Nutrition 78 (2020) 110952

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Applied nutritional investigation

Plasma enterobacterial ClpB levels and ClpB- and α-MSH–reactive immunoglobulins in lung cancer patients with and without anorexia

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ARTICLE INFO

Article History:
Received 23 April 2020
Received in revised form 18 June 2020
Accepted 6 July 2020

Keywords:
Gut microbiota
Cancer anorexia
Caseinolytic peptidase B
α-MSH
Autoantibodies

ABSTRACT

Objectives: Anorexia represents a common and debilitating clinical problem in patients with several forms of cancer, in particular lung cancer, but its mechanisms are not completely understood. Recently, the caseinolytic-protease-B (ClpB) homologue protein, produced by common gut bacteria, such as *Escherichia coli*, was identified as an antigen-mimetic of α-melanocyte-stimulating hormone (α-MSH), an anorexigenic neuropeptide. ClpB was previously detected in human plasma and displayed satietogenic properties; however, its possible relevance to cancer anorexia has not yet been investigated.
Methods: To address this question, we analyzed plasma ClpB concentrations as well as levels and affinities of anti-ClpB and α-MSH–reactive antibodies in patients with lung cancer with and without anorexia as compared with body mass index–matched healthy controls with normal appetite.
Results: We found that plasma ClpB concentrations were significantly lower in non-anorexic patients with cancer than those of the control group ($P = 0.028$). In contrast, patients with cancer and anorexia had lower levels of anti-ClpB immunoglobulins (Ig)M ($P < 0.0001$) and of both α-MSH IgM and IgG ($P < 0.05$) with respect to controls. Moreover, in patients with cancer and anorexia, anti-ClpB IgG showed a trend of lower affinities compared with non-anorexic patients ($P = 0.05$).
Conclusions: Taken together, the results revealed a reduced humoral immune response to ClpB in patients with cancer and anorexia, which may lead to an enhanced satietogenic effect of this enterobacterial protein contributing to the mechanisms of reduced appetite.

Introduction

Anorexia (i.e., the loss or low desire to eat), represents one of the most common symptoms during the course of several chronic conditions, including cancer, end-stage renal disease, cirrhosis, and aging [1,2]. In particular during cancer, the loss of appetite represents a severe debilitating symptom underlying malnutrition and worsening the clinical course of the disease [3,4]. It represents a relevant challenge to most patients with cancer. Taste and smell alterations, early satiety, meat aversion, or nausea and vomiting may all characterize disease-associated anorexia [5,6].

We have previously shown a key role of the hypothalamus in the pathophysiology of cancer anorexia in humans, observing the different effects of a standard meal in modulating central nervous system (CNS) activity between anorexic and non-anorexic patients with cancer and healthy controls [7]. Neuroinflammation mediates the progressive insensitivity of the hypothalamic neurons to the signals arising from peripheral tissues. Anorexigenic signals prevail on peripheral orexigenic drives promoting the progressive depletion of energy stores and the wasting of lean body mass and fat tissues [7–10].

However, the pathogenesis of cancer anorexia is multifactorial and not yet completely understood. Modifications to the CNS are associated with elevated hypothalamic concentrations of proinflammatory cytokines, which indicate that neuroinflammation may be an adaptive

SF is supported by the Inserm Transversal Microbiota Program, France and by the EU projects exploring the role of gut microbiota in eating disorders (ERAnet "MIGBAN") and in autism (H2020 "GEMMA"). SF declares the following financial interests/personal relationships, which may be considered as potential competing interests: co-founder and consultant of TargEDys SA. The remaining authors have no conflicts of interest to declare.

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https://doi.org/10.1016/j.nut.2020.110952
0899-9007/

response of the hypothalamus to peripheral challenges, including tumor growth [11–13]. Experimental evidence also suggests a crucial role of tumor-derived catabolic factors (such as myostatin, serotonin, transforming growth factor-β) in the loss of appetite during cancer, as well as other proinflammatory mediators including growth differentiation factor-15 and tumor necrosis factor-like weak inducer of apoptosis [14].

Recently, gut microbiota has been implicated in the physiologic regulation of appetite [15]. Therefore, it is conceivable that loss of appetite in several chronic conditions, such as cancer, may be associated with altered communication between gut microbiota-derived signals and host hunger and satiety pathways. In particular, the study by Tennoune et al. identified caseinolytic protease B (ClpB) homologue protein produced by the family of Enterobacteriaceae as an antigen mimetic of α-melanocyte-stimulating hormone (α-MSH), an anorexigenic neuropeptide [16]. Autoimmune response to ClpB was postulated to play a key role in the pathophysiology of anorexia nervosa and bulimia, the psychiatric eating disorders [17]. Moreover, enterobacterial ClpB was shown to display a direct satietogenic effect in mice leading to the reduction of the body weight gain as well as activation of the intestinal and central satiety pathways [18,19].

ClpB protein is naturally present in plasma of both rodents and humans and its plasma levels can be elevated in some patients with eating disorders, suggesting its role both as a satietogenic signal and as an antigen leading to production of α-MSH cross-reactive immunoglobulins (Ig) G and IgM autoantibodies (auto-Abs) [19,20]. Whether ClpB protein and α-MSH auto-Abs can be relevant to cancer anorexia has not yet been studied. Currently, no data are available on the modulation of plasma enterobacterial ClpB levels and ClpB- and α-MSH–reactive Igs in patients with cancer and their potential effect on appetite.

