

## Comparing the effects of whey and casein supplementation on nutritional status and immune parameters in patients with chronic liver disease: a randomised double-blind controlled trial

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### Abstract

Protein supplementation may be beneficial for patients with chronic liver disease (CLD). This study compared the effects of whey protein isolate (WP) and casein (CA) supplementation on nutritional status and immune parameters of CLD patients who were randomly assigned to take 20 g of WP or CA twice per d as a supplement for 15 d. Body composition, muscle functionality and plasmatic immunomarkers were assessed before and after supplementation. Patients were also classified according to the model for end-stage liver disease (MELD) into less (MELD < 15) and more (MELD ≥ 15) severe disease groups. Malnutrition, determined by the Subjective Global Assessment at baseline, was observed in 57.4% and 54.2% of patients in the WP and CA groups, respectively ( $P = 0.649$ ). Protein intake was lower at baseline in the WP group than in the CA group ( $P = 0.035$ ), with no difference after supplementation ( $P = 0.410$ ). Both the WP and CA MELD < 15 groups increased protein intake after supplementation according to the intragroup analysis. No differences were observed in body composition, muscle functionality, most plasma cytokines (TNF, IL-6, IL-1 $\beta$  and interferon- $\gamma$ ), immunomodulatory proteins (sTNFR1, sTNFR2, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor) or immunomodulatory hormones (adiponectin, insulin and leptin) after supplementation in the WP groups at the two assessed moments. WP supplementation increased the levels of interferon- $\gamma$ -induced protein-10/CXCL10 ( $P = 0.022$ ), eotaxin-1/CCL11 ( $P = 0.031$ ) and monocyte chemoattractant protein-1/CCL2 ( $P = 0.018$ ) and decreased IL-5 ( $P = 0.027$ ), including among those in the MELD ≥ 15 group, for whom IL-10 was also increased ( $P = 0.008$ ). Thus, WP consumption by patients with CLD impacted the immunomodulatory responses when compared with CA with no impact on nutritional status.

**Key words:** Chronic liver disease: Whey protein: Supplementation: Nutritional status: Immunomodulation

Patients with chronic liver diseases (CLD) of any aetiology present high mortality rates related to different factors, with malnutrition<sup>(1–4)</sup> playing a key role. Malnutrition is highly prevalent in this population due to impaired nutrient metabolism, early satiety and poor dietary intake, among others<sup>(5)</sup>. In particular, decreased protein intake affects the synthesis of all body

proteins, including those related to the immune response<sup>(6,7)</sup>, and muscle mass, leading to poorer outcomes<sup>(8)</sup>, as deleterious effects on energy metabolism regulation, immune function and systemic inflammation can be expected<sup>(9)</sup>.

The liver is constantly exposed to a wide range of products, including those with inflammatory potential<sup>(10,11)</sup>, requiring

**Abbreviations:** BDNF, brain-derived neurotrophic factor; CA, casein; CLD, chronic liver disease; GDNF, glial cell line-derived neurotrophic factor; IP-10, interferon- $\gamma$ -induced protein-10; MCP-1, monocyte chemoattractant protein-1; MELD, model for end-stage liver disease; WP, whey protein isolate.

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physiological processes to control and/or resolve inflammatory responses<sup>(12,13)</sup>. However, multiple overlapping pathways in various liver diseases mediate the dysregulation of the liver inflammatory response, leading to chronic inflammation, which is a hallmark of chronic infection and autoimmune and malignant diseases<sup>(14)</sup>. Therefore, therapeutic strategies targeting the liver should restore its homeostasis, which means resolving any inflammatory and/or immune dysregulation<sup>(11)</sup>. To favour the synthesis of indispensable proteins responsible for muscle mass maintenance/recovery and the immune response, the choice of high nutritional quality proteins, that is, those that present an adequate amino acid profile, digestibility and bio-availability, might be an alternative therapy for patients with liver disease.

Cows' milk protein, of which casein and whey proteins make up 80 and 20%, respectively, has received increasing attention as a potential health-promoting nutrient source<sup>(15)</sup>. Bovine casein consists of  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins, which represent 37, 10, 35 and 12% of whole casein, respectively. Bovine whey protein contains approximately 50%  $\beta$ -lactoglobulin, 20%  $\alpha$ -lactalbumin and 10% albumin and lactoferrin, with lactoperoxidase making up the remaining source<sup>(16)</sup>.

Although there is a growing body of intervention studies evaluating the effects of these compounds on health<sup>(17–19)</sup>, some authors have suggested that whey proteins could be more effective than casein, as the former present higher digestibility and are rich in essential amino acids, especially the branched-chain amino acids<sup>(20,21)</sup>. Furthermore, bioactive peptides derived from whey proteins seem to exert effects on nutritional status recovery and immune modulation<sup>(22–24)</sup>. While the positive effects on nutritional status are well recognised<sup>(25,26)</sup>, the immunomodulatory effects of whey proteins, especially under disease conditions, have been less frequently reported in clinical trials. Moreover, most of the studies assessing their immune modulatory effects on cell stimulation, proliferation and cytokine secretion have been conducted with whey protein-specific components or peptides<sup>(27–29)</sup> rather than the whole protein as a supplement.

