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IL-2, TNF-α, and Leptin: Local Versus Systemic Concentrations in NSCLC Patients

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One recent line of cancer research shows increasing interest for biological factor such as IL-2, TNF-α, and leptin, which have been found to participate in the development and progression of non-small cell lung cancer (NSCLC). The aim of this study was to measure IL-2, TNF-α, and leptin concentrations in the airways and in the systemic circle of patients with NSCLC, investigating the role of these factors in the lung tumors. We enrolled 32 patients (17 men, 71 ± 7 years) with a histological diagnosis of NSCLC and 20 healthy ex-smoker controls, negative for computed tomography of the chest (14 men, 69 ± 8 years). IL-2, TNF-α, and leptin levels were measured in the serum, the urine, the bronchoalveolar lavage, the induced sputum, and exhaled breath condensate (EBC) of patients enrolled by means of a specific enzyme immunoassay kit. Higher concentrations of IL-2, TNF-α and leptin were found in NSCLC patients than in controls ($p < 0.0001$). A statistically significant increase of IL-2, TNF-α, and leptin concentrations was observed in patients from stage I to stage III of NSCLC. These findings suggest that IL-2, TNF-α, and the leptin play an important role in the cancerogenesis of NSCLC. Their measure in the EBC could be proposed as noninvasive markers for an early detection of NSCLC and in the follow-up of this tumor.

Key words: IL-2; TNF-α; Leptin; Non-small cell lung cancer (NSCLC); Exhaled breath condensate; Systemic circle

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the most common cause of death in Western Europe and in North America (1). Lung cancer often is not diagnosed until it is symptomatic and usually when it is in advanced and incurable stages (2). However, Henschke et al. recently demonstrated that annual spiral computed tomography can detect lung cancer that is curable (3). Although surgical resection can provides lung cancer patients with the hope of a cure, the long-term survival rate, even in surgical treated cases, remains unsatisfactory (4). Nowadays, researchers' efforts are based on the fact that early diagnosis could identify early lesions in their natural history, and that early tumors may be biologically less aggressive than late-stage tumors (5).

A number of biological factors are not only involved in the development and course of malignant diseases (6), but may also serve as prognostic markers for survival and as predictive markers for the efficacy of a specific therapeutic strategy (7).

Many authors demonstrated that different cytokines, such as interleukin-10 (IL-10), IL-2, IL-6, and tumor necrosis factor-α (TNF-α), may play a key role in NSCLC. These markers have been largely studied either in invasive or noninvasive samples and their possible usefulness in the screening of subjects at risk and in the follow-up of patients with NSCLC has been investigated (7).

IL-2 is typically associated with a T Helper (Th) 1 response that is established to be an effective antitumor response (8) and it is found to exhibit antitumor activity in NSCLC (9). The circulating levels of IL-2R could be confirmed as an indirect marker of tumor activity (10).

TNF-α is a pleiotropic cytokine produced by activated macrophages (11) and mediates numerous physio-

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logical and immune reactions, which are manifested in various biological effects (12).

Leptin is an adipocyte hormone that functions as the afferent signal in a negative feedback loop regulating body weight (13), and weight loss is frequently observed in advanced lung cancer (14).

Until now IL-2, TNF-α, and leptin levels have been largely studied in peripheral blood. Nevertheless, carcinogenic alterations became evident early in the primary airways (primary site of the disease) and appear later in the systemic samples, such as peripheral blood and urine. To our knowledge, only a few studies investigated these oncogenetic factors in airways and none compared their local to systemic levels.

The aim of this study was to measure IL-2, TNF-α, and leptin concentrations in the airways and in the systemic circle of patients with NSCLC, investigating the role of these factors in the lung tumors.