Thus, in the present study, we aimed to investigate plasma levels of enterobacterial ClpB and also to characterize plasma levels and affinities of anti-ClpB and α-MSH–reactive IgG and IgM in a cohort of patients with cancer with and without anorexia, before initiating antineoplastic treatments.

Materials and methods

Study design and participants

This was a two-center controlled study designed according to the Declaration of Helsinki and conducted with patients with lung cancer and healthy individuals enrolled at the Department of Translational and Precision Medicine (formerly Department of Clinical Medicine) and at the Oncology Unit, Sapienza-University of Rome. This study was approved by the local Ethical Committee (Azienda Policlinico Umberto I, Sapienza University of Rome, Italy).

Patients with confirmed diagnosis of lung cancer (non-small cell lung carcinoma [NSCLC] and small cell lung carcinoma [SCLC]) were considered for enrollment before the initiation of any anti-cancer treatments. This type of cancer was chosen because of the high prevalence (>40%) of anorexia at its first diagnosis [21]. The inclusion criteria were ≥18 y of age and the ability to provide informed consent. Patients with concomitant wasting disease, such as end-stage renal disease, liver cirrhosis, psychiatric disorders or cognitive impairment, or dysphagia or mechanical obstruction of the gastrointestinal tract were excluded. The control group included healthy volunteers working as personnel at the two study sites or individuals without evidence of diseases. All study procedures were performed in accordance with the ethical standards of the responsible institutional committee on human experimentation. In the fasting condition during the morning, venous blood samples were collected in EDTA tubes and stored on ice before centrifugation, after which plasma was separated and the sample stored at −80°C until transported on dry ice and then thawed for ClpB and auto-Abs assays as described later.

Nutritional assessment and other clinical characteristics

We registered each participant's current weight, usual weight, and current body mass index (BMI). We collected information on body weight loss (%) in the previous 6 mo and assessed serum biomarkers, such as albumin and C-reactive protein. These two parameters were used to calculate the Glasgow Prognostic Score. We also collected information on histology and staging of disease, comorbidities, and current medications. The presence or absence of anorexia was assessed by three different validated appetite assessment tools, as described in detail in the next section.

Anorexia tools

The Anorexia Questionnaire (AQ) was chosen to discriminate the presence of anorexia in patients with cancer [5,22]. The AQ consists of four questions that investigate different domains of anorexia (i.e., meat aversion, early satiety, nausea and/or vomiting, and smell alterations). If one or more of these symptoms were referred, the patient was considered to be anorexic [22]. The main reasons we considered the AQ as the "primary" measure of anorexia are represented by its reliability shown in patients with cancer [5,7,21], the reduced time needed to complete it, and the simplicity in dichotomizing patients with or without anorexia.

Additionally, we used the Functional Assessment of Anorexia/Cachexia Therapy (FAACT) questionnaire, which has been endorsed by the European Society for Clinical Nutrition and Metabolism as a reliable tool to assess disease-associated anorexia [6]. The FAACT score is based on a subset of the FAACT questionnaire, in particular, the anorexia/cachexia subscale-12 section and it consists of 12 questions related to appetite and food intake and allows a qualitative and quantitative diagnosis of anorexia. Each question is on a 5-point Likert scale (i.e., *not at all, a little bit, somewhat, quite a bit, very much*), each conferring a score from 0 to 4. As recently shown, a FAACT score ≤30 is indicative of the presence of anorexia [21] and we used this value to estimate the prevalence of reduced appetite by this tool.

We also used a visual analog scale (VAS) for appetite consisting of a line of 10 cm. The extremities of this line are anchored to *no hunger* (0 cm) and *hunger* (10 cm). Patients were asked to place a line on the VAS that corresponded to their current appetite. A VAS score ≤5 was considered indicative of poor appetite [6,23]. We used this value to estimate the prevalence of anorexia by the VAS.

Plasma ClpB assay

Concentration of enterobacterial ClpB protein was measured in plasma samples by the enzyme-linked immunosorbent assay (ELISA) as described elsewhere [19]. For this purpose, three antibodies were used: Rabbit polyclonal anti-*Escherichia coli* (*E. coli*) ClpB antibodies (Delphi Genetics, Gosselies, Belgium) as a capture antibody, mouse monoclonal anti-*E. coli* ClpB antibodies as a detection antibody (Delphi Genetics) and an alkaline phosphatase-conjugated goat anti-mouse revelation antibody (Jackson ImmunoResearch, Cambridgeshire, UK). The optical density of the ELISA reaction was measured at 405 nm using a microplate reader Infinite F200PRO (Tecan, Männedorf, Switzerland) and ClpB concentration was determined using a ClpB protein (Delphi Genetics) standard curve ranging from 0 to 5, 10, 25, 50, 70, 100, and 150 pM. Each determination was performed in duplicate.

