Prognostic Significance of Serum Adipokine Levels in Colorectal Cancer Patients

FIORELLA GUADAGNI[1], MARIO ROSELLI[2,3], FRANCESCA MARTINI[1], ANTONELLA SPILA[1], SILVIA RIONDINO[1], ROBERTA D'ALESSANDRO[1], GIROLAMO DEL MONTE[4], VINCENZO FORMICA[1,3], ANASTASIA LAUDISI[3], ILARIA PORTARENA[3], RAFFAELE PALMIROTTA[1] and PATRIZIA FERRONI[1]

[1]Department of Laboratory Medicine and Advanced Biotechnologies, [2]IRCCS San Raffaele, 00163 Rome; [3]Medical Oncology, Department of Internal Medicine, University of Rome 'Tor Vergata', Tor Vergata Clinical Center, 00133 Rome; [4]San Raffaele Rocca di Papa, 00040, Rocca di Papa, Rome, Italy

Abstract. *Background: Adipokines may significantly influence the growth and proliferation of tumor stroma and malignant cells within. Reduced adiponectin and increased leptin serum levels were found in colorectal cancer (CRC) patients. Recently, it has been demonstrated that tumor necrosis factor-α (TNF-α) is able to induce dose-dependent changes in serum adipokine levels. Thus, aims of this study were to evaluate the possible associations between adipokines, TNF-α and clinicopathological variables of CRC patients and to analyze their possible prognostic value in predicting relapse-free and overall survival. Materials and Methods: Baseline leptin, adiponectin and TNF-α levels were analyzed in 90 patients with histologically diagnosed primary or newly diagnosed metastatic CRC treated at 'Tor Vergata' Clinical Center and followed up for a median period of 3 years. Results: Serum leptin levels were higher in CRC patients than in controls (p<0.0001). Conversely, serum adiponectin levels were lower in CRC patients than in controls (p<0.0001). Leptin inversely correlated with adiponectin (p<0.005). The leptin/adiponectin (L/A) ratio was eight-fold greater in CRC compared to controls (p<0.0001). Kaplan-Meier analysis of relapse-free and overall survival time showed that the L/A ratio was an independent predictor for adverse outcome in CRC. Conclusion: Serum adipokine levels might have a role in the biology of CRC and the combined measurement of leptin and adiponectin levels might provide useful prognostic information in the management of patients with CRC.*

Correspondence to: Fiorella Guadagni, MD, Ph.D., Department of Laboratory Medicine and Advanced Biotechnologies, IRCCS San Raffaele, Via della Pisana 235, 00163, Rome, Italy. Tel: +39 0666130425, Fax: +39 0666130407, e-mail: fiorella.guadagni@sanraffaele.it

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The prevalence of being overweight and obesity is dramatically increasing in western countries and can lead to obesity-related diseases (1). Among these, colorectal cancer (CRC) has been shown to have a certain relationship with obesity in some epidemiological studies of the past decades (2, 3).

It is generally accepted that disturbances in the production of adipocyte-derived hormones, leptin and adiponectin, may represent one of the causal links explaining the well-known relationship between obesity and increased prevalence of malignancies (4-6).

Leptin is a product of the *ob* gene involved in the control of food intake and energy expenditure (5, 7). Adiponectin is a 30-kDa protein hormone and cytokine secreted mainly by adipocytes and shares homologies with collagens, complement factors and tumor necrosis factor-α (TNF-α) (8).

Both these adipokines may significantly influence the growth and proliferation of tumor stroma and malignant cells within (4, 5). In particular, leptin may act as a potent mitogen (9-11) and antiapoptotic (12-15) cytokine in colon cancer and promotes the invasiveness of familial adenomatous colonic cells (16). Furthermore, it has been recently shown that leptin expression was dramatically increased from normal colonic mucosa to adenoma and adenocarcinoma, suggesting its involvement in multistep colorectal carcinogenesis (17). On the other hand, adiponectin exerts antiproliferative effects (acting on signal pathways involved in cell growth and proliferation) and is a direct angiogenesis inhibitor that preferentially induces apoptosis in activated endothelial cells in pathological neovascularization (4, 5). Many additional findings support the hypothesis that these adipokines are involved in the pathogenesis of gastrointestinal (GI) cancer (18,19), however, their role in carcinogenesis is still controversial (20).