MATERIALS AND METHODS

Study Population: Characteristics of Patients

The population of the study consisted of 32 patients (17 men, 71 ± 7 years), consecutively enrolled, who had a histological diagnosis of NSCLC at the Department of Thoracic Surgery, San Paolo Hospital, Bari and at the Department of Respiratory Disease of Foggia University. Twenty healthy ex-smoker controls, negative for computed tomography of the chest, were also enrolled (14 men, 69 ± 8 years, tobacco consumption estimated at 44 pack-years). Written informed consent was obtained from all subjects upon approval of the study by the Ethic Committees of the two institutions. All the patients were enrolled in the study immediately after cytohistological diagnosis when none of them had received any forms of anticancer therapy whatsoever, including primary surgery.

The patients underwent standard diagnostic and staging procedures consisting of a physical examination, serum chemistry analysis, brain, chest, and abdomen CT scans, radionuclide bone scan, and bronchoscopy. The diagnosis of NSCLC was made either by bronchoscopic biopsy or by transthoracic needle aspiration. Assessments of T and N status were based on the International Union Against Cancer TNM staging system (15). Overall, NSCLC patients were classified as stage I in 10 cases, stage II in 10 cases, stage III in 12 cases.

At the time of enrolment into the study, all patients with NSCLC were ex-smokers (tobacco consumption stopped from 7.6 ± 4.2 years) with a previous average tobacco consumption estimated at 46 pack-years (range 22–105). Ten healthy subjects enrolled were also ex-smokers (tobacco consumption stopped from 15.6 ± 6.8 years) with a previous average tobacco consumption estimated at 43 pack-years (range 19–100) while the others never smoked.

Patients and healthy subjects with concomitant diseases (infectious, autoimmune disease, etc.) were excluded from the study. All patients and the healthy subjects went to the clinic twice after enrollment. During the first day they were required to have blood, urine, and BAL collection. The second day they underwent breath condensate collection, followed by induced sputum.

Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) was performed following a standardized protocol according to current National Heart, Lung and Blood Institute guidelines (16). Subjects were premedicated intramuscularly with diazepam and atropine 30 min before the procedure. Lidocaine (5 ml or less) was used for topical anesthesia of the upper airways. A flexible fiberoptic bronchoscope was wedged into a segment or subsegment of the right middle lobe, and three fractions, 50 ml each, of saline preheated at 37°C were introduced. Each aliquot of fluid was recovery by gentle suction with a syringe and collected into 50-ml tubes and stored at −70°C.

Induced Sputum

Sputum was collected and processed according to the method of Spanevello et al. (17). All sputum cell counts and measurements were performed blind to the clinical details. Definition of an adequate selected sputum was one in which there were fewer than 20% squamous cells and viability >50%. A specific enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) was used to measure TNF-α and IL-6 concentrations in supernatant of induced sputum.

Exhaled Breath Condensate

Exhaled breath condensate was collected by using the EcoScreen (Jaeger, Wurzburg, Germany). The subjects breathed through a mouthpiece and a two-way nonrebreathing valve, which also served as a saliva trap. They were asked to breathe at a normal frequency and tidal volume, wearing a nose clip, for a period of 10 min. If subjects felt saliva in their mouth they were instructed to swallow it. Condensate, at least 1 ml, was collected as ice at −20°C, transferred to Eppendorf tubes, and immediately stored at −70°C. Samples were analyzed within 3 months from collection. To exclude saliva contamination amylase activity was analyzed in EBC.

IL-2, TNF-α, and Leptin Measurements

A specific enzyme immunoassay kit (Cayman Chemical) was used to measure IL-2, TNF-α, and leptin con-

centrations in exhaled breath condensate, urine, blood, supernatant of induced sputum, and BAL. The intra-assay and interassay variability were ≤10% for each assay and the detection limit of the assays was 1.5 pg/ml, 1 pg/ml, and 1 ng/ml, respectively. The reproducibility of repeated IL-2, TNF-α, and leptin measurements was assessed by the Bland and Altman method and the coefficient of variation (18). Reproducibility of exhaled IL-2, TNF-α, and leptin measurements was assessed in 10 nonsmoking normal subjects. The coefficient of variation for IL-2, TNF-α, and leptin was 10%, 5%, and 9%, respectively.

