Biochemical, cellular and proteomic profile from head and neck cancer patients submitted to postoperative immunomodulating therapy

Perfil bioquímico, celular e proteômico de pacientes com câncer de cabeça e pescoço submetidos à terapia imunomoduladora pós-operatória

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ABSTRACT

Introduction: Head and neck cancer is one the most prevalent forms of cancer in Brazil and worldwide. Immunomodulating enteral nutrition therapy (ENT) can benefit head and neck cancer patients by modulating immune and inflammatory response. Objective: The purpose of this study was to draw a biochemical, cellular and proteomic profile of plasma from head and neck cancer patients submitted to postoperative immunomodulating ENT for 3-5 days. Material and Methods: The cohort was predominantly male, elderly and malnourished. Plasma proteins were quantified and submitted to electrophoresis, depletion chromatography and mass spectrometry. Results: The most highly expressed proteins were C-reactive protein and serum amyloid A1 and A2 (before ENT) and Factor B of the complement system (after ENT). Six protein tumor markers were identified: AAS, plasmin, zinc-α2-glycoprotein, α-1 antichymotrypsin, leucine-rich α-2 glycoprotein 1 and 2, and heavy chain of IgM. Conclusion: In conclusion, the most highly expressed protein in response to postoperative immunomodulating ENT was Factor B of the complement system.

Keywords: Immunonutrition; Head and neck cancer; Proteomics.

RESUMO

Introdução: O câncer de cabeça e pescoço é uma das formas de câncer mais prevalentes no Brasil e no mundo. A terapia de nutrição enteral imunomoduladora (TNE) pode beneficiar pacientes com câncer de cabeça e pescoço, modulando a resposta imune e inflamatória. Objetivo: O objetivo deste estudo foi traçar um perfil bioquímico, celular e proteômico do plasma de pacientes com câncer de cabeça e pescoço submetidos à TNE imunomoduladora pós-operatória por 3-5 dias. Material e Métodos: A coorte foi

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predominantemente masculina, idosa e desnutrida. As proteínas plasmáticas foram quantificadas e submetidas a eletroforese, cromatografia de depleção e espectrometria de massa. **Resultados:** As proteínas mais expressas foram a proteína C reativa e a amiloides sérica A1 e A2 (antes da TNE) e o Fator B do sistema complemento (após TNE). Seis marcadores tumorais proteicos foram identificados: AAS, plasmina, zinco-α2-glicoproteína, α-1 antiprotease, α-2 glicoproteína 1 e 2 rica em leucina e cadeia pesada de IgM. **Conclusão:** Em conclusão, a proteína mais expressa em resposta à TNE imunomoduladora pós-operatória foi o Fator B do sistema complemento.

**Palavras-chave:** Imunonutrição; Câncer de cabeça e pescoço; Proteômica.

**INTRODUCTION**

Localized in the upper aerodigestive tract, head and neck cancer is one of the most important types of tumor in Brazil and worldwide with regard to incidence, prevalence and mortality (PRIMO et al., 2016). Malnutrition is observed in 30-40% of patients with early-stage head and neck cancer. It is associated with long hospital permanence, low response to treatment, high toxicity, loss of quality of life, poor prognosis, and low survival rates (LACH; PETERSON, 2017). It can also lead to significant immunological changes such as dysfunction and induction of lymphocytopenia, downregulation of monocyte expression and reduction of prostaglandin levels (VASSON et al., 2014). Toxicities induced by head and neck cancer treatment have a negative impact on nutritional status, compromising treatment tolerance and efficacy, increasing health care costs, and leading to increased risks of complications, death, and poor survival (CHANG et al., 2017). The persistent state of the chronic inflammatory response resulting from the cancer disease itself, contributes to the patient being more prone to different stages of nutritional status depletion, leading to progressive cachexia. Therefore, the monitoring of acute or chronic inflammation allows professionals greater tools for early nutritional assistance, allowing them to establish strategies such as the use of the immunomodulatory diet, in order to reverse and minimize the impacts caused by the pathology, leading to improved immunity and lower levels of postoperative morbidity and mortality (FRUCHTENICHT et al., 2018).

When supplied perioperatively, specific dietary components have been shown to boost immune function by modulating immune and inflammatory response *in vitro* and in patients with trauma, burns and oncological surgery (CASAS RODERA et al., 2012). Immunonutrients may be used to intervene therapeutically in the immune response autoregulation of oncological patients (OLIVEIRA; BONETI; PIZZATO,
Proteomics, an increasingly popular tool in this context, is a high-throughput technology used in comprehensive evaluations of clinically subtle phenotype changes (GALDOS-RIVEROS et al., 2010; CALDER et al., 2013). Nutritional proteomics is a fundamental tool in the study of nutrition therapy-induced changes in tumor incidence and behavior (MILNER, 2004).