Therefore, the aim of this study was to evaluate the effect of whey protein consumption on the nutritional status of end-stage CLD patients and the role of whey protein in the immune modulatory response. We hypothesised that patients supplemented with whey protein would present improvement of their nutritional status, as well as a more effective immune response, further improving their clinical outcomes.

## Methods

### Subjects

This randomised, double-blind, parallel-arm, clinical trial was carried out with patients with CLD on the waiting list for liver transplantation who were recruited at the Hospital das Clínicas, Universidade Federal de Minas Gerais, Brazil, from July 2014 to August 2016. The study was conducted according to the guidelines laid down in Declaration of Helsinki guidelines, and all procedures involving human patients were approved by the local

Institutional Review Board (Comitê de Ética em Pesquisa - COEP/UFMG), under the number CAAE- 27430714.8.0000.5149. Written informed consent was obtained from all patients. This study was also registered at [ClinicalTrials.gov](https://clinicaltrials.gov) under the number NCT02901119 (<https://clinicaltrials.gov/ct2/show/NCT02901119?term=NCT02901119&draw=2&rank=1>).

The inclusion criteria were age equal to or older than 18 years, patients under regular medical care at the study centre and patients on the waiting list for liver transplantation. Patients who were younger than 18 years old, illiterate, pregnant or breast-feeding; patients with chronic kidney failure or allergies to cows' milk proteins; and those who had previously undergone transplantation were excluded.

Patients and investigators were blinded to the randomisation. Patients were randomised into the whey protein isolate (WP) group (intervention) and the casein (CA) group (control) through block randomisation with a 1:1 allocation ratio. A random number sequence was generated by Microsoft Office Excel 12.0 (Office 2007) to designate the group. The randomisation and allocation were conducted by the blinded researcher. Patients were provided with sachets containing 20 g of WP or CA supplements, packed in bags with a code for each supplement and were asked to consume one in the morning and one in the evening for 15 d. They were also instructed to maintain their regular diet. Phone calls, at least twice a week, were used to monitor the participants and check if they were taking the supplements. Both patients and researchers were unaware of the treatment assignment, which was revealed only after all the established analyses had been carried out. Body composition, RMR, muscle functionality, food consumption and blood parameters were evaluated before and after the protein supplementation period.

### Nutritional status, body composition and RMR assessments

Nutritional status was determined at the beginning of the study through the Subjective Global Assessment, according to Detsky *et al.*<sup>(30)</sup>. Participants were grouped into categories, suspected malnutrition/moderately malnourished and severely malnourished, for comparison purposes. Anthropometric evaluation included the measurement of the mid-arm circumference (cm), obtained with an inelastic tape, and the triceps skinfold thickness (mm) using a Lange skinfold calliper (Cambridge Scientific Industries), and the mid-arm muscle circumference (cm) was calculated from mid-arm circumference and triceps skinfold thickness using the standard formula: mid-arm muscle circumference = mid-arm circumference – (3.1415 × triceps skinfold thickness); both measurements were conducted according to the established procedures<sup>(31,32)</sup>. The values obtained before and after protein supplementation were used to evaluate the intervention effect. Body composition was evaluated by bioelectrical impedance using a single-frequency (electrical current used in the measurement was 800 A and 50 kHz) bioelectrical impedance analyser (Quantum X, RJL Systems) as determined by the current protocols<sup>(33)</sup>. Fat-free mass (kg), fat mass (kg) and phase angle were determined. The RMR was measured



by indirect calorimetry in an open-circuit calorimeter (Cosmed) after an overnight fast. Finally, muscle functionality was evaluated by handgrip strength (kg) using a digital dynamometer (Jamar Handgrip Dynamometer, Preston) considering the best of three consistent readings, allowing about 1 min of recovery between each attempt, as per the protocol<sup>(32,34)</sup>. The 6-min walking test (m) was also performed according to the American Thoracic Society protocol<sup>(35)</sup>.

### Food consumption

Quantitative food intake data were obtained using a 24-h recall. The 24-h recall was completed according to the multiple pass method, whereby the interviewee is guided by five steps<sup>(36)</sup>. This method helps the individual remember the food and drink consumed on the day before the interview and to report them in detail, reducing errors in dietary measurement. Diet Pro4R® software (Agromidia Software) was used to calculate daily energy intake, proteins, carbohydrates and lipids.

### Clinical assessments

The severity of liver disease was graded using the model for end-stage liver disease (MELD) score in which values equal to or higher than fifteen indicate a more severe disease<sup>(37)</sup>. To better characterise the severity of the disease and the potential effect on the protein supplementation responses, patients were categorised according to the MELD cut-off values. This information and information related to the presence of ascites, oedema and encephalopathy were obtained from the medical records and information provided by the physician taking care of the patient.