Statistical Analysis

Data are expressed as means ± SD. A Mann-Whitney test was used to compare groups and correlations between variables were performed using Spearman's rank correlation test. Significance was defined as a value of $p < 0.05$. The reproducibility of repeated exhaled TNF-α, IL-6, and pH measurements was assessed in a separate experiment by the Bland and Altman method and the coefficient of variation determined (18).

RESULTS

IL-2

The IL-2 levels were correlated to the progression of the tumor. The IL-2 levels were higher in the exhaled breath condensate, urine, blood, induced sputum, and BAL of NSCLC patients (28.3 ± 3.7, 23.9 ± 3.0, 26.9 ± 2.5, 27.1 ± 3.6, 25.2 ± 3.7 pg/ml) (Fig. 1a) than in controls (19.2 ± 1.7, 17.5 ± 1.4, 17.3 ± 1.5, 16.2 ± 1.3, 17.3 ± 1.4 pg/ml, $p < 0.0001$). A positive correlation was observed between the IL-2 concentrations in the BAL, in the induced sputum, and in the breath condensate ($r = 0.6$, $p < 0.01$).

Progressively higher concentrations of IL-2 were found from stage I to stage III in all biological samples. However, significant differences between stage I and stage III were observed only in exhaled breath condensate, induced sputum, and BAL (Table 1). No difference was found between the histological types of NSCLC: patients with adenocarcinoma showed concentrations of exhaled IL-2 in exhaled breath condensate, urine, blood, induced sputum, and BAL (28.9 ± 5.0, 24.1 ± 5.1, 27.1 ± 3.7, 23.4 ± 3.6, 23.9 ± 5.4 pg/ml) (Fig. 1a) similar to those of patients with squamous cell cancer (30.1 ± 5.7, 21.2 ± 3.1, 26.6 ± 3.6, 30.8 ± 6.1, 26.4 ± 5.1 pg/ml) (Fig. 2a).

TNF-α

The TNF-α levels were correlated to the progression of the tumor. The TNF-α levels were higher in the exhaled breath condensate, urine, blood, induced sputum, and BAL of NSCLC patients (20.1 ± 1.5, 15.6 ± 1.4, 24.1 ± 8.9, 20.7 ± 1.5, 21.3 ± 1.5 pg/ml) (Fig. 1b) than in controls (12.1 ± 1.1, 11.7 ± 1.2, 11.8 ± 1.0, 12.1 ± 0.8, 11.4 ± 0.9 pg/ml, $p < 0.0001$). A positive correlation was observed between the TNF-α concentrations in the BAL, in the induced sputum, and in the breath condensate ($r = 0.5$, $p < 0.05$).

Progressively higher concentrations of TNF-α were found from stage I to stage III in all biological samples. However, significant differences between stage I and stage III were observed only in exhaled breath condensate, induced sputum, and BAL (Table 1). No difference was found between the histological types of NSCLC: patients with adenocarcinoma showed concentrations of exhaled TNF-α in exhaled breath condensate, urine, blood, induced sputum, and BAL (23.3 ± 2.5, 17.9 ± 2.2, 26.9 ± 2.6, 23.4 ± 2.4, 23.9 ± 2.6 pg/ml) (Fig. 1b) similar to those of patients with squamous cell cancer (16.9 ± 1.3, 13.3 ± 1.4, 21.3 ± 1.5, 17.9 ± 1.4, 18.7 ± 1.6 pg/ml) (Fig. 2b).

Leptin

The leptin levels were correlated to the progression of the tumor. The leptin levels were higher in the exhaled breath condensate, urine, blood, induced sputum, and BAL of NSCLC patients (5.1 ± 1.8, 5.6 ± 2.9, 45.3 ± 19.9, 5.7 ± 1.8, 5.7 ± 2.5 ng/ml) (Fig. 1c) than in controls (3.1 ± 0.4, 2.7 ± 0.6, 22.7 ± 0.4, 2.9 ± 0.6, 3.0 ± 1.1 ng/ml, $p < 0.0001$). A positive correlation was observed between the leptin concentrations in the BAL, in the induced sputum, and in the breath condensate ($r = 0.6$, $p < 0.01$).