ClpB- and α-MSH–reactive antibody assay

Plasma levels of anti-ClpB and α-MSH–reactive IgG and IgM were measured using ELISA according to a published protocol [24]. Briefly, α-MSH peptide (Bachem AG, Bubendorf, Switzerland) or ClpB protein (Delphi Genetics) was coated onto 96-well Maxisorp plates (Nunc, Rochester, NY, USA) using 100 μL and a concentration of 2 μg/mL in 0, 5M Na_2CO_3 and 0.5 M sodium bicarbonate buffer, pH 9.6 for 48 h at 4°C. The plates were washed three times in phosphate-buffered saline (PBS) with 0.05% Tween 200, pH 7.4, and then incubated for 3 h at 37°C with 100 μL of human plasma diluted 1:400 (IgM) or 1:800 (IgG) in PBS pH 7.4 or in PBS with 3 M sodium chloride + 1.5 M glycine pH 8.9. The plates were washed three times and incubated with 100 μL of alkaline phosphatase-conjugated antibodies in PBS (1:2000, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Following washing (three times), 100 μL of p-nitrophenyl phosphate solution (Sigma, St. Louis, MO, USA) was added as a substrate. After 40 min of incubation at room temperature, the reaction was stopped by adding 3N sodium hydroxide. The optical density (OD) was determined at 405 nm using a microplate reader Infinite F200PRO. Blank OD values resulting from the reading of plates without addition of plasma samples were subtracted from the sample OD values. Each determination was done in duplicate. The variation between duplicate values was <5%.

IgG purification

Total IgG were purified from plasma using Melon™ Gel Purification Kit (Life-Technologies, Carlsbad, CA, USA). Briefly, Mini Spin Columns were loaded with 500 μL of Melon™ Gel purification support and centrifuged for 30s at 5000g. After two washes with Melon™ Gel purification buffer under the same centrifugation conditions, 500 μL of plasma (diluted 1:4 vol. in purification buffer) was incubated in the columns for 5 min at room temperature on a roller mixer. To collect purified IgG, Mini Spin Columns were centrifuged for 30s at 5000*g* and samples were lyophilized for 48 h, resuspended in PBS or HBS-EP buffer (GE Healthcare, USA) for affinity kinetics analysis and other experiments and conserved at −80°C. IgG concentrations were evaluated using NanoDrop 2000C (ThermoFisher Scientific, Waltham, MA, U,S,A,) with PBS or HBS-EP buffer as blanks.

Affinity kinetics of IgG for α-MSH

Affinity kinetics of plasma-extracted IgG for α-MSH and ClpB were analyzed by biospecific interaction analysis (BIA) based on surface plasmon resonance (SPR) phenomenon on a BIAcore 1000 instrument (GE Healthcare), according to a previously published protocol [25]. α-MSH peptide (Bachem) or *E. coli* ClpB (Delphi Genetics) were diluted in 10 mM sodium acetate buffer, pH 5.0 (GE Healthcare, final concentration = 0.5 mg/mL) and were covalently coupled on the CM5 sensor chip (GE Healthcare). A multi-cycle affinity kinetic analysis method was run with five serial dilutions of purified IgG: 3360, 1680, 840 (in duplicate), 420, 210 nM in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M sodium chloride, 3 mM EDTA, and 0.005% surfactant P20, GE Healthcare). Two HBS-EP buffer-only blanks were performed at the beginning and end of each cycle. A cycle included 120s of analyte injection and 300s of dissociation with a 30 μL/min flow speed at 25°C. Between sample injections, the binding surface was regenerated with 10 mM sodium hydroxide, resulting in a return of baseline level of the sensorgram. Affinity kinetic data were analyzed using BiaEvaluation 4.1.1 program (GE Healthcare) and fitted with the Langmuir's 1:1 model after blank values subtraction.

Statistical analysis

This was a case–control study conducted for the first time in the clinical setting of cancer-associated anorexia. We described patient characteristics using mean ±SD for continuous normally distributed values, whereas median with interquartile range (IQR) was used for variables non-normally distributed. Normality was evaluated by Shapiro–Wilk or Kolmogorov–Smirnov tests. We expressed categorical variables as number (%). Differences among the three groups (patients with cancer and anorexia, patients with cancer but no anorexia, and controls) were analyzed by analysis of variance (ANOVA) with Tukey's post-tests or when data were not normally distributed by the Kruskal–Wallis test with Dunn's post-tests. Two-tailed *t* test or Mann–Whitney were used between two groups according to normality test. We treated the AQ as dichotomous and the FAACT and VAS scores as continues variables to assess linear correlations with anti-ClpB and α-MSH Ig levels using Pearson's and Spearman's tests, as appropriate. $P < 0.05$ was considered statistically significant. Considering the relatively low number of study participants, a post hoc power analysis was performed to estimate the significance. Data were analyzed and graphs were plotted using the GraphPad Prism 5.02 (GraphPad Software Inc., San Diego, CA, USA).

Results

Participant characteristics

Fifty participants were enrolled in this study. Of the 50 participants, 34 had lung cancer and 16 were healthy controls. Thirty-three patients were diagnosed with NSCLC and one with SCLC; their baseline characteristics are reported in Table 1.

