



Randomized control trials

Influence of a fermented protein-fortified dairy product on serum insulin-like growth factor-I in women with anorexia nervosa: A randomized controlled trial



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SUMMARY

Background & aims: Patients with anorexia nervosa (AN) have low serum IGF-I levels that may contribute to a lower bone mineral mass. We investigated the effects of a fermented, protein-fortified, dairy product on serum IGF-I levels in patients with AN during an in-hospital refeeding program.

Methods: In this multicenter, randomized, double-blind, placebo-controlled, clinical trial conducted at 3 university hospitals and 3 private clinics in France and Switzerland, 62 women recently admitted with confirmed AN and with a baseline low serum IGF-I level were randomized to 2 daily isocaloric fresh cheese pots containing either 15 g/150 g or 3 g/150 g (controls) of protein for 4 weeks. The primary outcome was the change in IGF-I levels.

Results: In the primary intention-to-treat analysis, mean serum IGF-I levels increased during the intervention phase from 22.9 ± 1.5 to 28.6 ± 1.3 nmol/L (means \pm SEM) (+20.2%) in the intervention group and from 20.2 ± 1.2 to 25.7 ± 1.5 nmol/L (+16.8%) in controls. In a preplanned analysis of covariance with repeated measures, the between-group difference was close to statistical significance ($P = 0.071$). In a post-hoc mixed-regression model analysis, the difference was statistically significant (4.9 nmol/l increase; $P = 0.003$), as was the change of the ratio IGF-I/IGF-BP3 ($P = 0.004$). There was no between-group difference in biochemical markers of bone turnover (osteocalcin, P1NP, CTX) or in serum parathyroid hormone level. Serum calcium levels slightly increased during the intervention phase in the higher protein group ($P = 0.02$). IGF-BP2 decreased significantly more in the intervention group during the follow up period at week 4 after supplements cessation ($P = 0.019$).

Conclusions: Intake of a fermented, protein-fortified, isocaloric dairy product during 4 weeks may slightly increase serum IGF-I levels in women with AN, without significant changes in bone turnover markers.

Clinical Trial Registration Number: NCT01823822 (www.clinicaltrials.gov).

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1. Introduction

Anorexia nervosa (AN) is associated with low bone mass [1] and increased fracture risk [2]. In 80% of women with AN, bone mineral

density (BMD) is reduced by at least 1.0 SD at one or more skeletal sites, and by at least 2.5 SD in 17% [1]. Potential mechanisms of bone loss include a decrease in gonadal steroid concentrations [3] and/or in serum IGF-I levels [4,5]. In patients with AN, altered bone turnover results from an imbalance between normal or higher bone resorption related to estrogen deficiency [6] and lower bone formation resulting from reduced serum IGF-I. The latter is correlated to body weight and body mass index (BMI) [5,7]. IGF-I levels are consistently found lower in AN [5,8–12], with values below the lower limit of normal range in 58% of the cases [1], or -1.74

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to -4.98 standard deviation scores as compared with means in age-matched healthy controls [9,10]. A study showed that IGF-I represents a biochemical marker of malnutrition and a sensitive index of nutritional repletion in patients with eating disorders [9].

In humans, serum IGF-I concentrations are markedly lowered by energy and/or protein deprivation [13]. This suggests that low IGF-I may play an important role in the pathogenesis of bone loss in women with AN.

Higher protein intakes prevent the decrease in IGF-I observed in hypocaloric states [14]. Recombinant human IGF-I increased markers of bone turnover in adult women with AN. At low dose, the bone formation marker procollagen type 1 amino-terminal propeptide (P1NP) increased, but without changes in markers of bone resorption [5]. Recombinant human IGF-I potentiated the effects of oral contraceptives on BMD [6].

In rats, an isocaloric low protein diet was associated with decreased IGF-I levels, estrogen deficiency, and lower markers of bone formation with unchanged markers of bone resorption, suggesting an uncoupling in bone turnover, accounting for the decreased BMD and bone strength [15]. While protein restriction reduced serum IGF-I levels [15], protein replenishment through essential amino acid supplements increased IGF-I, bone formation markers, BMD, and bone strength in adult ovariectomized rats fed an isocaloric low protein diet [16]. Though refeeding of undernourished patients increases IGF-I [17–19], the effects of increasing protein intake on serum IGF-I levels in women with AN remain unknown.

