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ORIGINAL ARTICLE

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Serum levels of leptin and proinflammatory cytokines in patients with advanced-stage cancer at different sites

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Abstract Leptin is a recently identified hormone produced by the adipocyte *ob* gene which acts as a negative feedback signal critical to the normal control of food intake and body weight. A number of proinflammatory cytokines, such as interleukin (IL) 1α, IL-6, tumor necrosis factor (TNF) α and interferon (IFN) γ, have been proposed as mediators of cancer cachexia. These data suggest that abnormalities in leptin production/release or in its feedback mechanism play a role in cancer patients. To elucidate this we studied the relationship between total serum leptin and serum cytokines IL-1α, IL-6, TNFα as well as the production of leptin and cytokines by peripheral blood mononuclear cells (PBMC) isolated from cancer patients. Sixteen advanced cancer patients (mainly stage IV) with tumors at different sites were included in the study. The serum levels of leptin in cancer patients were significantly lower than those of healthy individuals at all times (7 a.m., noon, 3 p.m.). No significant differences were found in circadian rhythm between patients and controls. Serum levels of IL-1α, IL-6, and TNFα were significantly higher in cancer patients than in healthy individuals. An inverse correlation between serum levels of leptin and IL-6 was found in cancer patients. The production in culture of leptin by unstimulated PBMCs and those stimulated by phytohemagglutinin M or by phorbol myristate acetate isolated from cancer patients was very low; no differences were observed in comparison with leptin production by PBMCs from healthy individuals.

Keywords Leptin · Proinflammatory cytokines · Cancer anorexia/cachexia · Nutritional status · Immunological aspects

Abbreviations *BMI:* Body mass index · *CACS:* Cancer-related anorexia/cachexia syndrome · *CRH:* Corticotropin-releasing hormone · *IFN*: Interferon · *IL:* Interleukin · *mAb:* Monoclonal antibody · *PBMC:* Peripheral blood mononuclear cells · *PHA:* Phytohemagglutinin · *PMA:* Phorbol-12 mirystate-13acetate · *TNF:* Tumor necrosis factor

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Introduction

Leptin is a recently identified hormone produced by the adipocyte *ob* gene which acts as a negative feedback signal critical to the normal control of food intake and body weight. Leptin shares striking structural similarities with members of the long-chain helical cytokine family [1, 2].

Leptin released by adipocytes binds to a specific receptor found in the hypothalamus [3, 4, 5], the brain center responsible for satiety [5], i.e., for controlling food intake and energy metabolism. In humans most of the circulating leptin is bound to two serum molecules, while a minor proportion is present as free leptin [6]. The ratio of free to total leptin may be unbalanced due to variations in metabolic and/or nutritional status. The biological effects of leptin/receptor interaction lead to a decrease in food intake and an increase in energy expenditure [3, 4, 7, 8] mediated by a leptin-induced decrease in the hypothalamic biosynthesis and release of the neuropeptide Y [4, 9] responsible for appetite stimulation, increase in food intake, insulin release, enhanced lipoprotein lipase activity, and reduced energy expenditure [10, 11]. Circadian variations are characterized by nyctohemeral rhythm, with serum leptin levels being highest between midnight and early morning and lowest around noon to middle afternoon [12]. Superimposed on the circadian rhythm, total circulating leptin levels show a pattern of pulsatile release with a pulse duration of approximately 30 min [13, 14].

In patients with advanced-stage cancer one frequently observes a characteristic clinical picture of anorexia, tissue wasting, loss of body weight accompanied by a decrease in muscle mass and adipose tissue, and poor performance status; this is known as the cancer-related anorexia/cachexia syndrome (CACS) [15, 16, 17, 18]. The pathophysiology of CACS is multifactorial and not yet well understood. A possible role has been suggested for intermediary metabolites (e.g., lactate, ketones, oligonucleotides) that accumulate along an abnormal metabolic pathway in cancer patients and for other substances released by the tumor itself or by normal cells in response to the tumor [17]. A number of proinflammatory cytokines, such as interleukin (IL) 1, IL-6, tumor necrosis factor (TNF) α, and interferon (IFN) γ, have been proposed as mediators of the cachectic process. High serum levels of IL-1 [19, 20, 21, 22, 23], IL-6 [19, 20, 22, 23, 24], and TNFα [19, 20, 21, 22, 23, 25] have been found in cancer patients, even in absence of "cachexia" [22, 23], and the levels of these cytokines seem to be correlated with tumor progression [26, 27, 28, 29].