In summary, the cancer group included 27 men and 7 women, whose age and BMI were 68 ± 12 y and 23.8 ± 3.91 kg/m^2, respectively. Additionally, in the 6 mo before the study, the patients with cancer showed an involuntary body weight loss (median) of 6.7% (IQR, 0.1%, 11%). The majority of comorbidities registered were hypertension (38%), dyslipidemia (12%), and diabetes (9%; Table 1). The control group included 7 men and 9 women, who were younger (60 ± 10 y of age) than the patients with cancer ($P = 0.02$) but did not differ in terms of BMI (25.4 ± 4.2, kg/m^2; $P = 0.21$). Arterial hypertension and dyslipidemia were present in 6 of 16 (37.5%) and in 3 of 16 (18.8%) controls, respectively. The number and type of these comorbidities did not differ between patients and controls ($P > 0.05$). None of the control group participants presented low appetite based on the appetite tools used and none reported involuntary body weight loss in the previous 6 mo. In controls, mean serum albumin levels (4.01 ± 0.54 g/dL) did not differ compared with the patient group ($P = 0.07$). None of the participants reported use of antibiotics and/or probiotics prior to the 4 wk before the study or at the time of the study.

Prevalence of anorexia according to the AQ and its correlation with other appetite tools

According to the AQ, the prevalence of anorexia in the patient group was 68% (23 of 34). The mean FAACT score was 29 ± 9, whereas VAS (cm) was 6 ± 3. By using the FAACT score, the prevalence of anorexia was 59% (20 of 34) and by VAS it was 44% (15 of 34). The AQ correlated well with FAACT score ($r = -0.6$, $P < 0.0001$), as well as with VAS ($r = -0.49$, $P = 0.0002$). The mean values of both VAS and FAACT scores in the two subgroups of patients and in controls are shown in Figure 1A. Additionally, as expected, the FAACT and VAS scores correlated with each other (Spearman's $r = 0.6$, $P < 0.0001$; Fig. 1B.

Table 1
Characteristics of patients with lung cancer (N = 34)

Patients	Cancer anorexia (n = 23)	No anorexia (n = 11)
Mean age, y	66.6 ± 13	70.8 ± 9.8
Men, %	82.6	72.7
Usual weight, kg	72.48 ± 12.49	76.64 ± 14.97
Actual weight, kg	65.22 ± 11.60	75.14 ± 13.60
Height, m	1.70 ± 0.09	1.68 ± 0.09
BMI, kg/m^2	22.49 ± 3.44	26.63 ± 3.42
Body weight loss, %	8.75 (6.55, 14.67)	0 (0, 3.09)
Albumin, g/dL	3.6 ± 0.66	3.8 ± 0.42
Hemoglobin, g/dL	12.63 ± 1.64	13.15 ± 1.72
C-reactive protein, mg/dL	6.30 (3.36, 12.00)	5.20 (1.65, 6.30)
GPS ≥ 1	10	2
FAACT score	28 (24, 30)	38 (38, 40)
VAS, cm	5.0 ± 3.0	7.0 ± 1.0
Stage		
II	2	1
III	3	2
IV	18	8
Comorbidities, n		
Hypertension	8	5
Diabetes	2	1
Dyslipidemia	3	1

BMI, body mass index; FAACT, Functional Anorexia Assessment Cachexia/Therapy; GPS, Glasgow Prognostic Score; IQR, interquartile range; VAS, visual analog scale
Median (IQR) shown for variables non-normally distributed.

Plasma ClpB concentrations

In the patients, median ClpB concentrations were significantly lower compared with controls (median ± SEM, 2 ± 1.1 vs 5.42 ± 2, $P = 0.018$; Fig. 2A). Indeed, many of the patients showed ClpB levels below the detection limit—6 of 11 (54.5%) of patients without anorexia and 8 of 23 (34.8%) with anorexia versus only 1 of 16 (6.3%) in controls. When the patients were stratified into two groups according to the presence or absence of anorexia, lower plasma ClpB concentrations were found only in the group of patients without anorexia when compared with controls (Fig. 2B). Although median ClpB levels were more elevated in patients with cancer and anorexia with respect to patients without the eating disorder (median ± SEM, 3.28 ± 1.5 versus 0 ± 1.1), this difference did not reach significance ($P = 0.24$). Nevertheless, post hoc analysis showed insufficient power varying between 7.7% and 37.3%, to make a strong conclusion about the observed differences.

Plasma anti-ClpB and α-MSH–reactive Igs

Comparison of patients and controls for their plasma anti-ClpB antibodies revealed lower levels of anti-ClpB IgM in patients with anorexia compared with healthy controls, as well as with respect to patients without anorexia (Fig. 3A). Post hoc power of 86.3% confirmed the validity of such differences. Plasma levels of anti-ClpB IgG (Fig. 3B) did not show significant differences between patients and controls ($P = 0.65$).