Progressively higher concentrations of leptin were found from stage I to stage III in all biological samples. However, significant differences between stage I and stage III were observed in all samples except BAL (Table 1). No difference was found between the histological types of NSCLC: patients with adenocarcinoma showed concentrations of leptin in exhaled breath condensate, urine, blood, induced sputum, and BAL (4.7 ± 1.6, 6.1 ± 3.2, 44.2 ± 27.2, 5.2 ± 1.5, 5.1 ± 2.5 and ng/ml) (Fig. 1c) similar to those of patients with squamous cell cancer (5.5 ± 1.9, 5.1 ± 1.6, 46.3 ± 8.9, 6.2 ± 1.9, 6.3 ± 2.5 ng/ml) (Fig. 2c).

DISCUSSION

In this study we found that IL-2, TNF-α, and leptin levels are detectable in systemic samples (blood and urine) as well as in those from airways (exhaled breath condensate, supernatant of induced sputum, and BAL). These markers were elevated in all biological samples of NSCLC patients compared to healthy controls. Con-

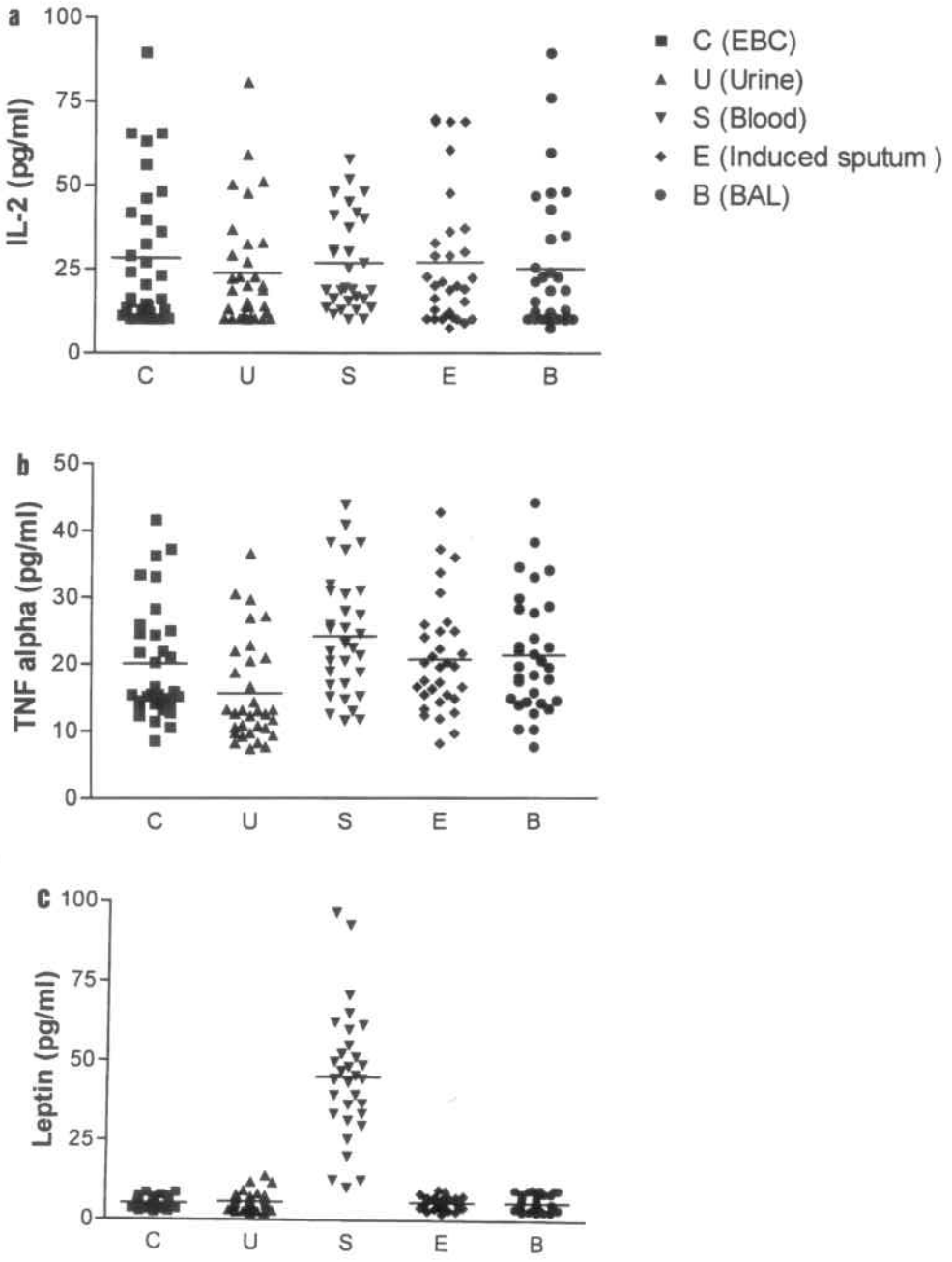

Figure 1. (a) IL-2 concentrations, (b) TNF-α concentrations, and (c) leptin concentrations in exhaled breath condensate, urine, blood, induced sputum, and BAL of NSCLC patients.

centrations of IL-2, TNF-α, and leptin in BAL, induced sputum, and breath condensate correlated with each other. Furthermore, the IL-2 or TNF-α and leptin concentrations were correlated with tumor progression. No differences were observed between adenocarcinoma and squamous carcinoma.

This is the first study in which IL-2, TNF-α, and leptin levels have been investigated in five biological samples involved in both systemic and local response of patients with NSCLC. IL-2 has recently generated increasing interest in biological factors involved in oncogenesis (9).