### Assessment of peripheral biomarkers

Blood was collected in tubes containing heparin or EDTA after an overnight fast before and after protein supplementation. The tubes were centrifuged at 3000 rpm for 10 min at room temperature. The plasma samples were aliquoted in microtubes and stored at  $-80^{\circ}\text{C}$  until the analyses. IL-1 $\beta$ , IL-5, IL-6, IL-10, interferon- $\gamma$ , interferon- $\gamma$ -induced protein-10 (IP-10), TNF- $\alpha$ , eotaxin, monocyte chemoattractant protein-1 (MCP-1), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), leptin and insulin were measured using the Human Premixed Multi-Analyte kit – Magnetic Luminex® Screening Assay (R&D Systems). Briefly, microparticles pre-coated with specific antibodies were added to each well with standards or plasma samples, and they were incubated for 60 min at room temperature in the dark under agitation (900 rpm). After washing the plate (three times with wash buffer), the detection antibody solution was added and the plate was incubated for 30 min at room temperature in the dark. The washing procedure was repeated. A mix with streptavidin-PE solution was added to the plate for 30 min. The washing procedure was repeated again. The samples were resuspended using wash buffer and acquired using the MagPix instrument (Luminex) and xPONENT software (version 3.1; Luminex). The results were converted into pg/ml using the mean fluorescence intensity from the curve standards. The final analysis

was carried out with Analyst software 5.1 (Merck Millipore). The concentration of each molecule was calculated using five-parameter regression models.

The plasma adiponectin and the soluble receptors I and II for TNF (sTNFR1 and sTNFR2) were measured by ELISA sandwich according to the procedures supplied by the manufacturer (R&D Systems).

### Statistical methods

A preliminary sample estimate ( $n$  29 patients) was determined based on a reported handgrip strength mean improvement (before and after the intervention, respectively) in outpatients with decompensated alcoholic liver disease who received 34 g protein supplementation as a casein-based enteral nutrition product in a controlled trial study<sup>(38)</sup>. Accordingly, a power of 80% and a 5% significance level were adopted. Data related to the sampling process were analysed by the Gpower 3.1.

Descriptive analysis included frequency distribution for categorical variables and measurements of mean and standard deviation for quantitative variables since there was a normal distribution according to the Shapiro–Wilk test. Pearson's  $\chi^2$  test was performed to compare the withdrawal causes according to the treatment group.

The intervention effect was obtained by comparing the means between the WP and CA groups after supplementation using the generalised estimating equation model. The aim of this model was to compare the mean values of the plasma biomarkers and anthropometric variables and the prevalence of oedema, ascites and encephalopathy after the intervention.

The categorical outcome variables were treated as having a binomial distribution with log link function, while the quantitative outcome variables were treated as having a gamma distribution with log link function.

OR, non-standardised coefficients ( $\beta$ ) and their respective 95% CI were calculated for the categorical- and quantitative-dependent variable models. All models were adjusted for baseline, including sex and age.

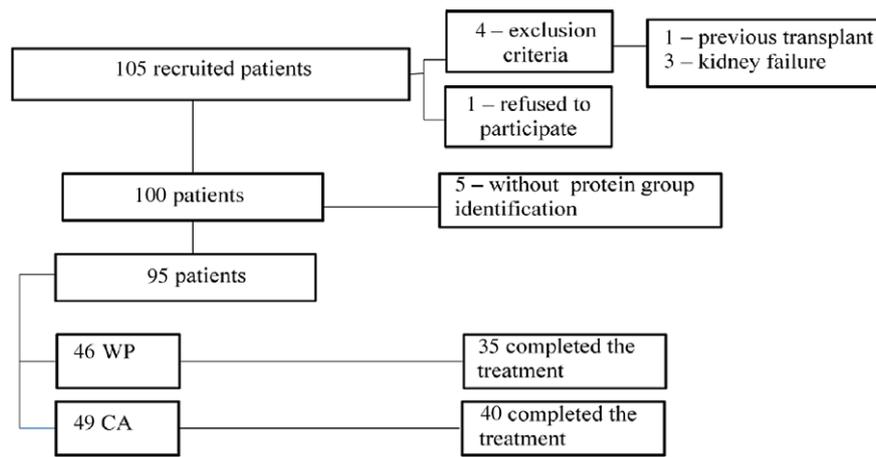
The working correlation matrix used was unstructured and exchangeable for categorical and quantitative variables, respectively, and its robust estimator covariance matrix was also used.

$P$  values  $<0.05$  were considered statistically significant. All analyses were carried out by the Statistical Package for the Social Sciences (version 20.0, SPSS).

### Results

A total of 105 patients with CLD were invited to participate in the study. One subject refused to participate, and four did not meet the inclusion criteria. Of the 100 patients who initiated protein supplementation, five were excluded due to technical problems with the labelling of the sachet supplements. Thus, forty-six patients were allocated to the WP group, and forty-nine were allocated to the CA group, of whom thirty-five and forty patients, respectively, completed the treatment. Study dropout was due to different reasons, such as lack of adherence, intolerance, clinical complications of the disease, liver transplantation and death, without differences between groups ( $P=0.948$ ) (Fig. 1).





**Fig. 1.** Flow chart of patient recruitment and distribution in the study. WP, whey protein isolate; CA, casein.

In both groups, the male/female distribution was similar (61.7 and 38.3%, respectively, in the WP group and 69.4 and 30.6%, respectively, in the CA group;  $P=0.428$ ) as was age ( $52 \pm 9$  years in the WP group and  $53 \pm 11$  years in the CA group;  $P=0.631$ ).

Alcohol consumption and viral infections were the main causes leading to liver failure in both groups, with similar proportions (66 and 61% in the WP and CA groups, respectively;  $P=0.663$ ), followed by schistosomiasis (*Schistosoma mansoni*), cancer, and autoimmune and cryptogenic aetiologies.