In this randomized controlled trial, we assessed the effects of an oral fermented, protein-fortified, dairy product compared to an isocaloric lower protein containing identical content on serum IGF-I levels and biochemical markers of bone turnover in adult women with AN.

2. Subjects and methods

2.1. Study design

The study was a multicenter, randomized, placebo-controlled, double-blind, trial in 6 centers in France and Switzerland. The trial was conducted between June 2008 and April 2009, in agreement with the principles of the Declaration of Helsinki and the requirements of Good Clinical Practice (clinical Trial Registration Number: NCT01823822 (www.clinicaltrials.gov)). The protocol and its amendments were approved by the institutional review boards of each center. All patients provided written informed consent.

2.2. Participants

Study participants were women aged 18–40 years with a DSM-IV- (Diagnostic and Statistical Manual – Revision IV) confirmed diagnosis of AN (either restricting or binge-eating/purging type) of at least 6 months' duration, with baseline serum IGF-I level below the 25th percentile of age-dependent reference values, and recently admitted to hospital for weight and nutritional rehabilitation. Women having started estro-progestagen therapy before the onset of AN were also included. This may explain the absence of amenorrhea in some patients. Contraception with either progestagens or non-hormonal methods was required by protocol during the study period. All women were already regular consumers of dairy products. If applicable, they discontinued calcium and vitamin D supplements 1 week before the start of the study. Patients were hospitalized during the entire duration of the 4-week supplementation phase. Exclusion criteria were: primary amenorrhea; lactose intolerance; any metabolic disease affecting bone metabolism; epilepsy; current or past use of bisphosphonates,

calcitonin, or glucocorticoids; and exposure to oral or intravenous nutritional supplements within 2 weeks prior to study inclusion.

2.3. Study setting and inpatient refeeding protocol

Participants were admitted to one of 4 centers in France (2 university hospitals and 2 private clinics) or one of 2 in Switzerland (1 university hospital and 1 private clinic). All centers were units specialized in the care of AN patients. Of 62 women included, 49 (79%) were recruited at the center in Lyon, France. Each center followed its own standard oral refeeding protocol. High-energy liquid supplement drinks were not allowed during the study. No subject received nasogastric tube therapy. A nurse was present during all meals and snacks. Diets started with a low amount of calories and increased progressively during hospital stay to avoid a refeeding syndrome. The amount of calorie intake provided by meals during the study was not recorded.

2.4. Intervention

The test product was composed of 2 pots of 75 g of sweetened, flavored fresh cheese rich in milk protein (15 g/150 g) given as mid-morning and afternoon snacks at least 5 days/week for 4 consecutive weeks. The control product was 2 pots of 75 g of sweetened, flavored fresh cheese with low protein content (3 g/150 g). Both study products (supplied by Danone Company, Palaiseau, France) were isocaloric by adapting carbohydrate content, and with similar organoleptic characteristics (supplemental content). They provided additional 219 kcal/d, 5.5 g/d lipids, 350 mg/d calcium, 5 µg/d (200 IU) vitamin D and 2.5 mg/d zinc.

2.5. Study conduct

As shown in Fig. 1, patients were screened at a selection visit (SV), 1 week before inclusion. If serum IGF-I was below the 25th percentile for age and all inclusion criteria were met, patients attended a baseline visit (T0) and were randomized to either the test (active group) or the control product (controls). Thereafter, 4 bi-weekly evaluations were scheduled: 2 during the 4-week intervention phase, and 2 during the 4-week follow-up. Height was determined with a stadiometer. Weight was measured with a calibrated scale to the nearest 0.1 kg. Height, blood pressure, and cardiac frequency (after 3-min sitting) were recorded at SV, and body weight at each study visit.

2.6. Randomization and blinding

At each site, patients were recruited and randomized 1:1 through a computer-generated list randomization using permutation blocks of 4, and stratified by study center. Patients were randomized at T0 using an Interactive Web Response System procedure and assigned a randomization number according to chronological order of inclusion. Double blind was maintained during the entire study in both groups with regard to study products (similar energy content and organoleptic characteristics, same packaging, labeling, administration, and dosage). At the end of the study, a blind review meeting of data was performed before the database was frozen and the analyses started.