Epidemiological studies have suggested that leptin is directly (21-23), whereas adiponectin is inversely (24, 25) correlated with the risk of CRC associated with obesity and

insulin resistance, the association being independent of body mass index (BMI), waist circumference and physical activity (23, 26). Accordingly, increased leptin (22) and reduced adiponectin (27, 28) serum levels were found in GI cancer patients, although low or undetectable leptin concentrations were observed in other studies (29-31). Furthermore, it has been recently shown that adiponectin might represent a prognostic parameter in risk prediction for CRC recurrence (28) and that the leptin/adiponectin (L/A) ratio is positively associated with CRC risk (32)

It is well known that tumor cells and tumor-associated leukocytes may produce inflammatory cytokines, such as TNF-α (33). The role of TNF-α has been linked to all steps involved in cancer initiation and progression, including cellular transformation, promotion, survival, proliferation, invasion, angiogenesis and metastasis (for review see (34)). Moreover, circulating levels of this cytokine have been associated with the disease status of CRC patients (35-39). Recently, it has been demonstrated that TNF-α administration induced a prompt and dose-dependent increase in serum leptin levels (40, 41) and a significant reduction of adiponectin expression and secretion (42).

Thus, the aim of this study was to evaluate the possible associations between leptin, adiponectin, TNF-α and clinicopathological variables of CRC patients at time of diagnosis of the primary tumor. Since leptin and adiponectin have been found to be inversely correlated, the index of increased leptin concentration was also corrected by reduced adiponectin values (L/A ratio) (32) and analyzed in a follow-up study designed to investigate the possible prognostic value of L/A ratio in predicting relapse-free and overall survival of patients with CRC.

Patients and methods

Patients and sample collection. Ninety consecutive patients with primary (n=76, 15 with resectable synchronous metastasis) or newly diagnosed metastatic (liver n=7, peritoneum n=6, multiple n=1) CRC entered into the study. All patients with primary cancer underwent surgical resection with curative intent at the University of Rome 'Tor Vergata'. Clinical features of CRC patients are summarized in Table I. As control group, in a 3:1 ratio, 30 control individuals (13 males, 17 females; mean age 59±12 years, ranging from 37 to 80 years) were also evaluated. Diabetes mellitus (fasting blood glucose level>115 mg/dl or treatment with a hypoglycemic agent), body mass index>28, history of alcohol or drug abuse, impaired liver (bilirubin level>1.5 mg/dl) or renal (creatinine level>1.5 mg/dl) function and a Karnofsky performance status lower than 90% were considered as exclusion criteria. All patients were followed from the time of diagnosis for at least 3 years or until the event date. No patient was lost to follow-up. The study was performed under the appropriate institutional ethics approvals and in accordance with the principles embodied in the Declaration of Helsinki. Written informed consent was obtained from each participant.

Table I. *Clinical features of colorectal cancer patients*.

Variable		Colorectal cancer N=90
Age (years)	Mean±SD	63±11
	Range	36-80
Males	N (%)	49 (54)
Site of primary tumor*	N (%)	
Colon		23 (30)
Sigma		17 (22)
Rectum		36 (48)
Grading*	N (%)	
1		11 (15)
2		45 (59)
3		20 (26)
Dukes' stage	N (%)	
A		7 (8)
B		34 (38)
C		20 (22)
D†		15 (17)
MET‡		14 (15)
	Total:	90
Length of follow-up* (Months)	Median (range)	36.5 (0.8-69.1)
Type of recurrence		
Local	N (%)	7 (9)
Distant		29 (38)

*Including 76 patients with primary colorectal cancer; †including 15 patients with resectable synchronous metastasis; ‡patients with newly diagnosed metastatic disease.

Blood samples from CRC patients were drawn within 1 week before surgery, or prior to neoadjuvant chemotherapy and/or irradiation. Samples from patients with newly diagnosed metastatic disease were obtained at the time of clinical diagnosis and prior to any treatment. After an overnight fast and a rest of at least 20 minutes, blood was drawn from each consenting participant by venipuncture of the antecubital vein using a 20G needle. Blood was allowed to clot and then centrifuged at 2000×g for 10 minutes at 4˚C. Serum samples were aliquoted, coded and stored at –80˚C until the assays were performed. Storage conditions were carefully maintained, and all aliquots were limited to one freeze-thaw cycle.

Immunoassay. Serum leptin and adiponectin levels were determined by commercially available enzyme immunoassays (dbc-Diagnostics Biochem Canada Inc., Ontario, Canada for leptin measurements and BioVendor Laboratory Medicine, Inc., Brno, Czech Republic for adiponectin measurements) according to the manufacturers' instructions. Intra- and interassay coefficients of variation were below 5% and 10%, respectively, for both assays. The minimum detectable levels were 0.17 ng/ml and 0.2 μg/ml, respectively.