The MELD scores were similar at baseline (WP group mean MELD = 15, 95% CI 14, 17; CA group mean MELD = 16, 95% CI 14, 17;  $P=0.796$ ) and at the end of supplementation ( $\beta=0.002$ ; 95% CI  $-0.03, 0.04$ ,  $P=0.930$ ), even when the patients were stratified by disease severity (Table 1). The proportion of patients with more severe disease was similar in the WP and CA groups (72.3 and 67.3%, respectively,  $P=0.594$ ).

Clinical parameters, that is, ascites, oedema and encephalopathy, were statistically similar in both groups before and after supplementation. Although there was a significant difference in MELD  $\geq 15$  groups regarding encephalopathy after the intervention, this was related to a lower percentage of patients with this adverse event in the casein group (Table 1).

According to the Subjective Global Assessment, malnutrition was observed in 57.4 and 54.2% of patients in the WP and CA groups, respectively ( $P=0.649$ ). There was no difference in the nutritional status of patients in terms of the MELD scores: malnutrition was seen in 38.5 and 33.3% ( $P=0.778$ ) of patients in the WP and control groups, respectively, with MELD scores  $<15$  and in 64.5 and 62.5% of those in the WP and control groups, respectively, with the higher MELD scores ( $P=0.868$ ) (Table 2).

The protein intake (g/kg of weight) of patients in the WP group was lower at baseline (mean 0.75 g/kg; 95% CI 0.63, 0.86 g/kg) than that of those in the CA group (mean 0.95 g/kg; 95% CI 0.80, 1.09 g/kg;  $P=0.035$ ), but there was no difference after supplementation ( $\beta=-0.12$ ; 95% CI  $-0.42, 0.17$ ,  $P=0.410$ ). When patients were stratified by MELD score, similar protein intake was seen in both disease severity groups; however, a significant increase after intervention among those with less severe disease (WP and CA MELD  $<15$  groups) was observed in the

intragroup analysis (Table 2). No differences were observed in energetic intake, even when patients were stratified by MELD score (Table 2).

No differences were observed in gastrointestinal symptoms as loss of appetite, nausea and diarrhoea between before (at baseline) and after protein supplementation, in either group or according to the MELD stratification (online Supplementary Table S1).

The RMR increased after WP supplementation among patients with less severe disease (MELD  $<15$ ) compared with the RMR in the CA group ( $\beta=0.29$ ; 95% CI 0.12, 0.46,  $P=0.001$ ), with no differences in this parameter between the WP and CA groups in patients in the MELD  $\geq 15$  category (Table 2).

There were no differences in the body composition parameters (mid-arm muscle circumference, fat-free mass, fat mass and phase angle) or muscle functionality (handgrip strength and 6-min walking test) between before (at baseline) and after protein supplementation in either group or in the MELD-stratified groups (online Supplementary Table S2).

Most peripheral cytokines (TNF, IL-6, IL-1 $\beta$  and interferon- $\gamma$ ) (online Supplementary Table S3), immunomodulatory proteins (sTNFR1, sTNFR2, BDNF and GDNF) and immunomodulatory hormones (adiponectin, insulin and leptin) (online Supplementary Table S4) were similar in both groups at the two assessed time points. Although IL-10 presented a higher level at baseline in the WP group (mean 14.53 pg/ml; 95% CI 11.54, 17.52 v. CA group, mean 10.81 pg/ml; 95% CI 8.79, 12.82,  $P=0.044$ ), this difference was not maintained after protein supplementation ( $\beta=0.15$ ; 95% CI  $-0.10, 0.41$ ,  $P=0.241$ ) (Table 3).

Differences were observed in the WP group for all measured chemokines. Increased levels of IP-10/CXCL10 ( $\beta=0.43$ ; 95% CI 0.06, 0.81,  $P=0.022$ ), eotaxin-1/CCL11 ( $\beta=0.22$ ; 95% CI 0.02, 0.42,  $P=0.031$ ) and MCP-1/CCL2 ( $\beta=0.35$ ; 95% CI 0.06, 0.64,  $P=0.018$ ) and a decreased level of IL-5 ( $\beta=-0.17$ ; 95% CI  $-0.31, -0.01$ ,  $P=0.027$ ) were observed in the WP group at the end of treatment (Table 3). The same pattern of effects was observed in patients with more severe disease supplemented with WP, for whom increased levels of IP-10/CXCL10 ( $\beta=0.64$ ; 95% CI 0.23, 1.06,  $P=0.002$ ), eotaxin-1/CCL11 ( $\beta=0.42$ ; 95% CI 0.13, 0.72,  $P=0.004$ ) and MCP-1/CCL2 ( $\beta=0.68$ ; 95% CI 0.25, 1.12,  $P=0.002$ ) and lower levels of IL-5 ( $\beta=-0.28$ ; 95% CI  $-0.52,$

**Table 1.** Model for end-stage liver disease (MELD) score and clinical parameters of patients with chronic liver disease before and after protein supplementation ( $\beta$ -Coefficients and 95 % confidence intervals; odds ratios)