2.7. Laboratory variables

Blood collection and analytical assays were performed in accordance with Good Laboratory Practice. All patients had 6 blood samples taken under fasting conditions (one at SV, T0, and evaluation (E) visits E1, E2, E3, and E4). All analyses were performed in a

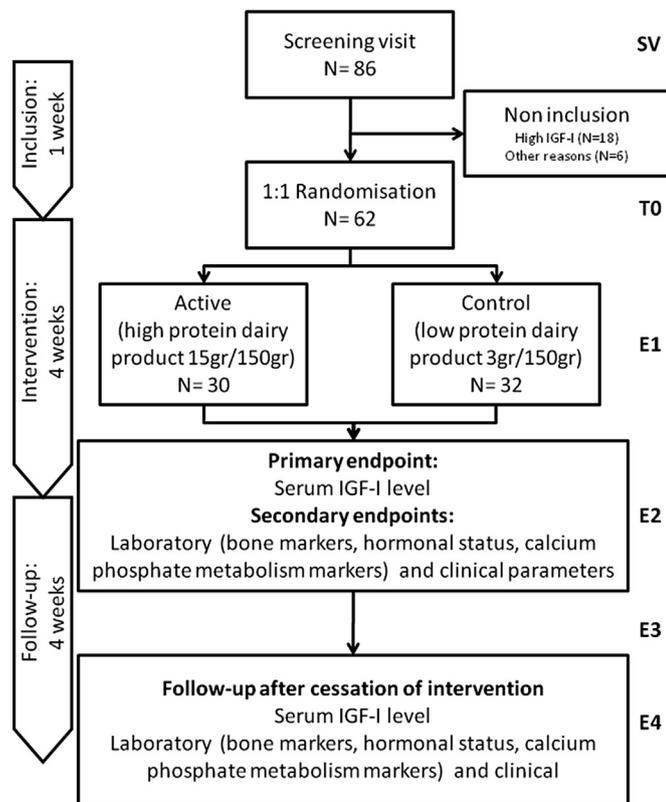


Fig. 1. Patient flow, study groups, and endpoints.

central laboratory. IGF-I was measured by a chemiluminescence-based immunoassay (Liaison[®], Diasorin, Saluggia, Italy) with intra- and interassay coefficients of variation lower than 4.4%. For IGF-I, the samples were processed as follows: SV samples were sent frozen immediately and analyzed at reception; T0 and E1–E4 samples were frozen at -20°C on site, sent to the central laboratory in dry ice, stored at -80°C immediately upon reception, and until the assays were performed batchwise.

Plasma and urinary calcium, phosphate, and creatinine were measured with an automatic analyzer. Plasma calcium was adjusted for albumin levels (adjusted calcium = measured calcium [mmol/L] + (40 - albumin [g/L]) \times 0.02). The marker of bone formation, P1NP, was determined by radioimmunoassay (Immunodiagnosics, Inc., Boldon, UK); osteocalcin was measured by chemiluminescent immunoassay (Liaison[®], Diasorin, Saluggia, Italy), and bone-specific alkaline phosphatase [BSAP] by chemiluminescent assay (Access[®], Beckmann Coulter, Marseille, France). The serum bone resorption marker, beta-C-terminal telopeptide of type I collagen [CTX], was measured by enzyme-linked immunosorbent assay (Immunodiagnosics, Inc.). IGF-BP-2 and IGF-BP-3 were measured with an ELISA (Mediagnost, Reutlingen, Germany). Intact parathyroid hormone [iPTH] was determined by paramagnetic-particle chemiluminescent assay (Access[®]). Serum 25-hydroxyvitamin D3 (25-OH-D3) was determined by chemiluminescence assay (Liaison[®]), while calcitriol was measured by radioimmunoassay (Immunodiagnosics, Inc.).