A tendency of a decrease in both α-MSH–reactive IgM and IgG levels was observed in patients with anorexia compared with

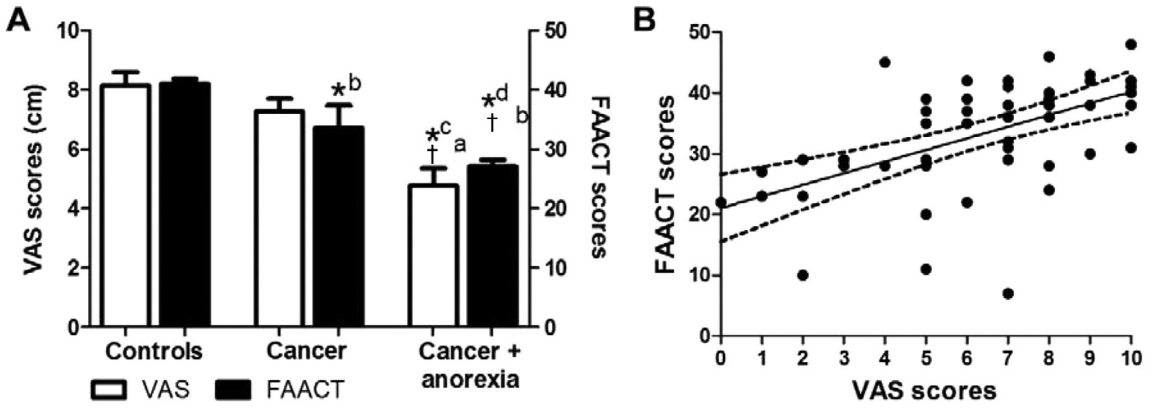

Fig. 1. Assessment of appetite using the VAS and FAACT. (**A**) Mean (± SEM) levels of VAS and FAACT scores in healthy controls and in patients with or without anorexia. VAS, ANOVA, $P < 0.0001$, Tukey's post-tests $^{*}P < 0.05$, $^{\dagger}P < 0.001$, ^{a}vs controls, ^{c}vs cancer. FAACT, ANOVA, $P < 0.0001$, Tukey's post-tests $^{*}P < 0.05$, $^{\dagger}P < 0.001$, ^{b}vs controls, ^{d}vs cancer. (**B**) Correlation between FAACT and VAS scores in the participants. Linear regression (line), slope 1.9 ± 0.4, $P < 0.0001$, dashed lines, 95% CI. ANOVA, analysis of variance; FAACT, Functional Assessment of Anorexia/Cachexia Therapy; VAS, visual analog scale.

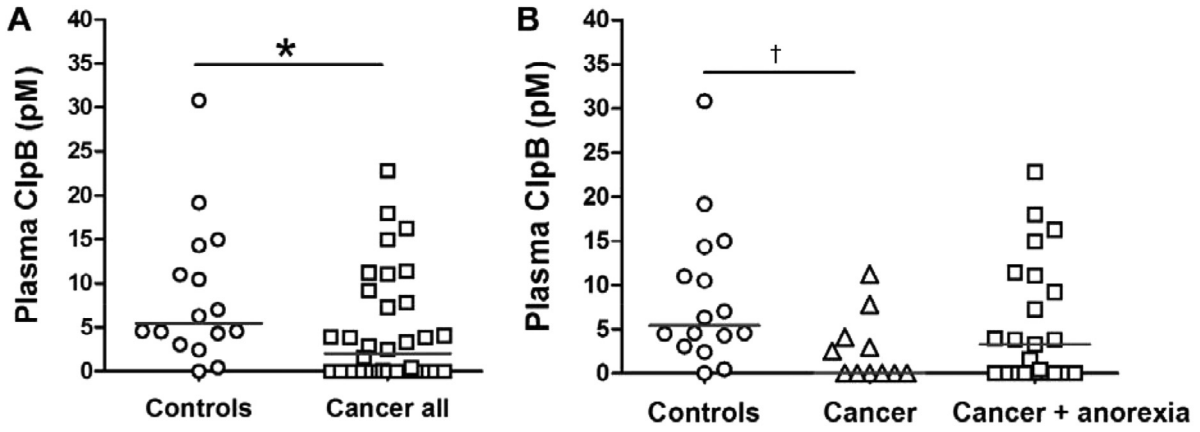

Fig. 2. Plasma ClpB concentrations. (**A**) Controls vs all cancer patients. *Mann–Whitney test $P = 0.018$. (**B**) Controls and cancer patients with or without anorexia. Kruskal–Wallis test $P < 0.05$, †Dunn's post-test $P = 0.028$. ClpB, caseinolytic-protease-B.

healthy control (Fig. 3C, D). Affinity kinetics analysis of plasma extracted IgG toward ClpB showed median value around the micromolar range with a strong tendency of a lower affinity in patients with anorexia compared with those without ($P = 0.05$; Fig. 4A). The constants of association and dissociation did not significantly differ among the groups. No significant differences in affinity kinetics was found for α-MSH IgG (Fig. 4B).

Appetite score correlations

To further analyze the relevance of ClpB levels and ClpB and α-MSH antibodies to the appetite scores in the patient group, we analyzed the correlation between these biomarkers and the FAACT and VAS scores. No significant correlations between these scores and ClpB concentrations were found. Both FAACT (Spearman's $r = 0.32$, $P = 0.01$) and VAS scores (Spearman's $r = 0.43$, $P = 0.001$) correlated positively with plasma levels of ClpB IgM antibodies; whereas FAACT scores correlated with α-MSH IgG (Spearman's $r = 0.31$, $P = 0.02$) and VAS with α-MSH IgM (Pearson's $r = 0.37$, $P = 0.005$; Fig, 5A, B). To estimate the functional potency of plasmatic ClpB in each participant, its level was divided by the plasma levels of anti-ClpB IgM and IgG, the latter adjusted to the affinity values. We found that the mean values of the ClpB potency in the patients with anorexia were 354 times higher than in patients without the disorder ($P < 0.05$; Fig. 5C). A difference in the ClpB potency in patients with anorexia versus healthy controls (1.5 times) was not significant.

