible marker of neutrophil dysfunction in type 2 diabetes. *J Clin Endocrinol Metab* **94**, 4036–4044.
 31. Cani PD, Amar J, Iglesias MA, *et al.* (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* **56**, 1761–1772.
 32. Wakabayashi H, Takase M & Tomita M (2003) Lactoferricin derived from milk protein lactoferrin. *Curr Pharm Des* **9**, 1277–1287.
 33. Takeuchi T, Kitagawa H, Harada E, *et al.* (2004) Evidence of lactoferrin transportation into blood circulation from intestine via lymphatic pathway in adult rats. *Exp Physiol* **89**, 263–270.
 34. Fischer R, Debbabi H, Blais A, *et al.* (2007) Uptake of ingested bovine lactoferrin and its accumulation in adult mouse tissues. *Int Immunopharmacol* **7**, 1387–1393.
 35. Yagi M, Suzuki N, Takayama T, *et al.* (2008) Lactoferrin suppresses the adipogenic differentiation of MC3T3-G2/PA6 cells. *J Oral Sci* **50**, 419–425.
 36. Moreno-Navarrete JM, Ortega FJ, Ricart W, *et al.* (2009) Lactoferrin increases (172Thr) AMPK phosphorylation and insulin-induced (p473Ser)AKT while impairing adipocyte differentiation. *Int J Obes (Lond)* **33**, 991–1000.
 37. Crawford SE & Borensztajn J (1999) Plasma clearance and liver uptake of chylomicron remnants generated by hepatic lipase lipolysis: evidence for a lactoferrin-sensitive and apolipoprotein E-independent pathway. *J Lipid Res* **40**, 797–805.
 38. Hofmann SM, Zhou L, Perez-Tilve D, *et al.* (2007) Adipocyte LDL receptor-related protein-1 expression modulates post-prandial lipid transport and glucose homeostasis in mice. *J Clin Invest* **117**, 3271–3282.