2.8. Outcomes

The primary outcome was serum IGF-I changes over the 4-week supplement consumption period (between T0 and E2). IGF-binding protein 2 (IGF-BP2) and IGF-BP3 were measured and the serum

IGF-I/IGF-BP3 ratio calculated. Secondary outcomes included IGF-I changes during the 4 weeks after supplements cessation, changes over 4 week supplementation in IGFBP2 and IGFBP3, and in bone metabolism parameters (P1NP, BSAP, osteocalcin, and CTX, 25OHD3, iPTH, total serum calcium, and albumin corrected-calcium), and during the follow-up phase (E2 to E4, and over the entire 8-week observation phase (T0 to E4), together with the safety of protein supplements.

2.9. Sample size calculation

To detect an expected difference of 45% for serum IGF-I (50% reported in elderly patients receiving a protein supplement [17]) with a power of 70% and a two-tailed P -value of 0.05, 29 assessable patients were required per group. With a 15% drop-out rate and a 25% screening failure, 92 patients were planned to be screened for 68 randomized. Because of the limit of the fermented dairy products production date, only 62 subjects could be enrolled.

2.10. Statistical methods

Descriptive statistics were computed as means \pm SEM for continuous variables or as number and percentages for categorical variables. Normality was verified by using Shapiro-Francia tests. Imbalance between groups at baseline was examined using t -tests or Wilcoxon rank sum tests for continuous data, and χ^2 or Fisher's exact tests for categorical data.

The primary analysis was per intention-to-treat (ITT), which included all randomized subjects having taken at least one dose of the study product. Missing data were replaced by the Last Observation Carried Forward (LOCF) method using the last post-baseline value per phase (intervention or follow-up phase). The type I error was two-sided and fixed at 5% maximum for all tests. A per protocol (PP, $n = 51$) secondary analysis was also preplanned.

The main analysis consisted in an analysis of covariance (ANCOVA) model with repeated measures at T0, E1, and E2 visits on all normally distributed parameters with the study group as fixed effect and covariates (baseline value, center as a random effect, and age). Secondary analyses, including secondary outcomes, used a similar methodology extended to datasets between T0 and E4 and E2 and E4. Data were also analyzed as follows: 1) without adjustment for baseline covariates; 2) by a PP analysis of study completers without imputation; and 3) using a post-hoc linear mixed-effects regression model analysis (with Stata "xtmixed" command) to predict the primary end point, with visit, group, intervention, age, and visit and center by intervention interaction as exploratory variables. In addition, we analyzed the effect of estrogen use and baseline protein intakes on the primary outcome. Mixed-effects regression takes into account all measures, including the fact that they are repeated within the same subject, without the need for imputation. It allows to also dissociate group (determined by randomization) and intervention effects (a variable), which specifies if the treatment was given at a given visit.

3. Results

Eighty-six women attended the screening visit (Fig. 1). Sixty-two aged 18–37 years (22.5 ± 0.5 years) with AN since 4.7 ± 0.5 years met the inclusion criteria and were randomized to the active ($n = 30$) or control ($n = 32$) groups. Duration of secondary amenorrhea was 24.3 ± 3.9 months, BMI was 16.1 ± 0.3 kg/m², and daily calcium and protein intake were 896 ± 56 mg and 52 ± 4 g, respectively. Eighteen women (29%) were taking oral sex hormones. Only 5 patients had a fracture history with 2 non-traumatic fractures among the 16 prevalent fractures. Characteristics were

Table 1
Patient baseline characteristics.

	Active high protein dairy product (15 g/150 g)	Controls low protein dairy product (3 g/150 g)
Number	30	32
Age (years)	22.2 ± 0.6	22.8 ± 0.9
Weight (kg)	42.7 ± 1.2	43.1 ± 1.5
BMI	16.1 ± 0.4	16.0 ± 0.5
Anorexia nervosa duration (years)	4.4 ± 0.7	5.0 ± 0.8
Time since last menstrual period (months)	22.8 ± 4.4	25.6 ± 6.2
Calcium intake (mg/day)	908 ± 94	884 ± 64
Total protein intake (g/day)	57 ± 6	47 ± 4
Women on sex hormones (%)	8 (27%)	10 (31%)

Values are means ± SEM. BMI = body mass index.

well balanced between study groups (Table 1). Mean product exposure was 26.8 ± 0.5 days with comparable compliance between the active and control groups (90 ± 4% and 94 ± 3%, respectively). Mean study duration was 57.3 ± 0.2 days. Five patients of the active group and 3 patients of the control group took at least one other unauthorized alimentary or nutritional product during the study.