Weight loss occurs because of an imbalance between nutrient intake and requirements. There are two main metabolic situations associated with weight loss: first, starvation or hypocaloric feeding resulting in a lack of nutrient intake and, second, pathological weight loss resulting from cancer, AIDS, sepsis, trauma, or hypermetabolic states [30]. The body weight/height relationship is often used in evaluating nutritional status. Various indices of the weight/height relationship have been constructed. To date the most useful and the most widely used is the body mass index (BMI), or Quételet index [31], calculated dividing the weight (in kilograms) by the square of the height (in meters). A nomogram has been constructed for the calculation of BMI [32]. The various levels of obesity are related to respective risks of mortality.

Regarding the mechanism of leptin activity it can be hypothesized that abnormalities in leptin production and/or release or in its feedback mechanism play a role in cancer patients, particularly in CACS. Moreover, evidence has been provided that *ob* gene expression is upregulated by proinflammatory cytokines such as IL-1 and TNFα, producing prompt, dose-dependent increases in serum leptin concentrations [25, 33, 34, 35], and that high leptin levels are involved in CACS [35, 36].

To elucidate this we studied the total serum levels of leptin and serum cytokines IL-1α, IL-6, TNFα, and their correlations in a population of noncachectic but advanced-stage cancer patients and in age-sex-matched healthy individuals. Additionally, we studied the production of cytokines and leptin by peripheral blood mononuclear cells (PBMC) isolated from cancer patients. The PBMCs are the main source of circulating cytokines, while leptin is reported to be produced primarily by adipocytes or by tumor cells. Nonetheless, our purpose was to determine whether there is a relationship between the production and/or release of proinflammatory cytokines and leptin at the source cell level.

Patients and methods

Patients

The protocol was consistent with the Declaration of Helsinki (1989). Informed consent was obtained from all patients. The study included 16 patients advanced-stage cancer (10 stage IV, 6 stage III) and tumors at various sites. There were eight men and eight women, with a mean age of 58.3 years (range 41–71), mean weight of 55.8±11.5 kg (range 37–75), and mean height of 1.59±0.11 m (range 1.42–1.77). Their clinical characteristics are reported in Table 1. The patients were characterized by nonsignifi-

Table 1 Patient's clinical characteristics (*ECOG* Eastern Cooperative Oncology Group)

	n	%
Performance status (ECOG)		
0	4	25
1	5	31.25
2	6	37.5
4	1	6.25
Stage		
IIIA	3	18.75
IIIB	2	12.5
IIIC	1	6.25
IV	10	62.5
Cancer site		
Ovary	5	31.25
Oral cavity	3	18.75
Endometrium	2	12.5
Bladder	1	6.25
Cervix uteri	1	6.25
Esophagus	1	6.25
Larynx	1	6.25
Lung	1	6.25
Oropharynx	1	6.25

Table 2 BMI scale according to Thomas et al. [32]

	Women	Men
Underweight	<19	<20
Ideal weight (obesity 0)	19–24	20–25
Obesity I	25–29	26–30
Obesity II	30–39	31–40
Obesity III	>40	>41

cant weight loss compared to the preillness weight and a body weight not far from the customary one. Sixteen age-sex-matched normal-weight healthy individuals were used as controls. For this study we considered as reference the BMI scale of Thomas et al. [32] (Table 2).

Leptin serum levels

After an overnight fast of 12 h blood was collected by venipuncture at 7 a.m., at noon, and at 3 p.m. in evacuated blood collection tubes. The tubes were directly centrifuged for 10 min in a refrigerated (10°C) table-top centrifuge at 500 *g*. Serum samples were immediately stored at –30°C until analysis. Serum levels of leptin were measured by a double-antibody "sandwich" enzyme-linked immunosorbent assay using a monoclonal antibody specific for human leptin, an assay that measures total (i.e., free plus bound) leptin. The following procedure was used for measurement. Microtiter plates were coated overnight with monoclonal antibody for human leptin at 4°C in a humidity chamber. Plates were then rinsed three times with diluted wash solution, 300 µl/well (DRG Instruments, Germany). Of the diluted second antibody (polyclonal rabbit anti-human leptin-antibody; DRG Instruments) 100 µl was dispensed into each well and incubated for 1.5 h at room temperature. The wells were rinsed three times with diluted wash solution (300 µl/well). Of an enzyme complex (goat anti-rabbit IgG conjugated to horseradish peroxidase) 100 µl was dispensed into each well and incubated for 45 min at room temperature. The wells were rinsed three times with diluted wash solution, and 100 µl substrate solution (tetramethyl benzidine and hydrogen peroxide in citrate buffer) was added to each well and incubated for 20 min at room temperature. The reaction was stopped by adding 50 µl stop solution (H_2SO_4 0.5 M) to each well, and the optical density (absorbance) was measured at 450±10 nm in a plate reader. The lowest level of detection was 0.8 ng/ml and the upper limit 25 ng/ml, the intra- and interassay variations were 5% and 7%, respectively. The leptin levels of normal weight healthy individuals ranged from 2 to 43 ng/ml at 7 a.m., from 7 to 45 ng/ml at noon, and from 3 to 43 ng/ml at 3 p.m.