Serum TNF-α levels (R&D Systems, Minneapolis, MN, USA) were measured by an enzyme-immunometric assay according to the manufacturers' instructions. Intra and interassay coefficients of variation were below 5% and 10%, respectively. The lower detection limit of the assay was 4.4 pg/ml.

Table II. *Serum leptin, adiponectin and TNF-α levels in patients with colorectal cancer and healthy controls.*

	No. cases	Leptin (ng/ml) Median (IQR)	Adiponectin (μg/ml) Median (IQR)	TNF-α (pg/ml) Median (IQR)
Healthy controls	30	1.1 (0.25-3.75)	13.1 (12.2-15.6)	0.2 (0.1-1.95)
Colorectal cancer				
Primary	73	8.4 (3.8-17.2)	8.3 (5.5-9.5)	9.3 (2.7-20.1)
Metastatic	17	14.0 (3.9-24.2)	7.0 (6.1-8.2)	14.1 (9.7-23.7)
Total	90	8.8 (3.8-17.6)*	8.1 (5.7-9.3)*	12.6 (2.9-21.9)†

*Mann-Whitney *U*-test controls *vs*. CRC: *$p<0.0001$; †$p<0.001$. IQR, Interquartile range.

Serum carcinoembryonic antigen (CEA) determination was performed using two-step chemiluminescent microparticle immunoassays on an ARCHITECT i2000 System (Abbott Labs, Chicago, IL, USA). The analytical sensitivity of the CEA assay was calculated to be better than 0.5 ng/ml at the 95% level of confidence. The cut-off limit chosen for sample evaluation was 5 ng/ml, as commonly recognized.

Measurements were carried out blinded. All samples were assayed in duplicate and those showing values above the standard curve were re-tested with appropriate dilutions.

Statistical analysis. Data analysis was performed by appropriate statistics. Data are presented as percentages, mean±SD, or median and interquartile ranges (IQR). Differences between percentages were assessed by chi-square test. Student's unpaired *t*-test and ANOVA test were used for normally distributed variables. Appropriate non-parametric tests (Mann Whitney *U*-test and Kruskal-Wallis ANOVA and median test) were employed for all the other variables. Univariate and multivariate linear regression analyses were performed to assess the possible associations between laboratory and clinicopathological variables. Survival curves were calculated by the Kaplan-Meier method and the significance level was assessed according to the Cox F test. For disease-free survival analyses, the time to the end point was calculated from the date of diagnosis of CRC until the date of the first CRC recurrence, with the event being any recurrence, locoregional or systemic. Global survival was calculated on an intention-to-treat basis from the day patients joined the study until the date of malignancy-related death or the latest day of follow-up. Only *p*-values lower than 0.05 were regarded as statistically significant. All calculations were made using computer software packages (Statistica 8.0, StatSoft Inc., Tulsa, OK, USA).

Results

As shown in Table II, serum leptin levels were higher in CRC patients than in controls ($p<0.0001$). As expected, adiponectin levels were significantly lower in CRC compared to controls ($p<0.0001$) and inversely correlated with leptin levels (Rho=–0.331, $p<0.01$). Thus, leptin concentrations were corrected by adiponectin values (L/A ratio), as also suggested by Stocks *et al*. (32). The median L/A ratio was eight-fold greater in CRC [1.090 (interquartile range, IQR: 0.427-2.611)] compared to controls [0.065 (0.020-0.260); $p<0.0001$]. In particular, patients with newly diagnosed metastatic disease had a higher, despite not being significant, median L/A ratio [1.942 (IQR: 0.491-4.448)] compared to patients with primary CRC [1.090 (IQR: 0.339-2.045)] (Figure 1).