3.1. Changes in serum IGF-I levels

In the primary ITT analysis (center as a random effect), mean serum IGF-I increased during the intervention phase (T0 to E2) from (mean ± SEM) 22.9 ± 1.5 nmol/L to 28.6 ± 1.3 nmol/L in the active group (+20.2%), and from 20.2 ± 1.2 nmol/L to 25.7 ± 1.5 nmol/L in controls (+16.8%), (between-group: $P = 0.071$; Table 2). These results were robust as confirmed by the secondary ITT analysis (center as fixed effect; $P = 0.074$) and the PP analysis (after exclusion of protocol violators; $P = 0.175$). During the follow-up phase (E2 to E4), mean serum IGF-I levels remained stable and reached 27.3 ± 1.8 nmol/L and 26.4 ± 1.8 nmol/L in the former active and control groups, respectively. A similar trend of statistical significance (ANCOVA) was observed with $P = 0.086$ in the ITT analysis and $P = 0.051$ in the PP analysis. Data were also analyzed using linear mixed-effects regression models to predict the primary endpoint when taking into account all available values during the 5 visits (Table 3). The effect of the intervention on serum IGF-I levels were significant with a 4.9 nmol/l increase during the intervention phase at visits T0 to E2, ($P = 0.003$) while adjusting for visit, group, intervention, age, and visit and center by intervention interaction. Adjusting for baseline protein intake or for estrogen consumption

Table 2
Changes in serum IGF-I: ANCOVA model with repeated measurements.

	Normal range	Active			Controls		
		High protein	Dairy product	(15 g/150 g)	Low protein	Dairy product	(3 g/150 g)
		T0 (baseline)	E2 (+4 weeks)	E4 (+8 weeks)	T0 (baseline)	E2 (+4 weeks)	E4 (+8 weeks)
Serum IGF-I (nmol/L)	14.0–61.0	22.9 ± 1.5	28.6 ± 1.3	27.4 ± 1.8	20.2 ± 1.2	25.7 ± 1.5	26.4 ± 1.8
Study product effect on Adjusted mean (nmol/L)*	serum IGF-I,	from T0 to E2,	center as random effect,	ITT set,	ANCOVA	model	
Raw change (nmol/L)			25.8 (0.9)			25.1 (0.9)	
Relative change (%)			4.3			3.6	
Study product effect**			20.2			16.8	
				$P = 0.071$			

Values are means ± SEM. ** ANCOVA model with repeated measurements on serum IGF-I values with study group as explicative factor, age ($P = 0.036$), baseline value ($P < 0.001$), and center as covariates (center as random effect, centers N° 1, 4, 5, 7, 8 being pooled). Best structure of covariance: repeated measurements: AR(1)-random effect: CS. Following interactions were tested: product*baseline, product*center ($P = 0.012$), product*visit ($p = 0.493$) and product*visit*center.

Table 3
Changes in serum IGF-I: mixed-effects regression model.

	Coef.	95% CI		P-value
Serum IGF-I baseline values (nmol/L)	0.756	0.646	0.866	<0.001
Intervention	4.907	1.627	8.186	0.003
Group	−0.832	−2.633	0.968	0.365
Age	−0.297	−0.492	−0.102	0.003
Study visit				
T0	0.000			–
E1	3.770	1.871	5.669	<0.001
E2	4.690	2.704	6.676	<0.001
E3	6.305	4.732	7.878	<0.001
E4	5.590	4.071	7.109	<0.001

Mixed-effects regression model coefficient (coef.) with their 95% confidence interval predicting IGF-I values with subjects as random factor, while adjusting for visit, group, intervention, age, visit by intervention interaction and center by intervention. N = 284 observations in 62 patients. Observation by patient: minimum 2, maximum 5 (average 4.6).

did not change the results (data not shown). There was no significant group effect over the 5 visits ($P = 0.365$).