Serum levels of IL-1α, IL-6, and TNFα

Cytokines were detected by a "sandwich" enzyme-linked immunosorbent assay (for IL-1α, Cytimmune Sciences, College Park, Md., USA; for IL-6 and TNFα, Biosource Europe, Belgium) using monoclonal antibodies (mAbs) for two different epitopes of the cytokine molecule. Samples were incubated in polystyrene microtiter strip wells coated with the first mAb. The cytokine was captured by the first mAb and detected by the second mAb specific for each cytokine, linked to acetylcholinesterase and directed against a second epitope of the molecule. After removing the unbound enzyme-conjugated anti-cytokine mAb by washing a chromogenic substrate was added to each well. The extent of color developed was directly proportional to the amount of cytokine present in the sample for IL-6 and TNFα and inversely proportional for IL-1α. The absorbance of the sample at 450 nm for IL-6 and TNFα and at 492 nm for IL-1α was measured with a spectrophotometer (Unidata, Rome, Italy). A standard curve was prepared by plotting the absorbance value of the standards vs. corresponding concentrations. The concentration of the cytokine in the sample was determined by extrapolating from the standard curve. Ranges of assay results were 0.2–200 ng/ml for IL-1α, 2–1500 pg/ml for IL-6, and 10–1500 pg/ml for TNFα. Intra-assay variations were 5% for IL-1α, 3% for IL-6, and 6% for TNFα. Interassay variations were 7% for IL-1α and TNFα and 8% for IL-6. Results were expressed in namograms per milliliter for IL-1α and picograms per milliliter for IL-6 and TNFα.

PBMC separation

PBMCs were separated on a Ficoll-Hypaque density gradient and washed three times in Hank's solution (Gibco, Paisley, UK). Cells were then kept in RPMI 1640 medium (Gibco) plus 20% fetal calf serum (Boehringer-Mannheim-Roche, Mannheim, Germany), 10 mg/ml gentamicin (Gibco), and 20 nM L-glutamine (Gibco). This medium is referred to as "complete medium."

Production in culture of leptin and cytokines IL-1α, IL-6, and TNFα by unstimulated and stimulated PBMCs isolated from cancer patients

PBMCs were cultured in flat-bottomed 96-microwell microtiter plates (Falcon, Oxnard, Calif., USA) at a concentration of 1×10^6 cells/ml in complete medium in a volume of 100 µl/well. The white blood cell count of cancer patients showed an absolute number of lymphocytes from 1×10^9/l to 2.2×10^9/l (mean 1.7×10^9/l) and that of healthy individuals showed a mean absolute number of lymphocytes of 1.5×10^9/l. To each well as stimulant was added 100 µl phytohemagglutinin (PHA-M; Boehringer-Mannheim-Roche), 5 µg/ml, or phorbol-12 mirystate-13acetate (PMA; Sigma Chemical, St. Louis, Mo., USA), 25 µg/ml. To the control wells was added 100 µl RPMI 1640 plus 10 mg/ml gentamicin (Gibco) and 20 nM L-glutamine (Gibco). The cultures were set up in triplicate and kept at 37°C in a 5% CO_2 atmosphere for 24 h. Cultures were centrifuged at 500 *g* for 10 min to remove the cells and the supernatants frozen at –30°C until assayed. Leptin and cytokines were detected according to the techniques described above.

Statistical analysis

Results were expressed as mean ±SD. The significance of between-group differences were analyzed by Student's two-tailed *t* test.