Immunomodulation is believed to benefit patients directly by improving immune response and nutritional status, thereby reducing the risk of postoperative complications. Improvement may be detected with proteomic analysis. The purpose of the present study was to draw a biochemical, cellular and proteomic profile of plasma from head and neck cancer patients submitted to postoperative immunomodulating enteral nutrition therapy (ENT).

**MATERIAL AND METHODS**

**Study design**

Prospective, descriptive laboratory study.

**Study location**

ENT and blood sampling were conducted at a referral center in Northeastern Brazil (Haroldo Juaçaba Hospital/Ceará Cancer Institute). The proteomic analyses were performed at the Laboratory of Proteomic Analysis (University of Fortaleza).

**Study population**

All patients hospitalized from August to November 2015 at the head and neck service undergoing exclusive enteral therapy were a total of 10 patients, corresponding to 100% of the sample.

**Clinical parameters**

Gender, age, baseline and endpoint nutritional status (body mass index), duration of hospital stay, intercurrencies, type of surgical procedure, tumor site, comorbidities and postoperative complications (hospital infection, fistula, sepsis).

**Inclusion criteria**
Age >18 years; diagnosis of head and neck cancer; postoperative immunomodulating enteral nutrition therapy; informed written consent.

**Exclusion criteria**

Chronic transmissible diseases (such as HIV), receiving pharmacological treatment; neurological or psychiatric conditions; incomplete medical records with regard to the study parameters.

**Ethical considerations**

Approved and filed under #965.632, the study protocol followed the guidelines set forth in Resolution #466/12 of the National Health Council (Brazilian Ministry of Health) and the guidelines of the research ethics committees of the University of Fortaleza (Unifor) and the Ceará Cancer Institute (ICC).

**Blood sampling**

Two blood samples (7 mL) were drawn from each patient: the first after one day of ENT without immunonutrients; the second after 3-5 days of ENT with immunonutrients.

**ENT with and without immunonutrients**

All patients received adequate volume (approximately 1.8L/day) to reach 100% of the caloric goals (mean: 1861.2 kcal/day), according to the pocket formula (energy recommendation of 25 to 35 kcal/kg/day; recommendation protein from 1.2 to 2.0 g/kg/day) (TALWAR et al., 2016), being infused intermittently. The immunonutrients included arginine, polyunsaturated fatty acids (DHA and EPA), nucleotides, chloride, pantothenic acid, biotin, vitamin B6, vitamin C, vitamin A, zinc, copper, selenium, chrome and molybdenum. Table 1 shows the amount of immunonutrients received by each patient per day.
Table 1- Amount of immunonutrients received by each patient.

<table>
<thead>
<tr>
<th>Patient</th>
<th>L-arginine (g)</th>
<th>Nucleotides (g)</th>
<th>Polyunsaturated fatty acids (g)</th>
<th>DHA, EPA (g)</th>
</tr>
</thead>
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<td>8,2614</td>
<td>6,6878</td>
</tr>
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<td>2</td>
<td>25,5</td>
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<td>8,568</td>
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<td>7,182</td>
<td>5,814</td>
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<tr>
<td>4</td>
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<td>2,04</td>
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<td>2,23344</td>
<td>7,81704</td>
<td>6,32808</td>
</tr>
</tbody>
</table>

EPA – Eicosapentaenoic acid; DHA- Docosahexaenoic acid.


Laboratory methods

**Protein quantification**

The samples were individually quantified by absorbance at 280 nm using a Nano Vue Plus spectrometer (GE Healthcare, USA). Four pools were created using equivalent amounts of protein (3 mg) from the 10 individual samples, with a final volume of 1 mL: Pool 1: before immunomodulating ENT, depleted of albumin and IgG; Pool 2: after immunomodulating ENT, depleted of albumin and IgG; Pool 3: before immunomodulating ENT, rich in albumin and IgG; Pool 4: after immunomodulating ENT, rich in albumin and IgG; Pools 1 and 2 were submitted to mass spectrometry.

**Electrophoresis of plasma protein**

Plasma protein was submitted to capillary electrophoresis at a local laboratory of clinical analysis (Dr. Perez Limardo).

**Depletion chromatography**

Plasma samples stored at -80°C were thawed and depleted. A 100-µL plasma aliquot was added to 200 µL buffer and filtered through a 0.22 µM membrane. The
filtered plasma (150 µL) was applied to a 1-mL albumin and IgG depletion column (HiTrap, GE Healthcare, USA) coupled to a FPLC system (ÄKTApurifier 10, GE Healthcare, USA).