3.2. Changes in other biochemical parameters

No significant between group difference was observed with regard to IGF-BP3. Using a linear mixed-effects regression model, we observed a significant effect of the intervention on the IGF-I/IGF-BP3 ratio ($P = 0.004$). During the whole study, IGF-BP2 decreased significantly more at the last follow up visit in the intervention group (Table 4, $P = 0.019$, interaction term (time × group): $P = 0.065$). During the intervention phase (T0 to E2), the mean albumin-adjusted total serum calcium change from baseline was +0.03 ± 0.02 mmol/L vs −0.01 ± 0.02 mmol/L in the intervention and control groups, respectively ($P = 0.015$). This difference was confirmed over the entire observation period of 8 weeks ($P = 0.003$). Mean albumin-adjusted serum calcium between T0 and E4 was 2.26 ± 0.01 vs 2.22 ± 0.01 mmol/L in the active and control groups, respectively ($P = 0.025$). Serum CTX levels changed from 1.08 ± 0.09 ng/mL to 0.96 ± 0.53 and from 1.11 ± 0.09 ng/mL to 0.98 ± 0.06 ng/mL in the active and control groups, respectively (NS). No statistically significant product effect was demonstrated on other laboratory parameters in the ITT or PP data sets (data not shown), in particular, hormone levels. (Table 4).

3.3. Changes in clinical parameters and safety

No product-related side effects were observed during the consumption phase or follow-up. Safety and tolerance were excellent,

Table 4
Changes in secondary outcome parameters.

	Normal range	Active			Controls		
		High protein	Dairy product	(15 g/150 g)	Low protein	Dairy product	(3 g/150 g)
		T0 (baseline)	E2 (+4 weeks)	E4 (+8 weeks)	T0 (baseline)	E2 (+4 weeks)	E4 (+8 weeks)
BMI		16.1 ± 0.4	16.4 ± 0.4	17.2 ± 0.4	16.0 ± 0.5	16.9 ± 0.5	17.2 ± 0.4
Weight (kg)		42.7 ± 1.2	43.3 ± 1.0	46.0 ± 1.1	43.1 ± 1.5	44.9 ± 1.5	46.4 ± 1.4
Serum FSH (IU/L)		4.1 ± 0.6	–	5.2 ± 0.7	3.5 ± 0.6	–	3.7 ± 0.5
Serum estradiol (pmol/L)		90.8 ± 15.8	–	115.2 ± 25.5	111.4 ± 23.8	–	160.4 ± 46.5
Serum IGF-I (nmol/L)	14.0–61.0	22.9 ± 1.5	28.6 ± 1.3	27.4 ± 1.8	20.2 ± 1.2	25.7 ± 1.5	26.4 ± 1.8
Serum IGF-BP2 (ng/mL) ^o	84–702	401 ± 42	274 ± 19	312 ± 25	429 ± 43	335 ± 26	378 ± 38
Serum IGF-BP3 (ng/mL)	2010–4950	3069 ± 100	3183 ± 77	3283 ± 88	3021 ± 85	3279 ± 112	3134 ± 110
IGF-I/IGF-BP3 ratio		0.057 ± 0.003	0.069 ± 0.003	0.064 ± 0.004	0.051 ± 0.003	0.061 ± 0.003	0.064 ± 0.004
Serum P1NP (µg/L)	19–83	48.7 ± 4.2	66.0 ± 4.9	75.0 ± 6.1	51.3 ± 4.4	65.0 ± 4.8	71.0 ± 5.0
Serum BSAP (µg/L)	2.9–14.5	7.85 ± 0.49	8.96 ± 0.47	10.35 ± 0.60	8.27 ± 0.71	9.32 ± 0.63	9.97 ± 0.66
Serum osteocalcin (nmol/L)	1.1–7.2	3.15 ± 0.22	3.73 ± 0.23	4.01 ± 0.24	3.77 ± 0.34	4.27 ± 0.38	4.33 ± 0.35
Serum CTX (ng/mL)	0.112–0.738	1.08 ± 0.09	0.94 ± 0.08	0.96 ± 0.11	1.11 ± 0.09	1.01 ± 0.08	0.98 ± 0.06
Serum 25OHD3 (nmol/L)	75–250	68.3 ± 5.6	70.0 ± 4.7	65.8 ± 4.4	67.3 ± 4.5	71.4 ± 4.2	70.1 ± 4.8
Serum intact PTH (pmol/L)	1.3–9.3	2.55 ± 0.21	2.73 ± 0.18	2.58 ± 0.17	2.81 ± 0.16	2.86 ± 0.18	2.98 ± 0.23
Serum calcium (mmol/L)*	2.15–2.55	2.35 ± 0.01	2.38 ± 0.02	2.39 ± 0.02	2.34 ± 0.02	2.33 ± 0.02	2.34 ± 0.02
Serum albumin (g/L)	34–48	43.17 ± 0.48	45.18 ± 0.67	45.33 ± 0.68	43.34 ± 0.75	44.43 ± 0.79	45.04 ± 0.88
Albumin-corrected serum calcium (mmol/L)**	2.15–2.55	2.27 ± 0.01	2.25 ± 0.01	2.26 ± 0.01	2.26 ± 0.01	2.23 ± 0.01	2.22 ± 0.01