We analyzed the concentrations of IL-2 in the systemic circulars (plasma, urine) and in the airways (breath condensate, induced sputum, and BAL) of NSCLC patients. Although studies on IL-2 measurement in serum and BAL are to date available, to our knowledge this is the first study in which IL-2 has been stud-

Table 1. Concentrations of IL-2, TNF-α, and Leptin in Stages I–III

	Stage I	Stage II	Stage III
IL-2 (pg/ml)			
EBC	14.9 ± 2.1	36.3 ± 7.4	33.5 ± 5.3*
Urine	18.9 ± 3.9	21.4 ± 6.7	25.6 ± 4.7
Blood	26.7 ± 4.7	26.3 ± 4.5	28.3 ± 4.5
Induced sputum	15.2 ± 2.2	27.3 ± 3.3	38.9 ± 7.6*
BAL	14.4 ± 3.8	19.9 ± 4.0	41.5 ± 6.9†
TNF-α (pg/ml)			
EBC	13.3 ± 0.8	15.9 ± 0.9	29.3 ± 1.9†
Urine	13.5 ± 1.9	12.5 ± 1.4	19.9 ± 2.7
Blood	22.0 ± 2.6	22.7 ± 2.7	27.1 ± 2.8
Induced sputum	13.8 ± 1.1	18.7 ± 1.2	30.5 ± 1.9
BAL	12.9 ± 0.9	18.7 ± 0.8	30.5 ± 1.9
Leptin (ng/ml)			
EBC	3.3 ± 0.2	5.4 ± 0.5	6.4 ± 0.4*
Urine	4.4 ± 0.7	4.5 ± 0.4	7.4 ± 1.1*
Blood	34.0 ± 6.3	42.8 ± 4.3	57.7 ± 6.1†
Induced sputum	3.9 ± 1.2	6.2 ± 0.5	6.9 ± 0.4*
BAL	5.7 ± 1.1	5.9 ± 0.7	5.6 ± 0.7

*Stage I versus stage III: $p < 0.05$.
†Stage I versus stage III: $p < 0.01$.

ied in the urine, in the induced sputum, and in the breath condensate. We found increased concentrations of this tumoral cytokine in all NSCLC patients' five biological samples analyzed compared to those of controls. Levels of IL-2 appear correlated with progression of the disease because they were higher from stage I to stage III. Our results agree with those of Orditura (8) and Chyczewska (19) in serum and the BAL.