Discussion

In cancer, disturbance in appetite is an important and frequent clinical problem and the present results showed a high prevalence of anorexia (68%, by AQ) in patients with lung cancer, confirming the data obtained in previous studies [21,26]. To our knowledge, this was the first study to evaluate enterobacterial ClpB protein as well as anti-ClpB and α-MSH–reactive Ig levels in cancer patients with and without anorexia, although analyzed in a limited number of participants using a post hoc power calculation. In particular, we found that in the present cohort, the patients had decreased plasma levels of ClpB compared with the healthy controls. Importantly, when stratified by the presence of anorexia, such decrease was significant only in those patients without anorexia. Moreover, low levels of anti-ClpB IgM and decreased affinity of anti-ClpB IgG further differentiated patients with anorexia, pointing to a deficient immune response to ClpB, which might lead to its increased functional potency. Thus, considering satietogenic properties of ClpB, our results, although exploratory, may indicate an increased satietogenic effect of ClpB in patients with cancer and anorexia. Indeed, the FAACT and VAS tools, indicating a minimum (low appetite) and a maximum (high appetite) score, were useful for testing

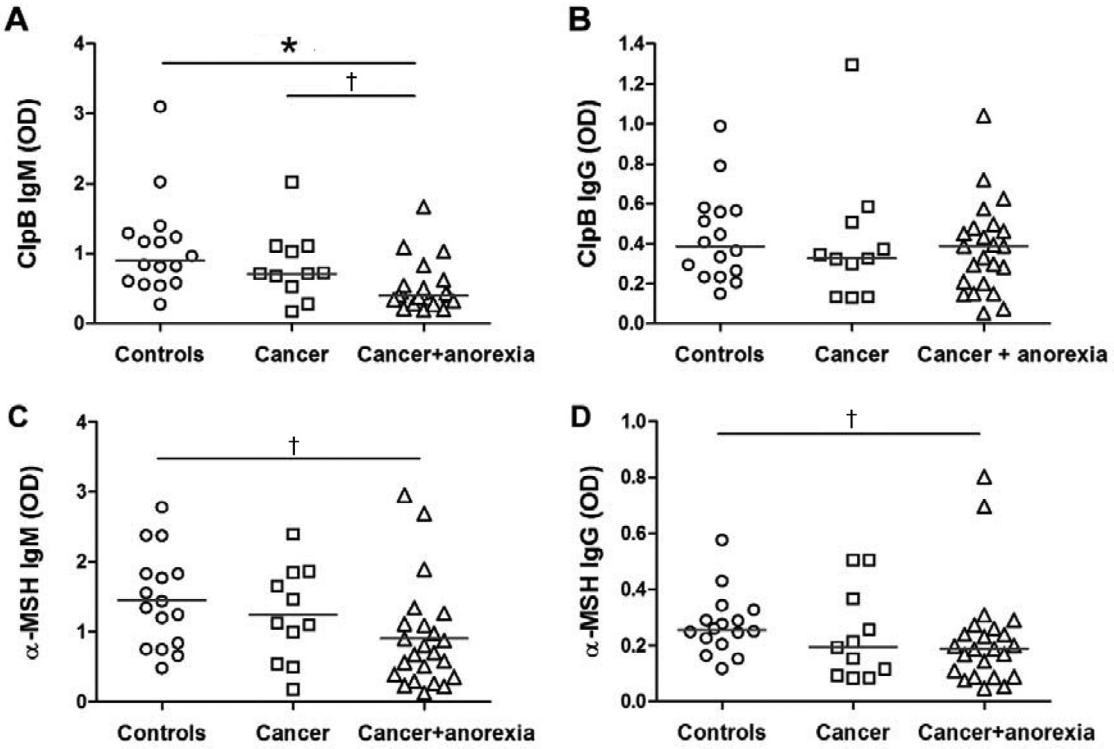

Fig. 3. Plasma levels (including median values) of anti-ClpB antibodies (**A, B**) and of α-MSH–reactive immunoglobulins (**C, D**) in healthy controls and in cancer patients with or without anorexia. IgM (**A, B**) and IgG class (**B, D**) of antibodies. **A**, Kruskal–Wallis test $P = 0.0009$, *Dunn's post-test $P < 0.0001$, †Mann–Whitney tests $P < 0.05$. ClpB, caseinolytic-protease-B.