	All patients					Patients with MELD < 15					Patients with MELD $\geq$ 15				
	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI
<b>MELD score</b>															
Before protein supplementation			0.796					0.413					0.223		
Mean	16	15				11	12				18	17			
95 % CI	14, 17	14, 17				10, 12	11, 13				17, 19	16, 18			
After protein supplementation			0.93	0.002	-0.03, 0.04			0.599	-0.01	-0.07, 0.04			0.287	0.01	-0.01, 0.05
Mean	15	15				11	11				18	17			
95 % CI	14, 16	14, 16				10, 12	10, 12				17, 18	16, 18			
<i>P</i> (intra group)		0.829				0.280	0.766				0.111	0.943			
<b>Ascites</b>															
	CA	WP	<i>P</i> (inter group)	OR†	95 % CI	CA	WP	<i>P</i> (inter group)†	OR†	95 % CI	CA	WP	<i>P</i> (inter group)†	OR†	95 % CI
Before protein supplementation (%)	44.7	27.3	0.174			50	11.1	0.390			41.7	33.3	0.274		
After protein supplementation (%)	36.8	27.3	0.081	1.43	0.95, 2.14	42.9	11.1	0.327	0.95	0.87, 1.04	33.3	33.3	0.147	0.95	0.88, 1.01
<i>P</i> (intra group)	0.250	1.000				1.000	1.000				0.500	1.000			
<b>Oedema</b>															
Before protein supplementation (%)	80.0	78.1	0.854			64.3	75.0	0.717			90.5	79.2	0.614		
After protein supplementation (%)	74.3	65.6	0.382	0.70	0.32, 1.53	64.3	37.5	0.068	0.18	0.02, 1.13	81.0	75	0.477	1.37	0.56, 3.41
<i>P</i> (intra group)	0.625	0.125				1.000	0.250				0.500	1.000			
<b>Encephalopathy</b>															
Before protein supplementation (%)	14.3	15.2	0.898			6.3	23.1	0.191			18.2	12.1	0.562		
After protein supplementation (%)	2.6	11.8	0.385	1.48	0.60, 3.62	0.0	10	1.00	1.00	1.00-1.00	4.2	12.5	0.008‡	1.18‡	1.04, 1.35‡
<i>P</i> (intra group)	1.000	0.500				0.800	0.107				0.800	1.000			

CA, casein; WP, whey protein isolate.

\* Comparison between CA and WP groups after intervention for quantitative variables.

† Comparison between CA and WP groups after intervention. Significance level  $P < 0.05$ . Generalised estimating equations adjusted by sex and age.

‡ Significant difference.

**Table 2.** Nutritional status, diet intake and RMR of patients with chronic liver disease before and after protein supplementation ( $\beta$ -Coefficients and 95 % confidence intervals; odds ratios)

	All patients					Patients with MELD < 15					Patients with MELD $\geq$ 15				
	CA	WP	<i>P</i> (inter group)*	OR	95 % CI	CA	WP	<i>P</i> (inter group)*	OR	95 % CI	CA	WP	<i>P</i> (inter group)*	OR	95 % CI
<b>Malnutrition by SGA</b>															
Before protein supplementation (%)	54.2	57.4	0.649			33.3	38.5	0.778			62.5	64.5	0.868		
After protein supplementation (%)															
<i>P</i> (intra group)															
<b>Protein (g/kg weight)</b>															
Before protein supplementation			0.035‡					0.210					0.078		
Mean	0.95	0.75				0.89	0.73				0.97	0.76			
95 % CI	0.80, 1.09	0.63, 0.86				0.71, 1.08	0.56, 0.90				0.78, 1.16	0.61, 0.91			
After protein supplementation			0.410	-0.12	-0.42, 0.17			0.653	-0.10	-0.55, 0.34			0.476	-0.13	-0.52, 0.24
Mean	1.28	0.89				1.40	1.03				1.22	0.82			
95 % CI	1.08, 1.48	0.72, 1.06				1.08, 1.71	0.75, 1.30				0.97, 1.47	0.62, 1.03			
<i>P</i> (intra group)	0.005‡	0.129				0.015‡	0.032‡				0.084	0.566			
<b>Energy content (kcal/24 h)§</b>															
Before protein supplementation			0.201					0.273					0.420		
Mean	22.94	20.38				24.64	20.93				22.08	20.17			
95 % CI	20.45, 25.42	17.44, 23.32				19.75, 29.53	16.61, 25.25				19.36, 24.81	16.43, 23.90			
After protein supplementation			0.941	0.008	-0.215, 0.232			0.572	0.10	-0.25, 0.46			0.747	-0.04	-0.32, 0.23
Mean	24.49	21.95				24.44	23.04				24.51	21.37			
95 % CI	21.43, 27.56	18.68, 25.23				18.15, 30.73	18.25, 27.83				21.15, 27.86	17.26, 25.48			
<i>P</i> (intra group)	0.351	0.408				0.954	0.415				0.168	0.632			
<b>RMR (kcal/24 h)§</b>															
Before protein supplementation			0.113					0.092					0.586		
Mean	1553	1399				1722	1387				1446	1400			
95 % CI	1385, 1721	1305, 1492				1343, 2100	1260, 1515				1332, 1560	1280, 1520			
After protein supplementation			0.089	0.09	-0.01, 0.20			0.001‡	0.29	0.12, 0.46			0.531	-0.03	-0.15, 0.07
Mean	1494	1480				1527	1654				1481	1381			
95 % CI	1390, 1598	1363, 1598				1298, 1756	1385, 1924				1370, 1591	1284, 1478			
<i>P</i> (intra group)	0.331	0.179				0.070	0.019‡				0.532	0.773			

Whey and casein in liver failure patients

MELD, model for end-stage liver disease; CA, casein; WP, whey protein isolate; SGA, Subjective Global Assessment.