All values are means ± SEM. BMI = body mass index; IGF-BP = IGF binding protein; P1NP = procollagen type 1 amino-terminal propeptide; BSAP = bone-specific alkaline phosphatase; CTX = carboxy-terminal collagen crosslinks. * Difference between groups T0 to E2: $P = 0.015$, interaction term (time × group): $P = 0.02$; Difference between groups T0 to E4: $P = 0.003$, interaction term (time × group): $P = 0.716$; ** Difference between groups T0 to E4: $P = 0.025$, interaction term (time × group): $P = 0.358$. ^o Difference between groups T0 to E4: $P = 0.019$, interaction term (time × group): $P = 0.065$.

without any serious adverse event or withdrawal due to adverse events.

4. Discussion

This is the first randomized controlled trial comparing the effects of higher (15 g per day) vs. lower (3 g per day) protein supplements in the form of fermented dairy products on biochemical variables of bone metabolism in adult women with AN. The increase in the primary endpoint serum IGF-I levels showed a trend in favor of the higher protein supplement.

IGF-I levels are consistently found lower in AN [5,8,10–12], with values below the lower limit of normal range in 58% of the cases [1]. Low serum IGF-I levels are a key contributor to bone loss/low bone mass and altered trabecular microarchitecture in women with AN [20]. In addition, a low serum IGF-I level is a risk factor for osteoporotic fracture in post-menopausal women [21]. Low serum IGF-BP-3 and maintained serum IGF-BP-2 levels may also contribute to bone loss [22]. A reduction in dietary protein leads to lower calcium absorption, secondary hyperparathyroidism [23], and increased bone turnover [24]. Protein restriction reduces serum IGF-I levels through a resistance to the action of growth hormone at the hepatic level [13,25] and an increased IGF-I metabolic clearance rate [26]. In rats, an isocaloric low protein diet decreases BMD and bone strength. This is associated with an early decrease in serum IGF-I and of bone formation with unchanged, or followed by increased bone resorption [15]. Essential amino acids administration increases serum IGF-I as well as bone formation, and decreases bone resorption markers [16]. Furthermore, protein supplements were shown to increase serum IGF-I levels and to attenuate proximal femur bone loss in patients with a recent hip fracture [17].

Under these conditions, we postulated that stimulating endogenous IGF-I secretion through a higher protein intake may improve bone health in women with AN. Serum IGF-I levels increased in both the lower and higher protein supplement groups during the 4-week intervention phase and the difference between groups almost reached statistical significance ($P = 0.071$) with an ANCOVA, but was significant with a linear mixed-effects regression model

($P = 0.003$). Interestingly, all these values remained constant during the follow-up phase, i.e., 4 weeks after intervention cessation. This could be related to the increase in body weight over the 8-week study period, which similarly increased in both groups. Indeed, body weight recovery leads to an increase in serum IGF-I levels followed by a change in biochemical markers of bone turnover and BMD [22,27–32]. Over the 4-week supplementation and 4-week follow-up, there was no difference in the biochemical markers of bone turnover osteocalcin, P1NP and CTX, or in PTH levels, suggesting that the slight change in IGF-I was not accompanied by some biological response. However, whether longer duration supplementation and/or higher amounts of supplements may translate into changes of bone turnover, BMD or even fracture risk, would deserve further investigation. The increase in weight and BMI may correspond to an improved general nutritional status during hospitalization and may have blunted the difference in serum IGF-I changes between groups. The expected difference used for the power calculation was extrapolated from results obtained in malnourished elderly patients with hip fractures receiving protein supplements [17] and may be larger than the difference that should be expected in young female patients with AN.