**Mass spectrometry**

Initially, the dialyzed and concentrated pool fractions containing serum proteins were submitted to tryptic digestion. The tube was immersed in a dry bath at 60°C. After incubation for 30 min, 5 µL 300 mM iodoacetamide was added to alkylate the cysteins. The samples were stored for 30 min in a dark place at room temperature to allow for alkylation, followed by the addition of 1 µg trypsin and digestion in an incubator for ~16 hours at 37°C. Finally, the sample was centrifuged at 12000 g for 30 min and the supernatant transferred to appropriate vials. Tryptic peptides of yeast alcohol dehydrogenase (ADH, UniProt P00330) were added to the digested proteins to serve as standard in the absolute quantification of each sample. Chromatography was performed on a nanoACQUITY C18 UPLC BEH (1.7 µm, 100 µm × 10 cm) reversed-phase column with 130 Å pores. A 200 ng aliquot of sample containing the peptides obtained by protein digestion was used. All analyses employed electrospray ionization in ESI(+) mode powered by a NanoLockSpray source. The data were managed with the software MassLynx v4.1 and ProteinLynx v2.4, while database searches were performed with the package ProteinLynx Global Server (PLGs) v.2.4, containing the software Expression E v.2.4. Searches in the databases UniProtKB/Swiss-Prot 57.1 and UniProtKB/TrEMBL 40.1 were conducted specifying the taxonomy of *Homo sapiens*. Only proteins above the 95% confidence limit were considered acceptable results in database searches. The before/after immunonutrition ratios of the 3x3 filter were <0.66 for ‘down’ and >1.5 for ‘up’.

**Statistical analysis**

The data obtained for the biochemical, cellular and proteomic plasma profile was submitted to the Kolmogorov-Smirnov test. Mean values of parametric variables were compared with Student’s *t* test at the 95% level of significance. Bilateral *p*-values under 0.05 were considered statistically significant in the comparison of means. Pearson’s correlation coefficient was used to compare the variables “days on immunomodulating diet” vs. “leukocytes, neutrophils and segmented cells”. The closer
to 1 (negative or positive), the stronger the correlation. The significance of $r$ was determined with Student’s $t$ test at the 5% level of probability (the correlation was significant when $p \leq 0.05$).

**RESULTS**

*Demographic, clinical and nutritional profile*

The patients were predominantly male (70%) and elderly (mean: 66.7 years). Half (50%) were malnourished and diagnosed with oral cancer (Figure 1). Tumors were equally distributed between the larynx, the mouth and the tongue. The most common surgical procedures were total laryngectomy and glosso-pelvi-mandibulectomy. The mean duration of hospital stay was 9 days, with 80% discharge and 20% rehospitalization. This is less than the mean time (14 days) informed by the hospital department responsible for monitoring procedures and quality. In general, no postoperative complications (hospital infection and sepsis) were registered, with the exception of fistula in a patient with severe malnutrition. Most (70%) experienced intercurrences during hospitalization, such as diarrhea, constipation and (most frequently) dysphagia and/or anorexia. Most patients had no comorbidities, according to the results: 70% had no comorbidities, 20% had arterial hypertension and 10% had drug allergy.

*Biochemical and cellular profile before and after immunomodulating ENT*

The protein parameters measured in patients before and after the use of immunomodulatory nutritional therapy showed that the data did not present differences between the averages before and after immunonutrition. Compared to reference values, albumin, transferrin, immunoglobulins and total proteins were lower than metabolic changes caused by tumor and malnutrition. Alpha 2 values and albumin/globulin ratio were within the reference values, while the values of the alpha 1 fraction protein components and C3 complement were above. The parameters such as leukocytes, neutrophils and segmented showed a difference between the mean ($p < 0.05$). Compared to the reference values, the values of red blood cells, hemoglobin, hematocrit were inferior, while lymphocytes, platelets, potassium, sodium and urea were within the reference values. Regarding creatinine, for men were below and for women were within normal. Leukocyte values without immunonutrients were above the reference value,
while with the use of immunonutrients was within normal. Neutrophils and segmented were above the reference values.

**Chromatographic profile before and after immunomodulating ENT**

As shown in Figure 2, protein concentrations expressed before and after immunomodulating ENT were similar for pools rich in albumin and IgG (peak 1). For pools depleted of albumin and IgG, concentrations were higher after immunomodulating ENT (peak 2).