\* Comparison between CA and WP groups after intervention.

† Comparison between CA and WP groups after intervention for quantitative variables. Significance level  $P < 0.05$ . Generalised estimating equations adjusted by sex and age.

‡ Statistical difference.

§ To convert kcal to kJ, multiply by 4.184.

**Table 3.** Plasma immunomodulatory and chemokine proteins of patients with chronic liver disease before and after protein supplementation ( $\beta$ -Coefficients and 95 % confidence intervals)

	All patients					Patients with MELD < 15					Patients with MELD $\geq$ 15				
	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI	CA	WP	<i>P</i> (inter group)*	$\beta$	95 % CI
<b>IL-10 (pg/ml)</b>															
Before protein supplementation			0.044†					0.095					0.460		
Mean	10.81	14.53				9.88	14.50				11.97	13.69			
95 % CI	8.79, 12.82	11.54, 17.52				8.08, 11.69	9.35, 19.64				9.04, 14.91	10.24, 17.15			
After protein supplementation			0.241	0.15	-0.10, 0.41			0.346	-0.25	-0.79, 0.27			0.008†	0.38	0.10, 0.66
Mean	11.63	18.28				14.00	15.86				10.67	17.90			
95 % CI	9.51, 13.75	14.36, 22.19				10.36, 17.64	11.45, 20.26				8.01, 13.32	14.00, 21.80			
<i>P</i> (intra group)	0.450	0.017†				0.012†	0.715				0.260	0.008†			
<b>Eotaxin (pg/ml)</b>															
Before protein supplementation			0.443					0.337					0.565		
Mean	868	1103				821	1010				974	1082			
95 % CI	707, 1030	845, 1361				620, 1022	685, 1335				739, 1210	799, 1365			
After protein supplementation			0.018†	0.35	0.06, 0.64			0.318	-0.17	-0.52, 0.17			0.004†	0.42	0.13, 0.72
Mean	877	1390				937	965				903	1540			
95 % CI	727, 1028	1019, 1761				717, 1156	719, 1212				696, 1109	1046, 2033			
<i>P</i> (intra group)	0.865	0.014†				0.351	0.680				0.091	0.030†			
<b>IL-5 (pg/ml)</b>															
Before protein supplementation			0.852					0.200					0.421		
Mean	6.48	6.64				6.77	5.59				6.34	7.33			
95 % CI	5.44, 7.51	5.28, 8.00				5.71, 7.83	4.18, 7.01				4.91, 7.76	5.42, 9.25			
After protein supplementation			0.027†	-0.17	-0.32, -0.01			0.401	-0.06	-0.22, 0.09			0.022†	-0.28	-0.52, 0.04
Mean	7.03	6.07				7.44	5.74				7.13	6.21			
95 % CI	6.06, 7.99	5.19, 6.96				6.03, 8.85	4.16, 7.32				5.85, 8.40	5.23, 7.19			
<i>P</i> (intra group)	0.075	0.175				0.214	0.472				0.119	0.121			
<b>IP-10 (pg/ml)</b>															
Before protein supplementation			0.943					0.122					0.172		
Mean	234	231				194	283				260	196			
95 % CI	177, 291	192, 271				143, 245	182, 384				182, 338	153, 237			
After protein supplementation			0.022†	0.43	0.06, 0.81			0.892	-0.04	-0.61, 0.53			0.002†	0.64	0.23, 1.06
Mean	234	359				274	384				222	319			
95 % CI	191, 278	259, 459				208, 3398	265, 503				158, 286	208, 431			
<i>P</i> (intra group)	0.990	0.009†				0.026†	0.234				0.145	0.026†			
<b>MCP-1 (pg/ml)</b>															
Before protein supplementation			0.443					0.104					0.492		
Mean	355	325				379	289				348	313			
95 % CI	293, 418	277, 372				301, 457	214, 364				261, 436	263, 363			
After protein supplementation			0.018†	0.35	0.06, 0.64			0.982	-0.003	-0.294, 0.301			0.002†	0.68	0.25, 1.12
Mean	355	461				476	364				297	531			
95 % CI	296, 414	342, 579				365, 586	255, 473				240, 354	347, 715			
<i>P</i> (intra group)	0.994	0.013†				0.053	0.043†				0.236	0.019†			

MELD, model for end-stage liver disease; CA, casein; WP, whey protein isolate; IP-10, interferon- $\gamma$ -induced protein-10; MCP-1, monocyte chemoattractant protein-1.

\* Comparison between WP and CA groups after intervention. Significance level  $P < 0.05$ . Generalised estimating equations adjusted by sex and age.

† Significant difference.



-0.04,  $P=0.022$ ) were also found. Among WP patients with MELD  $\geq 15$ , IL-10 was increased at the end of supplementation period ( $\beta=0.38$ ; 95% CI 0.10, 0.66,  $P=0.008$ ) (Table 3).

Baseline values are demonstrated in supplemental tables (online Supplementary Tables S5 and S6).

## Discussion

Patients with end-stage liver disease waiting for liver transplantation who were supplemented with either casein or whey protein for 2 weeks presented no differences in nutritional status or several immune markers after the supplementation period. However, patients who received WP had a different response pattern in regard to IL-10 and chemokines.