Results

Leptin serum levels

Leptin levels in the healthy individuals whom we studied (mean 21.3 ng/ml, range 2–43) were consistent with those reported in the literature. Evaluation of serum leptin levels must consider that they have a very wide range of variation according to gender (women have higher values than men) [37] and age (elderly had higher values than younger persons [38], but leptin concentrations decline slightly in older women [39]) and in menopause [40], and in relation to nutritional status and body mass index (the obese have higher values than lean individuals) [41]. For the reference values of our healthy individuals we can use those reported by Wallace et al. [37] (18.7 ng/ml for women and 6.5 ng/ml for men) and by Salbach et al. [40] (18.8 ng/ml).

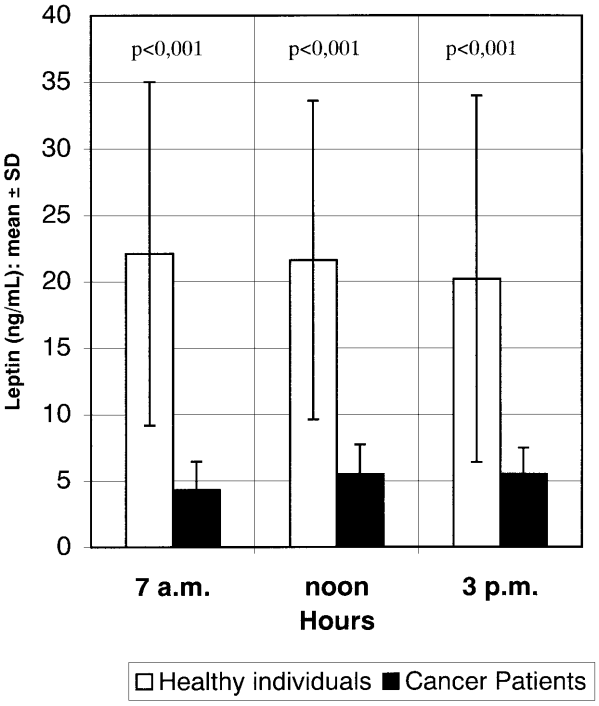

Fig. 1 Leptin serum levels. None of the differences was significant

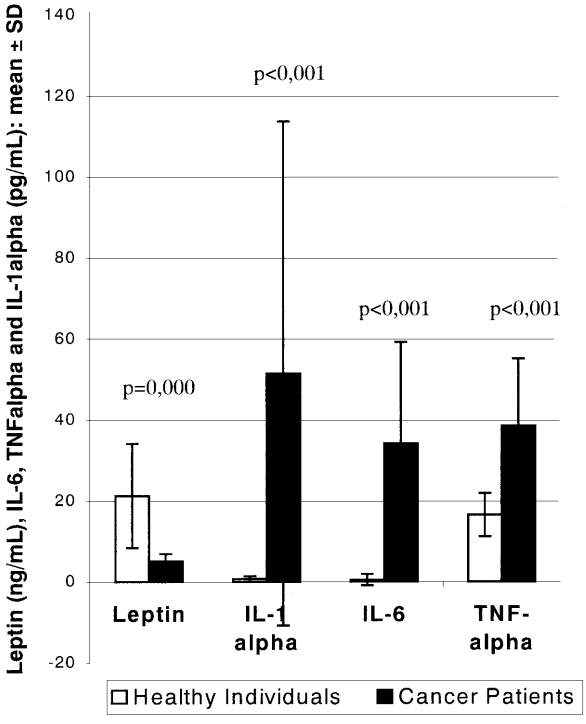

Fig. 2 Serum levels of leptin and proinflammatory cytokines in 16 cancer patients and 16 healthy individuals (see Table 4)

Table 3 Mean serum leptin levels (ng/ml) according to BMI group in 16 cancer patients and 16 healthy individuals

	Cancer patients		Healthy individuals	
	n	Leptin	*n*	Leptin
Underweight	1	4	2	1
Ideal weight	12	5	10	16
Obesity I	2	5	4	29
Obesity II	1	12	–	–
Total	16	4.9 (1–11)	16	16.7 (2–43)

Table 4 Serum levels of leptin and proinflammatory cytokines in 16 cancer patients and 16 healthy individuals (see Fig. 2)

	Leptin	IL-1α	IL-6	TNF-α
Healthy individuals	21.3±12.9	0.7±0.7	0.5±1.4	16.6±5.4
Cancer patients	5.1±1.8	51.5±62.3	34.2±25.1	38.6±16.6