**Proteomic profile before and after immunomodulating ENT**

The identification and quantification of serum proteins expressed before and after immunomodulatory nutritional therapy was a total of 29 proteins. The most highly expressed protein after immunomodulating ENT was Factor B of the complement system. C-reactive protein and serum amyloid A1 and A2 protein (characteristic inflammatory proteins) were the most highly expressed before immunomodulating ENT. All other protein levels remained unchanged. Blood clotting proteins were also detected, including β-2-glycoprotein I, plasmin, fibronectin, antithrombin III, hemopexin and fibrinogen, as were apolipoproteins E type and A-II type, and zinc-α2-glycoprotein. The identified globulins included α-1 antitrypsin, α-1 acid glycoprotein 1 and 2, α-1 antichymotrypsin, haptoglobin, α-2 macroglobulin, leucine-rich α-2 glycoprotein 1 and 2, vitamin D-binding protein, C3, C4, and immunoglobulin (heavy chain and heavy chain of IgM).

**Correlation between immunomodulation and clinical progress**

The relationship between leukocytes, neutrophils and segmented variables and immunonutrient diet days was negatively, that is, as the days of dietary supply increased the inflammatory response cells. There was no significance in this relationship.

**DISCUSSION**

In a multicenter study on perioperative immunonutrition in head and neck cancer patients conducted by Falewee et al. (2013), patients were predominantly male and over 65 years. Likewise, in a study by Vidal-Casariego et al. (2014), most of the patients were male and in the range of 55-63 years.
Half our patients (50%) were malnourished, a finding compatible with Vasson et al. (2014) who found 30-40% malnutrition among head and neck cancer patients. Malnutrition is the result of neoplasia which causes inefficient carbohydrate metabolism, accelerated protein catabolism and progressive lipid depletion, favoring the development of severe postoperative complications (STABLEFORTH; THOMAS; LEWIS, 2009). The tumor sites most frequently observed by Vidal-Casariego et al. (2014) and Felekis et al. (2010) were the oral cavity, the larynx and the pharynx. In Riso et al. (2000), 18.2% of the patients developed postoperative complications and the time of postoperative permanence was 11.6-25 days. Malnutrition is generally associated with poor healing, increased risk of infection, treatment toxicity and a greater incidence of fistulas (LENZA et al., 2013). The preliminary results of Talwar et al. (2016) suggest that perioperative ENT enriched with polyunsaturated fatty acids can improve nutritional parameters, including weight, lean body mass, the incidence of postoperative infections and the time of hospital permanence.

Contrasting with the present study, Vasson et al. (2014) reported an increase in albuminemia after immunomulating ENT, probably due to the extended time of treatment. Luis et al. (2009) found increased serum albumin and transferrin levels in head and neck cancer patients submitted to ENT enriched with arginine, when compared to the standard formula. The addition of arginine to the diet had no measurable effect on any other serum protein. The negative hematological effects of malnutrition include numerical deficiencies in most cell lines and changes in innate immune function (WAITZBERG et al., 2006).

Hypoalbuminemia is correlated with postoperative morbidity and mortality. Inflammation and malnutrition can significantly affect albumin levels. Reduced serum albumin concentrations may reflect a rise in the level of an acute-phase reagent (WAITZBERG et al., 2006).

Transferrin levels are low in acute-phase reactions, neoplasia and malnutrition, all of which were observed in our cohort. The β-1 fraction may also be reduced in inflammatory processes (BOTTINI, 2007). As for immunological changes, Riso et al. (2000) found significantly reduced levels of IgA and IgG in malnourished head and neck cancer patients. α-1 globulin levels rise in association with acute-phase reactions, metastatic carcinosis and use of corticoids and anti-inflammatory drugs, as observed in postoperative scenarios. C3, a weak and late acute-phase reagent (BOTTINI, 2007), was
elevated in our patients due to their physiological conditions following large surgeries. Casas Rodera et al. (2012) found no differences in plasma protein and lymphocyte levels in a study on immunonutrition. In a study by Dias et al. (2005), no significant differences were observed between the groups with regard to albumin, total proteins, hematocrits and hemoglobin, but total lymphocyte levels decreased significantly following immunomodulating ENT. Likewise, in Luis et al. (2007) no differences were detected between standard ENT and immunomodulating ENT with regard to plasma proteins and lymphocytes.

The observed decrease in erythrocytes, hemoglobin, hematocrits and creatinine in our cohort was likely due to surgical trauma, nutritional deficiency (iron, vitamin B12 or folate) and protein depletion associated with malnutrition in most patients. The observed increase in leukocytes, neutrophils and segmented cells suggests the presence of infection, chronic inflammation and/or acute hemorrhage, a common finding in this patient population (BOTTONI et al., 2000).