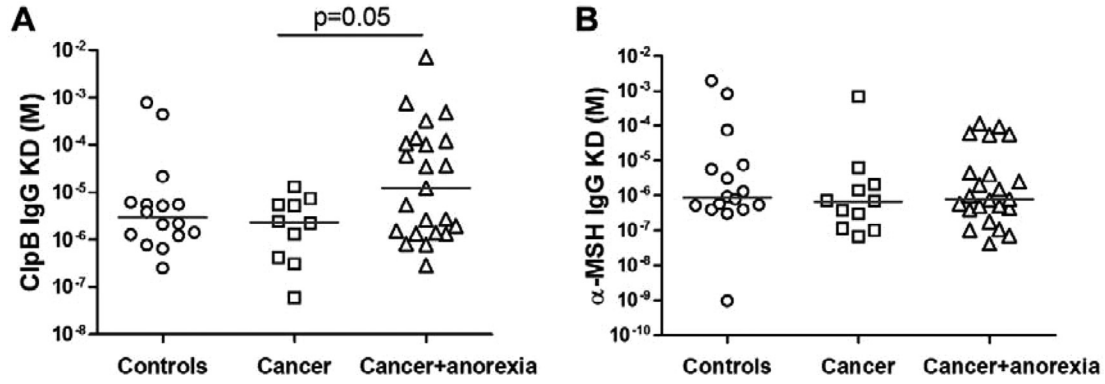

Fig. 4. Affinity kinetics of plasma IgG for ClpB (**A**) and for α-MSH (**B**). ClpB, caseinolytic-protease-B; Ig, immunoglobulin; MSH, melanocyte-stimulating hormone.

our hypothesis on the possible link between ClpB-related biomarkers and anorexia because these tools allowed to appreciate the degree of anorexia severity.

Previous studies showed that presence of ClpB protein was necessary to decrease food intake in both lean and obese mice receiving ClpB-producing *E. coli* [16,18]. Because ClpB with α-MSH-like epitope is produced by common gut bacteria (e.g., belonging to the Enterobacteriaceae family), its presence in the circulation is physiologic, and it was validated by the immunoassay in both rodents and humans [19,20]. Although the primary site of action of the enterobacterial ClpB should be a place of its production and release (i.e., the gut) where, for instance, it activates the secretion of peptide YY, a satiety hormone [27], the systemic effects of ClpB may include an action on the hypothalamus. Indeed, ClpB-activated anorexigenic proopiomelanocortin neurons of the arcuate nucleus may act on the hypothalamic feeding center [19].

As the plasmatic concentration of ClpB should reflect its bacterial production [19], a decrease in ClpB concentration in the entire group of patients may result from the modification in gut microbiota composition. Indeed, a decrease in relative abundance of *Escherichia-Shigella* and *Enterobacter* (i.e., some common genera of the Enterobacteriaceae family) was found in gut microbiota of patients with lung cancer [28]. Such changes in gut microbiota composition may underlie lower levels of enterobacterial ClpB protein in plasma of patients shown in the present study, suggesting that anorexia in patients with cancer cannot be a result of a mere increase of ClpB concentration. Moreover, although ClpB concentration was not significantly lower in patients with anorexia versus healthy controls, it was not increased either.

Nevertheless, the differences in ClpB concentration as well as in anti-ClpB IgM and IgG levels and properties, respectively, between patients with and without anorexia point to a possible increase of