Table 5 Correlation between serum levels of leptin and IL-6 in 16 cancer patients according to BMI (see Fig. 3)

	Leptin (ng/ml)	IL-6 (pg/ml)
Underweight[a]	4.3	86
Ideal weight	5.1±0.9	26.9±17.6
Obesity I, II	9.4±8.3	46.2±30.9

[a] *n*=1

The serum levels of leptin in cancer patients at all times (7 a.m., noon, 3 p.m.) were significantly lower (mean 5.1 ng/ml, range 1–11) than those of healthy individuals. No significant differences were found between any of the different times (7 a.m., noon, 3 p.m.) in either patients or controls; thus no a circadian rhythm of leptin release was found (Fig. 1). A direct correlation between BMI and leptin levels was found both in cancer patients and in healthy individuals, i.e., lowest BMI corresponded to lowest leptin levels (Table 3).

Serum levels of proinflammatory cytokines

The serum levels of IL-1α, IL-6, and TNFα were significantly higher in cancer patients than in healthy individuals. Figure 2 and Table 4 report serum levels of leptin and proinflammatory cytokines.

Correlation between serum levels of leptin and IL-6 in 16 cancer patients according to BMI

There was a direct correlation between BMI and serum leptin but an inverse correlation between BMI and IL-6; consequently the serum levels of leptin and IL-6 were inversely related (Fig. 3, Table 5). The lowest levels of leptin associated with highest levels of IL-6 are related to overt anorexia/cachexia symptoms, such that they can be considered clinically predictive of a bad prognosis by short time.

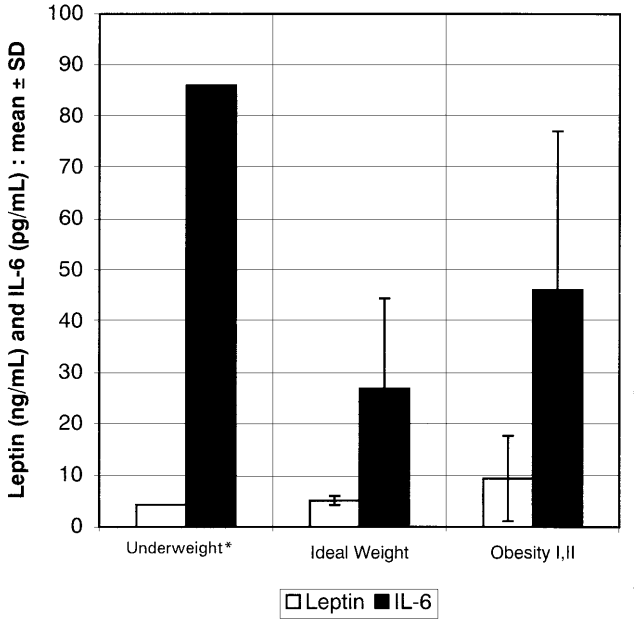

Fig. 3 Correlation between serum levels of leptin and IL-6 in 16 cancer patients according to BMI (see Table 5). *n=1

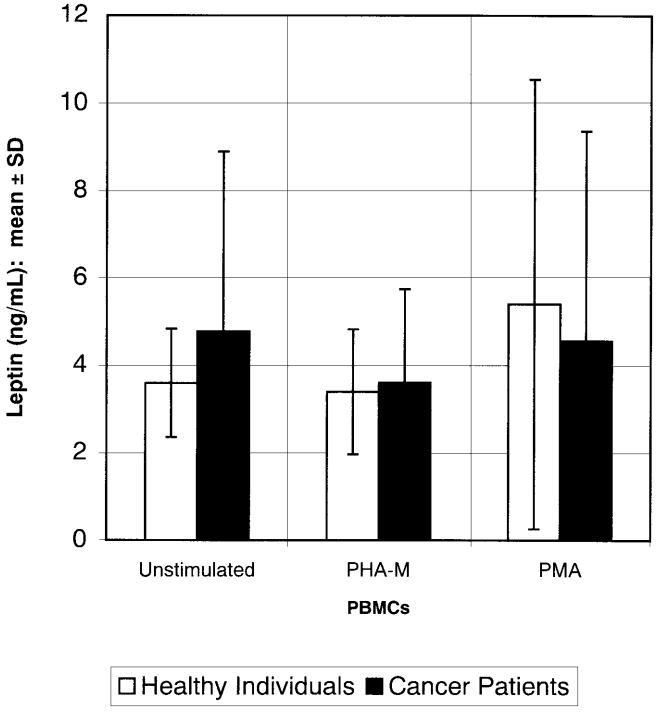

Fig. 4 Production in culture of leptin by unstimulated and PHA-M or PMA-stimulated PBMCs isolated from cancer patients and healthy individuals