Patients waiting for liver transplantation include those with more severe disease, justifying the elevated mean MELD score in the present study, in agreement with previous reports<sup>(4,39)</sup>. The more severe the disease, the greater the prevalence of malnutrition, which is frequently associated with clinical complications and immune dysfunction, increasing the mortality rate<sup>(40,41)</sup>. Our results revealed a prevalence of malnutrition of more than 50% among patients and more than 60% among those with more severe disease, similar to data reported by other authors<sup>(40,42)</sup>, although higher percentages have also been described<sup>(43,44)</sup>. The discrepancy in malnutrition frequency might be related to differences regarding the assessment methods of nutritional status<sup>(45)</sup>.

Malnutrition encompasses decreased nutrient intake, loss of muscle mass and functionality, and energy metabolism alterations<sup>(46)</sup>. Poor protein and energetic intake are frequently observed in cirrhotic patients due to restrictive diets and clinical complications such as ascites, anorexia and early satiety<sup>(47,48)</sup>. We found the same pattern of insufficient protein (<1.0 g/kg weight) and energetic intake (approximately 20 kcal (84 kJ)/kg weight) in both groups of patients before the supplementation trial. After supplementation, the protein intake was similar for both the WP and CA groups, including the MELD subgroups, but patients supplemented with WP were unable to reach the minimum protein recommendations for CLD patients (1.2 g/kg weight)<sup>(49)</sup>. Although we expected to observe effects on muscle mass after WP supplementation due to its higher concentration of branched-chain amino acids, especially leucine, and higher digestibility than CA<sup>(50,51)</sup>, these effects were not observed. WP supplementation might have further impacted early satiety due to its faster absorption<sup>(52-54)</sup>, preventing the patients from reaching adequate protein and energy intake. Both the WP and CA groups had insufficient energy intake before and after treatment, which could negatively affect protein synthesis<sup>(55)</sup>. Furthermore, the trial period (15 d of supplementation) may have been too short to assess such differences in addition to the methodological limitations regarding the body compartment assessment, such as anthropometry and bioelectrical impedance, which may not be sensitive enough to detect short-term muscle mass changes.

The effects of physical exercise and insulin on whole-body protein synthesis should be considered<sup>(49)</sup>. Our patients were physically inactive, and we did not find any effect of the

supplementation on insulin levels, although some authors have demonstrated the ability of WP to increase its secretion<sup>(52,54)</sup>. The maintenance of muscle and fat masses after protein supplementation may, in part, explain the lack of supplementation effects on insulin, adiponectin and leptin levels<sup>(56-59)</sup>.

Similar to the absence of the effects of supplementation on the amount of muscle mass, no effect was observed on muscle functionality. Our result was similar to that found at baseline by Aamann *et al.*<sup>(60)</sup>, who, in a prospective study, investigated whether resistance training would increase muscle strength and mass in patients with cirrhosis. Although they reported a positive effect of resistance training on both parameters, they stated that the results can only be generalised to patients with sufficient protein intake, established as 1.2 g/kg of body weight. Our patients did not reach this threshold; therefore, we speculate that as a result, no differences were seen after the intervention on the 6-min walking test. Most likely, for the same reason, no difference was observed in handgrip strength, although baseline values were in accordance with previous authors' data<sup>(61,62)</sup>.

Protein intake usually increases energy expenditure as a result of the energy required for the initial steps of metabolism, storage and oxidation, including urea synthesis<sup>(63)</sup>. However, both WP and CA supplementation did not impact the RMR of most patients, since only MELD < 15 patients supplemented with WP had increased values afterwards. This might be a consequence of the high digestibility of WP compared with that of casein, although this was not seen in the patients with more severe disease, and it could be speculated that these individuals presented with deranged absorption. An elevated amino acid concentration associated with high and fast digestibility implies a metabolic process, which readily increases thermogenesis and, consequently, the RMR<sup>(64-66)</sup>.

Immune dysfunction is well established in patients with cirrhosis, regardless of its aetiology. Stimulation of the immune system is a potential target for treatment to improve clinical conditions and nutritional status<sup>(67)</sup>. WP supplementation impacted immune markers, including those in patients with more severe disease. Our results showed an immunomodulatory effect after WP supplementation reflected by increased levels of eotaxin-1/CCL11, IP-10/CXCL10 and MCP-1/CCL2, including in patients with more severe disease. Proteins whose secretion was stimulated in the WP group are classically involved in the innate response and are mostly produced by macrophages, fibroblast cells, Kupffer cells, endothelial cells, dendritic cells and monocytes.