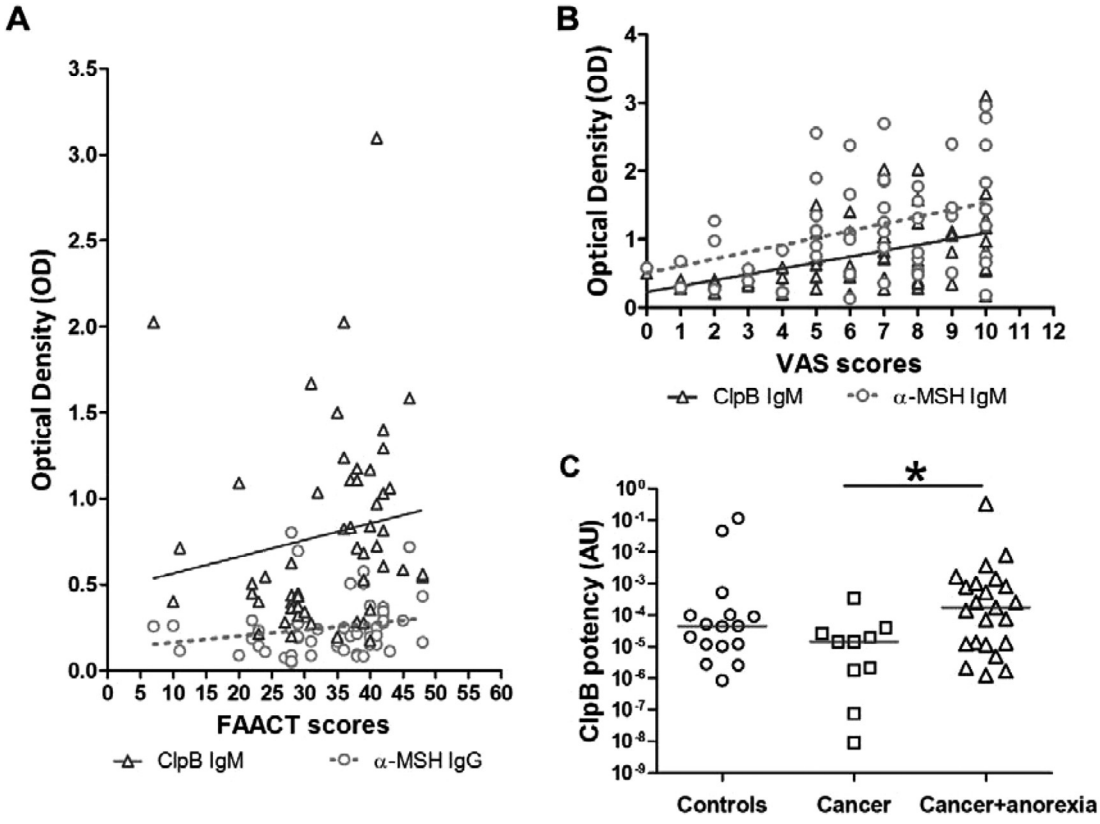

Fig. 5. (**A**) Correlations between FAACT scores and plasma levels of ClpB IgM (Spearman's $r = 0.32$, $P = 0.01$) or α-MSH IgG (Spearman's $r = 0.31$, $P = 0.02$) in study subjects. (**B**) Correlations between VAS scores for appetite and plasma levels of ClpB IgM (Spearman's $r = 0.43$, $P = 0.001$) or α-MSH IgM (Pearson's $r = 0.37$, $P = 0.005$) in participants. (**C**) ClpB functional potency in arbitrary units (AU) calculated as: ClpB conc. (pM) / ClpB IgM (OD) / ClpB IgG (OD) * 1 / KD (M). Kruskal–Wallis test $P = 0.048$, *Dunn's post-test $P < 0.05$. ClpB, caseinolytic-protease-B; Ig, immunoglobulin; MSH, melanocyte-stimulating hormone; OD, optical density.

ClpB signaling relevant to the reduced appetite in patients with anorexia. In particular, we found decreased levels of anti-ClpB IgM in patients with cancer anorexia, which distinguished them from both the controls and patients without anorexia. Because the IgM class of antibodies is induced by the recent antigen exposure, such deficiency may indicate an insufficient neutralization of newly produced ClpB protein by gut bacteria. Furthermore, although anti-ClpB IgG levels did not differ among the groups, the IgG affinity for ClpB was reduced in patients with anorexia, showing again an insufficient immune response to the antigen, whose continuous exposure should normally lead to an affinity maturation. Taken together, these changes in IgM and IgG anti-ClpB responses may underlie an increased functional ClpB potency even without increased production of ClpB by the gut bacteria. The statistical analysis of the ClpB potency, whereas the ClpB concentrations are divided by levels of anti-ClpB IgM and IgG (Fig. 5C) supports such possibility. The results showing significant positive correlations between anti-ClpB IgM levels and appetite, as found by both the FAACT and VAS tools for appetite assessment, also support this conclusion. The reason for a deficient immune response to ClpB is not yet known, it is possible that the natural IgM are mobilized to bind tumor cells [29,30].

We also found a decrease of plasma levels of α-MSH-reactive IgM and IgG in patients with cancer anorexia. This finding is not surprising considering the molecular mimicry between a ClpB motif and of the α-MSH peptide sequence. The functional significance of such a decrease is presently unclear, α-MSH–reactive IgG were shown to modulate activation of the melanocortin 4 receptor (MC4R) by forming an immune complex with the α-MSH peptide. In fact, a previous study showed that such immune complexes in patients with anorexia nervosa result in enhanced MC4R internalization (i.e., increased signaling upon the cells expressing these receptors, which may result in increased satiety signaling) [31]. However, in patients with anorexia nervosa, plasma levels of both α-MSH–reactive IgM and IgG were increased [31,32], differing them from the present results in patients with cancer anorexia. It suggests that in contrast to the autoimmune pathophysiology of eating disorders, whereas bacterial ClpB may play a role of an antigen responsible for production of pathologic α-MSH cross-reactive auto-Abs [17], the ClpB involvement in cancer anorexia can be related to its enhanced direct satietogenic effect due to insufficient immune control. Both α-MSH–reactive IgM and IgG levels correlated positively with the VAS and FAACT scores, respectively, showing that similar to anti-ClpB IgM, increased levels of these auto-Abs are associated with increased appetite. Interpretation of these results need further functional validation as changes in α-MSH immunoreactivity can be a mere reflection of anti-ClpB antibodies.

Finally, there were some limitations in our study. Although we considered the same type of cancer (lung) in our cohort, representing a homogenous sample, the entire number of patients and controls considered was small, particularly the number of patients with anorexia. In this light, the present results need to be confirmed selecting an equivalent number of patients in each group, also considering other type of cancer where anorexia is highly prevalent (ie, gastrointestinal cancer), and more importantly in larger cohorts. Anorexia may be associated with altered nutritional status, which in turn may impair immune response that might have affected the different immune reaction observed between patients with or without anorexia. We also

admit that the present study was descriptive and the functional significance of the observed changes need to be explored in in vivo and in vitro models.

Moreover, the often overlapping values of ClpB concentration and anti-ClpB antibody levels between patients and controls indicated that we should not overestimate the potential functional significance of these data, which should be taken in the context of other important anorexigenic signals present in cancer-associated anorexia

Conclusion

The present results showed that the decreased appetite observed in patients with lung cancer was associated with low levels of IgM directed against enterobacterial ClpB, a natural satietogenic protein produced in gut microbiota. Because the plasma concentrations of enterobacterial ClpB were decreased in patients with cancer, the present results suggested that an insufficient immune control of ClpB, rather than its increased production, may lead to its enhanced satietogenic effects contributing to the development of cancer anorexia in some patients.

Acknowledgments

The authors acknowledge TargEDys SA, France, for providing ClpB protein and antibodies.

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