Production in culture of leptin and cytokines IL-1α, IL-6, and TNFα by unstimulated and stimulated PBMC isolated from cancer patients

As would be expected, the production in culture of leptin by unstimulated and by PHA-M or PMA stimulated PBMC isolated from cancer patients was very low; no differences were observed in comparison with leptin production by PBMC from healthy individuals (Fig. 4). The production in culture of IL-1α by PBMC from cancer patients generally did not differ significantly from that of healthy individuals. The production in culture of IL-6 by unstimulated PBMC from cancer patients was higher than that of healthy individuals. The production in culture of TNFα by PHA-M stimulated PBMC from cancer patients was significantly higher than that of healthy individuals.

Despite the structural similarities between leptin and several cytokines and their respective receptors [42] we observed no relationship between the production and/or release of proinflammatory cytokines and leptin at the source cell level. This is probably because leptin and proinflammatory cytokines have a different cell source, i.e., adipocytes and monocyte/macrophages, respectively.

Discussion

The aim of the present study was to clarify whether abnormalities in circulating leptin concentrations and therefore in the leptin-dependent feedback pathway have a role in advanced stage cancer patients inducing the pathophysiological changes leading ultimately to CACS. Additionally, our purpose was to confirm findings previously reported by us and others on the increased serum concentrations of inflammatory cytokines in cancer patients which have been demonstrated to be implicated in CACS. Moreover, we looked for a possible regulatory activity of leptin on cytokine release or vice versa, i.e., the possible correlations between these two classes of agents both involved in CACS. As suggested above, our study was not able to demonstrate this regulatory activity, probably due to the different cell sources of leptin and proinflammatory cytokines.

We studied a population of noncachectic or precachectic cancer patients, although in advanced stage, because in our opinion the main primary causes leading to CACS, i.e., essentially inflammatory cytokines, are operative in cancer patients long before the onset of overt clinical CACS. Our opinion was supported by the evidence that the great majority (over 80%) of patients included in this study who were not cachectic had subsequently progressive uncontrollable disease and died in overt CACS within 6 months after the study. Moreover, when overt CACS occurs, the immunological, physical, and QL status of patients is so defective that an exhaustive evaluation of relevant parameters, such as leptin and cytokines, is often too difficult and even not reliable.

Simons et al. [43] found that elevated leptin concentrations are not involved in the development of lung cancer cachexia, and that the afferent part of the leptin feedback mechanism therefore does function normally. Similarly, Wallace et al. [37] showed that leptin concentrations in gastrointestinal cancer patients with weight loss are lower than those in healthy subjects, and that the lep-

tin concentrations are related to the predicted percentage of body fat.

Our results are consistent with those of Simons et al. and Wallace et al. Fasting, prefeeding, and postfeeding total serum leptin levels were homogeneously very low in the cancer patients studied. Additional information is provided by our study, which found no circadian rhythm of leptin release. Because the very low serum leptin levels were not reflected in increased appetite and food intake or in decreased energy expenditure, it may either be that the leptin feedback mechanism in cancer cachexia is altered at the hypothalamic level [43], or the release of proinflammatory cytokines is the cause of CACS, or both.

Although obtained in a cancer patient population not overtly cachectic, our results show that a simple dysregulation of leptin production and/or release cannot be involved in cancer-associated pathophysiological changes leading to cachexia, and therefore that the afferent pathway of the leptin feedback mechanism has a normal function [43].

We also investigated the production of leptin by PBMC of cancer patients, although there is no evidence reported in the literature that PBMC could be a significant source of leptin (see above). As expected, leptin production by PBMC was minimal both in cancer patients and controls.