In most studies in AN, the percentage of energy provided by proteins is preserved [33]. Nevertheless, the absolute protein intakes are decreased in the more severe forms of the disease, or at least insufficient to ensure a positive energy balance. However, AN patients tend to over-report protein-energy intake [34]. Despite a strict randomization procedure, there was a slight non-statistically significant imbalance in baseline spontaneous dietary protein intake in favor of the active group. This may have contributed to the trend to higher baseline IGF-I values in this group and to a blunted response to the higher protein supplement.

The isocaloric formulation of the fermented dairy products used in the present study was obtained by reducing the carbohydrate content of the protein-supplemented preparation. Protein intake requires the presence of adequate energy to raise serum IGF-I levels after fasting and the carbohydrate content of the diet is a major determinant of the responsiveness of IGF-I to growth hormone [13]. This might be an alternative explanation for the lower than

expected between-group serum IGF-I response difference. Furthermore, the difference in the protein content between the two formulations (12 g) may be too low to induce a significant difference in the IGF-I response.

In our study, higher compared to lower protein supplements increased serum albumin-corrected calcium levels during the intervention phase, statistically, but very likely without a clinical and/or biological relevance. This effect lasted for at least 4 weeks after the intervention cessation, while serum iPTH remained stable and non-significantly different between groups. Increased dietary protein intake, particularly through aromatic amino acids present in large amounts in dairy products, has been shown to increase intestinal calcium absorption [35], most likely through an IGF-I-dependent process [36] inducing thereby an increase in urinary calcium excretion [37,38]. We also observed during the follow up a larger decrease of IGF-BP2 in the intervention group. IGFBP-2 is a nutritionally controlled protein with an elevation during fasting or during dietary protein restriction [39]. It has a particular sensitivity to protein intake. In a study performed in a normal population, 6 days of protein restriction induced a marked elevation of serum IGFBP-2, while isolated caloric restriction did not change the values [39]. Another study found similar results. In addition renutrition with high protein content, decreased the serum levels of IGFBP-2, while no effect was observed with a refeeding program with a lower protein intake [40]. Moreover, it is known that diet-induced modifications of serum IGFBP-2 occurs after prolonged period [41] and this may explain why we observed in our study a significant decrease only during the last visit of the follow-up period.

The strengths of our study in this highly challenging patient group are the homogeneity of the study population and its conduct in a controlled environment. Including only inpatients ensured a better control of intake than under outpatient conditions. Only adult women under the age of 40 years with AN diagnosed according to DSM-IV criteria, admitted for refeeding, and presenting with lower than 25th percentile serum IGF-I levels at baseline, were included. The use of estrogen-progestagens as contraceptives was balanced between groups. To minimize the influence of milk protein allergy or lactose intolerance, we recruited mainly regular consumers of dairy products.

This study has several limitations. First, the apparent significant results (+20.2 vs + 16.8%) stemmed from a *post hoc* analysis. However, mixed-effects regression has advantages over classical ANCOVA in dealing with repeated measures [42]. It uses all available data of each subject, is unaffected by missing data and thus does not need data imputation. Second, we did not record dietary intakes during the trial and thus we do not know the absolute difference in protein consumed between the groups. We cannot exclude a modification of the diet during the study period. Finally, the changes in absolute IGF-I values were very small. It has still to be demonstrated whether these changes would be clinically meaningful.

5. Conclusion

In adult women with AN, isocaloric protein supplements, provided as fermented dairy products, during 4 weeks, tended to increase serum IGF-I.

Authors' contributions

AT participated to study design, data collection, statistical analysis and interpretation, and writing of the paper. EC participated to protocol design and data collection. AP participated to protocol design and data collection. FL participated to data collection and critical revision of the manuscript. JLV participated to data

collection and critical revision of the manuscript. FRH contributed to the statistical analysis and critical revision of the manuscript. RR participated in the study design, data statistical analysis and interpretation, and writing of the paper.

Competing interests

None declared.

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Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.clnu.2015.10.014>.

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