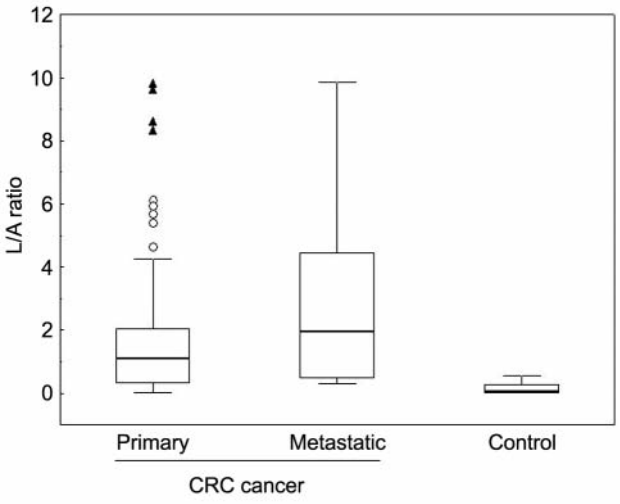

Figure 1. *Box-plot analysis of L/A ratios in primary and newly diagnosed metastatic CRC patients compared with L/A ratios of controls. Data are presented as median values (solid lines), 25th – 75th percentiles (columns) and non outlier range (whiskers). Open circles indicate outliers. Closed triangles indicate extreme values.*

Serum TNF-α levels of CRC patients were higher compared to controls ($p<0.001$) (Table II) and directly correlated with leptin concentrations (Rho=0.375, $p<0.01$). Therefore, to assess the possible determinants of serum leptin levels, a multiple regression analysis was performed in which adjustments were made on the following variables: age, gender, site of primary tumor, grading, Dukes' stage, CEA, adiponectin and TNF-α levels. The final model performed by forward stepping demonstrated that Dukes' stage of disease (regression coefficient=0.360, $p=0.04$) and serum TNF-α levels (regression coefficient=0.334, $p=0.05$) were the only independent predictors of increased leptin levels.

Table III. *Multiple regression analysis of variables associated with recurrence in primary colorectal cancer patients.*

Entire model				
Dependent variable	Explanatory variable	Standardized regression coefficient	Standard error	*p*-Value
Recurrence				
	Gender	0.026	0.177	0.883
	Age	0.137	0.161	0.405
	Site	0.208	0.187	0.281
	Grade	–0.280	0.168	0.114
	Dukes' stage	0.647	0.175	***0.002***
	TNF-α	–0.122	0.182	0.511
	L/A ratio*	0.294	0.170	0.101
	CEA	0.068	0.169	0.692
Forward stepwise method				
	Dukes' stage	0.655	0.151	***<0.001***
	L/A ratio*	0.365	0.141	***0.017***

*Categorized according to a cut-off value calculated on the median value observed in the overall cancer population. TNF-α, Tumor necrosis factor-α; L/A ratio, leptin/adiponectin ratio; CEA, carcinoembryonic antigen.

Clinical information on postoperative follow-up was available from all CRC patients. Over a median follow-up period of 36.5 months, 40 (53%) out of the 76 primary CRC patients remained free of disease, while 36 (47%) had relapsing disease (Table I). No differences were observed in age, sex, site of primary tumor or grading between patients with and without recurrence (data not shown). Patients were categorized as having a favorable (low, *i.e.* <1) or unfavorable (high, *i.e.* >1) profile on the basis of the median L/A ratio observed in all CRC patients. Multivariate analysis was, thus, performed in the 76 primary CRC patients including recurrence as the dependent variable and age, gender, site of primary tumor, grading, Dukes' stage, CEA, L/A ratio and TNF-α levels as the predictor variables. The final model by forward stepping showed that, beside stage of disease (beta=0.655, $p<0.001$), a high L/A ratio was the only independent predictor of recurrence (beta=0.365, $p=0.02$) (Table III). Analysis of disease-free survival time was performed for the 76 primary CRC patients. Figure 2 reports the Kaplan-Meier curves for disease-free survival in CRC patients. As shown in A, a low L/A ratio was associated with a reduced recurrence rate compared to patients with a high L/A ratio (Cox F=1.657; $p=0.06$). This association was even stronger when only stage B patients were included in the analysis. Indeed, a significantly higher disease-free survival rate was observed in stage B patients with low L/A ratio compared to those with high L/A ratio (Cox F=2.677; $p=0.05$) (Figure 2B).

Global survival was evaluated using intention-to-treat analysis from the time of patient inclusion in the study until the date of malignancy-related death or the latest day of follow-up. Survival analysis of the 76 CRC patients is reported in Figure 3A. As shown, a low L/A ratio was associated with an increased survival compared to patients with a high L/A ratio (Cox F test=2.260, $p=0.05$) (Figure 3A). Similar results were obtained when global survival was evaluated including patients with newly diagnosed metastatic disease in the analysis (Cox F test=2.991, $p=0.01$). Of interest, global survival of patients with metastatic CRC with low L/A ratios was greater than 90% compared to approximately 30% of patients with high L/A ratios (Cox F test=9.446, $p=0.002$) (Figure 3B).