For the first time we compared IL-2 concentrations in different histotypes of lung tumor and we did not find any difference, which led us to suppose that the production of this cytokine is not influenced by NSCLC histological nature.

TNF-α is a pleiotropic cytokine involved in cancerogenesis (12). This study suggests that a positive correlation exists between high TNF-α expression and favorable prognosis in NSCLC in terms of overall survival and disease-free interval (12). TNF-α showed a similar behavior to that of IL-2. In fact, an increase of TNF-α levels in all biological samples analyzed was observed. In agreement with Aleman et al. (13) and Chyczewska et al. (20), an increase of TNF-α in serum of NSCLC patients as well as in the BAL was found. We also found the highest concentrations of TNF-α in stage III, suggesting a strong correlation of this cytokine with the progression of the tumor. This evidence is in line with the defensive role of TNF-α in the killing of tumor cells (21).

Similar as for IL-2, for TNF-α we did not find any correlation between the cytokine level and the NSCLC histotypes in any sample.

The third biological factor we have considered is leptin. Leptin is produced by fat under cytokine action (13,14). Many researchers studied the relationship between leptin and proinflammatory cytokines in advanced-stage NSCLC (IIIB and IV). Tas et al. (22) and Aleman et al. (13) found decreased serum leptin levels in weight-losing patients with cancer. Their results led them to suppose that serum leptin concentrations in advanced lung cancer patients mainly depend on the total amount of fat (14). Nevertheless, these results are in contrast with our data, which evidenced increased leptin levels in all the samples and mainly in the blood. Leptin is produced by fatty tissue under inflammatory cytokine actions (14), and it appears earlier in the systemic circle than in the airways. This could explain the higher values of leptin in the blood that we observed in subjects at stage I (results not shown).

In our work, we measured IL-2, TNF-α, and leptin levels in three respiratory samples (BAL, induced sputum, and exhaled breath condensate) in the same patients. BAL and induced sputum are more or less invasive diagnostic tools, very important for providing cytological information of bronchial tree. However, they are not well tolerated by patients and therefore not suitable for the screening of healthy smokers at risk and for the follow-up of patients with lung tumor. The exhaled breath condensate is a fast collection method, completely noninvasive and well accepted even by patients affected by severe respiratory disease such as metastatic cancer (5). The use of the exhaled breath condensate, still limited to the research field, has recently been proposed for the diagnosis of patients with NSCLC and in the screening of smokers at risk (4).

In this study we aimed at verifying the validity of the exhaled breath condensate in the study of patients with lung cancer, comparing the dosage of tumoral markers in this sample with that in the BAL and in induced sputum, more commonly used in these patients. Although the exhaled breath condensate is not able to give information about the cytologic profile of airways, it appears to be useful in offering information on the oncogenesis though dosage of tumoral-soluble markers in airways. The same information found in the exhaled breath condensate and in the other two respiratory samples led us to confirm the specificity of the exhaled breath condensate.

In conclusion, our results provide evidence of an increase of IL-2, TNF-α, and leptin concentrations either in systemic samples or in those from airways of NSCLC patients, related with the progression of the tumor. The fully noninvasiveness of breath condensate collection and the important role of markers studied in lung carci-