Our results further confirm that serum levels of proinflammatory cytokines, such as IL-1α, IL-6, and TNFα, known to play a key role in the onset of both cachexia-related inflammation and CACS [44, 45], are abnormally high in advanced-stage cancer patients, even in the absence of overt CACS [22, 23]. Regarding their production in culture by PBMC isolated from cancer patients, it was not constantly higher than in controls [46]. However, it must be considered that most of the inflammatory cytokines are produced and released by monocytes/macrophages, which are only a minor component of cultured PBMC. Therefore, for the reasons cited above, a regulatory activity or a strict relationship between cytokines and leptin at the cell (PBMC) level could not be demonstrated in this study.

Our study provides evidence of some important clinical-biological correlations: (a) in cancer patients there is a direct correlation between BMI and serum leptin levels but an inverse correlation between BMI and proinflammatory cytokines. (b) Very low levels of leptin and high levels of proinflammatory cytokines, particularly IL-6, are correlated with short patient survival.

The possibility of an interrelationship between cytokines and leptin in the pathophysiology of CACS needs to be investigated. Several reports are available in the literature. A positive and independent association between the TNFα system and circulating leptin concentrations has been reported by some authors [47]. On the other hand, TNFα has been suggested to inhibit leptin secretion in a dose- and time-dependent manner [48]. In the mouse the administration of IL-1α and TNFα, which are known to induce anorexia and loss of body weight, produced a prompt and dose-dependent increase in serum leptin levels, suggesting that the leptin levels is one mechanism by which anorexia is induced during inflammation [35].

Since no relationship between high leptin concentrations and weight loss in patients with malignancies [37, 43], AIDS [25], and advanced age [49] has been found, an alternative explanation of CACS involves the corticotropin-releasing hormone (CRH) pathways. Several cytokines, such as IL-1α, IL-6, and TNFα, by increasing hypothalamic CRH gene expression, may activate the same CRH pathway as the leptin-catabolic effector system [42]. Considering that the leptin receptor is a member of the class I cytokine receptor family, and that leptin activates intracellular signal transduction pathways common to many cytokines, it is possible that some cytokines act as leptinlike factors and mimic the effect of leptin, decreasing energy intake and increasing energy expenditure [42].

In a series of previous reports we have demonstrated a significant functional defect in the cell-mediated immunity of cancer patients by a defective proliferative response to mitogens, anti-CD3, and rIL-2, a decreased cytolitic function, and a variety of alterations in ζ chain and $p56^{lck}$ signal transducing molecules [50, 51, 52]. The low leptin levels observed in this study may be partially responsible for the impaired cell-mediated immunity found in advanced-stage cancer patients, particularly those with CACS. Indeed, leptin has been shown to have a specific effect on T-lymphocyte responses, differentially regulating the proliferation of na and memory T-cells, increasing Th-1 and suppressing Th-2 cytokine production. These findings suggest a new relationship between nutritional status and cognate cellular immune function [53].

The discovery of leptin has led to the hypothesis of a feedback mechanism based on the following: An anorexia/cachexia-induced decrease in body fat mass, accompanied by low circulating insulin levels and high intracellular free fatty acids, may lead to a decreased adipocyte production and release of leptin [43, 54, 55, 56], resulting in increased hypothalamic production and release of neuropeptide Y, the production of which is normally suppressed by leptin after binding to a specific hypothalamic receptor [3, 4, 5, 9, 43]. Neuropeptide Y subsequently induces an increase in appetite and food intake, an increased release of insulin, and a decrease in energy expenditure [10, 11, 43], leading to restoration of body fat mass. Eventually leptin levels rise as a result of increasing body fat mass. In the presence of a normal leptin feedback mechanism cancer patients would be expected to have increased appetite and decreased energy expenditure in order to restore body fat mass. Instead, our findings as well as those of Simons et al. [43] and Wallace et al. [37] show that this is not the case. At least two alternative explanations may therefore be put forward: either the hypothalamus is insensitive to the low levels of leptin, or abnormalities in the hypothalamic neuropeptide circuitry occur, as has been demonstrated in tumor-bear-

ing animals developing anorexia-cachexia [57]. The above possibilities are based on the hypothesis that the physiological homeostatic mechanisms protecting body weight against losses have been overcome in CACS [11]. However, the exact physiopathological role played by either tumor- or host-derived cytokines [22, 23, 46] in overruling the normal weight homeostasis in CACS is not yet completely understood.

In conclusion, taking together the two relevant biological molecules, such as leptin and proinflammatory cytokines, and clinical features, we suggest that these are useful clinical markers of patient nutritional and performance status. Moreover, leptin and proinflammatory cytokines are predictive for patient survival.

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