Discussion

It is generally accepted that obesity and/or endocrine dysfunction of adipose tissue is related to the development and prognosis of colorectal cancer (35, 36). Numerous studies demonstrated that diets rich in fat that increase circulating leptin promote carcinogenesis by stimulating colon cell proliferation (43-45), while diets rich in dietary fibers that reduce leptin levels have an opposite effect (46). Fat itself, with its adipocytes and preadipocytes, can promote proliferation of colon cancer cells (39). Moreover, hormones produced predominantly by adipocytes of white adipose tissue [adipokines such as leptin (41) or adiponectin (28, 40)], are also associated with CRC risk. The results obtained in this study demonstrate the presence of an inverse correlation between increased leptin and lower adiponectin levels in CRC patients and, for the first time to our knowledge, suggest that the serum L/A ratio might represent a prognostic indicator in CRC patients. In fact, the negative prognostic value of a high L/A ratio with respect to disease-free and global survival suggests that adipokines might play an important role in the biology of CRC and that both leptin and adiponectin may represent an adjunctive tool in risk prediction for CRC recurrence. Moreover, a favorable adipokine profile (as indicated by a low L/A ratio) might identify a subset of patients with metastatic CRC characterized by longer survival and, possibly, better response to treatment. These results are in agreement with the findings recently reported by Stocks *et al.*, who demonstrated that high L/A ratios were associated with an increased risk of CRC, but suggests that only very high levels confer an increased risk (32).

The mechanisms responsible for regulation of adipokine levels have not been fully elucidated. Yet recent data suggest down-regulation of adiponectin (42) and up-regulation of leptin (40, 41) by insulin, as well as by TNF-α, a cytokine with functions of insulin resistance-inducing factor (47). In this respect, the finding that TNF-α was an independent predictor of increased leptin levels suggests that this cytokine could be involved in the regulation of leptin expression in CRC.

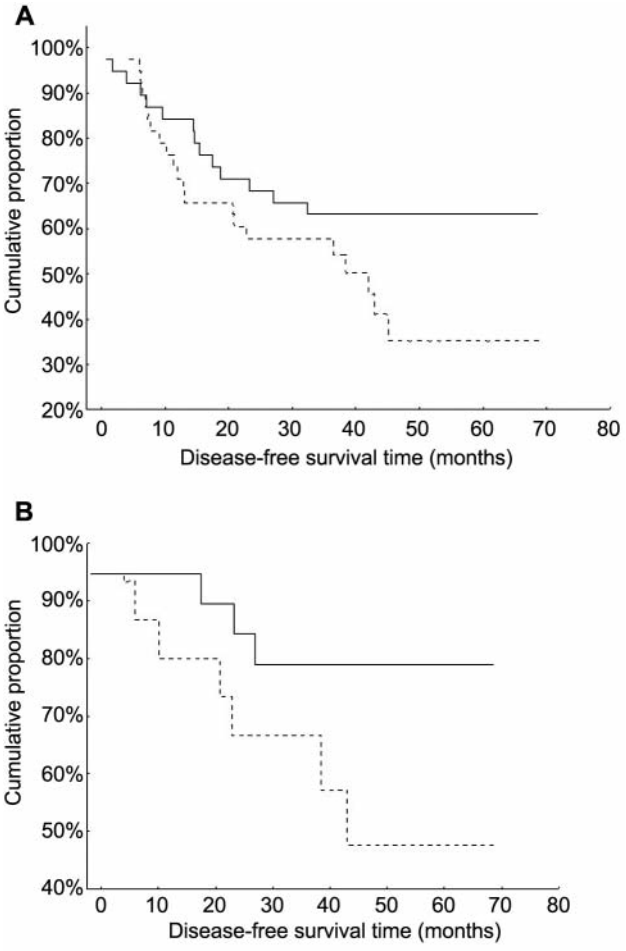

Figure 2. *Kaplan-Meier analysis of disease-free survival time of primary CRC patients (A, Cox F=1.657; p=0.06) and Dukes' stage B (B, Cox F=2.677; p=0.05) patients. Comparison between patients with low (solid line) and those with high (dotted line) L/A ratios.*