A pro-inflammatory profile was observed in the WP group. It is important to address that CLD patients have a spectral range from inflammatory to immunodeficiency responses<sup>(6)</sup>. Some authors have demonstrated that increased chemokine production can be harmful to CLD patients, as the exacerbation of inflammation contributes to fibrosis development and cirrhosis evolution<sup>(68,69)</sup>. On the other hand, chemokines, including IP-10/CXCL10, MCP-1/CCL2 and eotaxin-1/CCL11, are responsible for cell migration and the control of several infections, including liver infection<sup>(70-72)</sup>. In liver disease patients, both deleterious and beneficial effects exerted by IP-10/CXCL10<sup>(73-76)</sup>, MCP-1/CCL2<sup>(77,78)</sup> and eotaxin<sup>(79-82)</sup> have been described. Therefore, determining whether the increased production seen after WP intake will be harmful or protective for these patients

requires further assessments. Inflammatory chemokines contribute substantially to the recruitment of leucocyte populations and will regulate any non-leucocyte tissue cells (epithelial, mesenchymal and endothelial) important in defence mechanisms<sup>(83,84)</sup>. In contrast, IL-5 is frequently reported to be involved in liver lesion progression<sup>(85,86)</sup>. Thus, its decreased production in CLD patients after WP supplementation contributes to our hypothesis that WP plays an immunomodulatory role associated with the increase in IL-10. The latter cytokine has a general suppressive effect, preventing increased exacerbations of innate and adaptive immune responses, controlling detrimental pathological injury<sup>(87)</sup>. The reported effects of IL-10 have been mostly beneficial for CLD patients<sup>(88–90)</sup>.

The reason WP supplementation exerted these effects on CLD patients, including those with more severe disease, is not well understood since we could not find explanations for these specific findings in the literature. One potential explanation could be related to the effects exerted by whey protein on the liver by increasing apoptotic signals and inflammatory markers<sup>(91–94)</sup>. Additionally, potentially increased intestinal permeability due to the weak barrier observed in CLD patients<sup>(95)</sup> may increase the risk of food allergies<sup>(96)</sup> and overdosing nutrients, as observed under supplementation conditions<sup>(97)</sup>. Furthermore, protein consumption can modify the microbiota<sup>(98)</sup>, favouring chronic systemic inflammation as a result of precipitating events, for which antigen-like peptides could be responsible<sup>(99)</sup>. Kiewiet *et al.*<sup>(100)</sup> compared the immune effects and toll-like receptor activation and inhibition effects of whey and casein hydrolysates with different hydrolysis levels. These authors found that cells stimulated by intact WP and its hydrolysates were capable of inducing peripheral blood mononuclear cell cytokine secretion in healthy individuals, and the peptides of WP had a profound activating effect on toll-like receptor signalling, effects not observed for CA protein or its peptides. These effects were protein-type specific since none of the casein hydrolysates impacted toll-like receptor activation.

Systemic inflammatory diseases commonly cause alterations in a patient's behaviour, including the development of fatigue, anxiety and loss of appetite and social interest, collectively termed sickness behaviour<sup>(101)</sup>. In mice with liver inflammation, sickness behaviours were observed and the inhibition of cerebral monocytes in those animals was associated with the improvement of this condition<sup>(102)</sup>. Evidence suggests a link between neuroinflammation, predominantly modulated by microglia<sup>(103)</sup>, and impaired motor and cognitive functions during hepatic encephalopathy<sup>(104,105)</sup>. Additionally, recent studies have shown that eotaxin-1/CCL11 is able to influence neural progenitor cells and microglia<sup>(106)</sup>. Although the significant odds for encephalopathy among the severe CLD patients supplemented with WP was due to the reduction in this event among those supplemented with CA, we investigated brain alterations. Brain alterations are a consequence of both the immune response and increased protein consumption. By measuring neurotrophic factors and considering that WP increased MCP-1 and eotaxin-1/CCL11 levels, no differences were observed in either BDNF or GDNF levels. Both are important for central nervous system homeostasis, particularly after damage and/or inflammation<sup>(107)</sup>.

Based on the evidence that dairy products may have beneficial outcomes for neurocognitive health<sup>(108,109)</sup>, our expectation that milk protein supplementation could positively affect these neurotrophins was confirmed, not by an increase in their levels but by the observation that even under pro-inflammatory conditions, there was no reduction in their concentrations.

Although protein supplementation does not trigger gastrointestinal symptoms suggestive of protein intolerance, we cannot eliminate the possibility that WP may exacerbate pre-existing liver damage, but the increased IL-10 in patients with more severe disease supplemented with WP can contribute to preventing increased exacerbations of innate and adaptive immune responses, controlling pathological injury<sup>(87)</sup>. However, we could not determine the clinical meaning of this immunomodulation in liver disease or on the nutritional status of patients.

The present study has some limitations. The duration of the clinical trial was short. A relevant issue regarding clinical trials is the dropout rate, especially when enrolling patients with chronic severe diseases. Furthermore, diet and supplement intake were controlled basically by a retrospective data collection that required an extensive dependence on the recent memory of the study subject. In addition, patients' energy intake did not reach the recommended values, which may further compromise the impact of protein supplementation. Regarding the immune investigation, although we evaluated a wide panel of immune molecules, we did not evaluate cell function, which could enlighten us to better understand the clinical impact of WP supplementation. To our knowledge, this is the first clinical trial investigating the effect of WP supplementation on end-stage CLD patients, and our results have raised the need for further investigations to establish the clinical significance of WP intake on the nutritional and immunomodulatory status of these individuals.

In conclusion, our results showed that WP consumption by patients with CLD impacted immunomodulatory responses when compared with the intake of casein by increasing pro- and anti-inflammatory protein synthesis with no impact on nutritional status.

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All authors agree with all aspects of the work, ensuring that issues related to the accuracy or integrity of any part of the work have been properly investigated and resolved.

The authors declare that there are no conflicts of interest.

### Supplementary material

For supplementary materials referred to in this article, please visit <https://doi.org/10.1017/S0007114520003219>

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