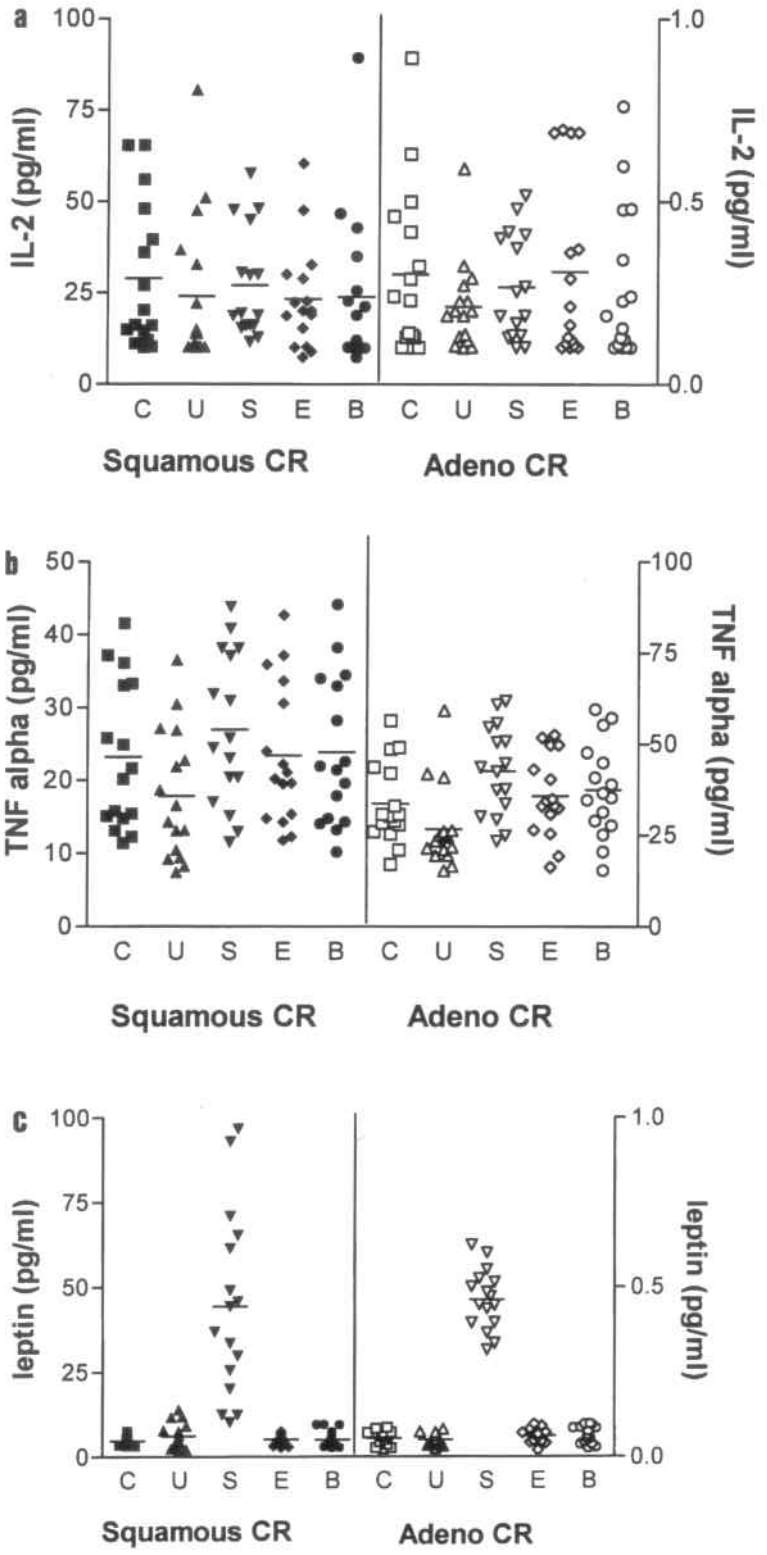

Figure 2. (a) IL-2 concentrations, (b) TNF-α concentrations, and (c) leptin concentrations in exhaled breath condensate, urine, blood, induced sputum, and BAL of patients with squamous lung cancer and patients with adeno lung cancer.

nogenesis make these results potentially relevant for the follow-up of NSCLC patients but also for the screening of high-risk populations.

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