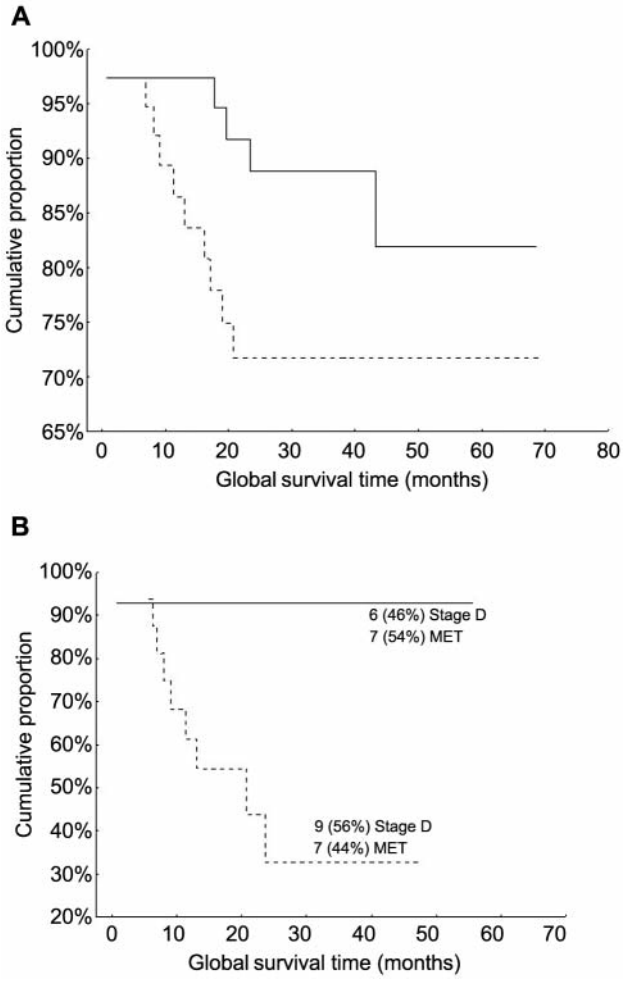

Figure 3. *Kaplan-Meier analysis of global survival of primary CRC patients (A, Cox F test=2.260, p=0.05) or patients with metastasis (B, Cox F test=9.446, p=0.002). Comparison between patients with low (solid line) and those with high (dotted line) L/A ratios.*

Although the source for increased TNF-α in cancer is still debated, it is well known that many tumors, including CRC, produce various inflammatory cytokines (21). Among them, TNF-α is frequently detected in biopsies from human cancer, produced either by epithelial tumor cells or stromal cells (33) and its production by tumors has been associated with a poor prognosis, loss of hormone responsiveness and cachexia/asthenia (48, 49). Clinically, several reports have associated the detection of abnormally high levels of circulating TNF-α in cancer patients with a wide range of tumor types (50) and circulating levels of this inflammatory cytokine have been associated with the disease status of cancer patients (36-39, 51-53). The finding here that TNF-α, together with tumor stage, was an independent predictor of increased leptin levels is in accordance with the cited studies. On the other hand, since both leptin and TNF-α are overexpressed in CRC patients compared to control subjects, another plausible explanation for the correlation observed might be that both biomarkers could be regulated in an autocrine fashion by factors elaborated by neoplastic cells in CRC patients.

As stated above, cytokine-mediated metabolic derangements have been considered among the candidates responsible for cachexia in cancer patients. Cancer cachexia is one of the most frequent effects of malignancy, is often associated with poor prognosis, and may account for up to 20% of cancer deaths (49). In particular, TNF-α plays a part in metabolic changes associated with chronic wasting (49). Previous studies showed a gender-dependent attenuation of expected physiological responses to weight loss among cancer cachexia patients, and suggested that impaired response of adiponectin and leptin may also play a role in the pathogenesis of cancer cachexia syndrome (54). The prompt and dose-

dependent increase in serum leptin levels observed following TNF-α administration (41), together with the significant association found between leptin and TNF-α in the present study, suggests that increased levels of circulating leptin might contribute to cancer cachexia.

One limitation of this study is that although all patients were in good performance status, detailed information on weight loss and anthropometric measures was not available to the investigators. Thus, we cannot presently formulate any hypothesis on the association between L/A ratio and cancer cachexia. Despite this limitation, the results reported here support the hypothesis that the L/A ratio might be regarded as a prognostic indicator in the management of patients with CRC, helping in the choice of more aggressive treatment and/or more strict follow-up procedures in subgroups of high-risk patients. We are aware that this hypothesis requires detailed experimental evaluation before its ultimate significance can be determined; nevertheless, we hope that our study will prompt investigators to design new studies to fully elucidate the mechanisms underlying adipokine effects and to better understand their significance in disease progression, as well as its contribution as a prognostic factor for CRC.

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