Factor B, the most highly expressed protein in mass spectrometry after immunomodulating ENT, is a thermolabile betaglobulin. As part of the alternative complement system, Factor B has been implicated in the proliferation and differentiation of preactivated B-lymphocytes, rapid propagation of peripheral blood monocytes, stimulation of lymphocyte blastogenesis and erythrocyte lysis. Along with antibodies, it is the main humoral mediator in inflammation and plays an important role in different types of immuno-inflammatory reactions (ITURRY-YAMAMOTO; PORTINHO, 2001).

C-reactive protein levels are usually high in acute and chronic infection, postoperative trauma, neoplasia, acute cardiovascular disease and non-infectious systemic inflammation (AGUIAR et al., 2013).

In patients submitted to immunomodulating ENT, plasma C-reactive protein levels are down-regulated possibly because of specific nutrients which act directly or indirectly on the immune system. This can lead to metabolic changes associated with systemic inflammation or multiple changes in circulating cytokines modulating the immune system by inhibiting neutrophil function, stimulating hormone production, releasing vasodilators, and activating lymphocytes and macrophages. In the study by Piovacari (2008), inflammatory response lessened and biochemical markers improved as acute-phase protein levels decreased.
Serum amyloid A (SAA), a major acute-phase protein with chemoattractive activity, was also detected. As shown by Knebel (2014), C-reactive protein and SAA are closely related. The latter modulates inflammatory processes and has recently been adopted as a marker of tumor progression due to the observed increase in SAA levels in human neoplasia and due to the fact that SAA is consistently produced in chronic processes, contributing to tumor genesis and progression.

In this study we identified six protein tumor markers: AAS, plasmin, zinc-α2-glycoprotein, α-1 antichymotrypsin, leucine-rich α-2 glycoprotein 1 and 2, and heavy chain of IgM. Plasmin degrades many blood plasma proteins, especially fibrin clots, and plays a role in tumor invasion and inflammation (ROSSIGNOL et al., 2004). Regulated by glucocorticoids, zinc-α2-glycoprotein has a range of important functions, including lipid mobilization. Due to structural similarities with antigen-presenting MHC class I molecules, zinc-α2-glycoprotein is involved in immune response and used as a tumor biomarker in several carcinomas (HASSAN et al., 2008).

α-1 antichymotrypsin, an acute-phase glycoprotein found in the α-1 globulin region, inhibits chymotrypsin-like proteinases in vivo. It is activated in the control of immune and inflammatory processes and may be used as as tumor marker (RUBIN et al., 1990). Leucine-rich α-2 glycoprotein 1 and 2, an acute-phase α-2 glycoprotein of the leucine type, is regulated by acute-phase response mediators. Concentrations of this marker increase during cancer, probably as a result of hepatic response to pro-inflammatory cytokines (YAMADA et al., 2004). Heavy chain of IgM plays an important part in primary defense mechanisms. It is produced in the early acute phase of diseases which induce humoral responses and is involved in the recognition and elimination of cancer/precancer lesions (TISCH; ROIFMAN; HOZUMI, 1988).

In a study by Bistrian (2004), immunomodulating diets designed to support the immune system were found to be modestly efficient at reducing postoperative infection rates in a cohort of patients with head and neck cancer.

Vasson et al. (2014) reported that immunomodulating ENT containing arginine, EPA, DHA and nucleotides was more efficient than the standard formula at preventing nutritional and functional deterioration, especially in malnourished patients. Moreover, Bharadwaj et al. (2016) showed that in large and stressful elective surgeries followed by inflammatory reactions, perioperative administration of immunonutrients
helps reduce infection rates, complications and hospital permanence, regardless of nutritional status.

The study has as limitations the sample size, the short time of administration of the immunomodulatory diet, the reduced number of anthropometric parameters to assess the nutritional status and how they could respond to the immunomodulatory diet. The fact that the immunological and inflammatory parameters were measured only at the end of the period of nutritional support without a comparison of values before surgery can also be pointed out.

CONCLUSIONS

In conclusion, in this study the most highly expressed protein after immunomodulating ENT was Factor B of the complement system. C-reactive protein and serum amyloid A (positive acute-phase proteins) were the most highly expressed before immunomodulating ENT. Biochemical and cellular parameters remained unchanged. However, further investigations with larger cohorts and longer immunomodulating ENT schemes are necessary to clarify the relation between expressed proteins and immunomodulating ENT.

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CONFLICT OF INTEREST

Authors certify that there is no conflict of interest.

ETHICS COMMITTEE APPROVAL

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REFERENCES


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