

## HEAD AND NECK

# IFN- $\gamma$ and other serum cytokines in head and neck squamous cell carcinomas

## *IFN- $\gamma$ e altre citochine sieriche nei carcinomi squamocellulari della testa e del collo*

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## SUMMARY

Altered immune responses have been reported in head and neck cancer, and some of these responses have been associated with poor clinical outcomes. A multiple-array technology platform was used to simultaneously evaluate the levels of 25 cytokines. Pre-treatment serum levels were evaluated in 31 HNSCC patients and 6 healthy controls. The levels of 8 cytokines, specifically IL-1ra, IL-2, IL-5, IL-6, IL-8, IL-17, IFN- $\gamma$  and IP-10, were significantly higher in patients than in controls. Among cancer patients we observed lower levels of IFN- $\gamma$  and IL-7 in cases with nodal metastases compared to those with cN0 disease. We observed increases in the levels of some serum cytokines in HNSCC patients, as well as reductions in selected cytokines associated with regional progression. These findings provide an intriguing perspective on the development and validation of novel markers for follow-up evaluations and predictions of regional spreading in HNSCC patients.

KEY WORDS: Head and neck SCC • Nodal metastasis • Predictive factors • Cytokines • Inflammation • IFN- $\gamma$

## RIASSUNTO

*È noto che nelle neoplasie della testa e del collo vi siano delle alterazioni della risposta immune, ed alcune di tali alterazioni sono state associate ad un peggioramento prognostico. Abbiamo utilizzato una piattaforma con una tecnologia multi-array per valutare simultaneamente i valori di 25 citochine nel siero di 31 pazienti, affetti da carcinomi squamosi della testa e del collo prima del trattamento, e di 6 controlli sani. I livelli di 8 citochine, IL-1ra, IL-2, IL-5, IL-6, IL-8, IL-17, IFN- $\gamma$  ed IP-10, sono risultati significativamente più elevati nei pazienti che nei controlli. Tra i pazienti i casi con metastasi linfonodali mostravano livelli significativamente inferiori di IFN- $\gamma$  ed IL-7 rispetto ai casi cN0. Pertanto alcune citochine tendono ad essere più elevate nei soggetti affetti da carcinomi squamocellulari della testa e del collo, ma nel contesto di tale gruppo di pazienti si osserva un calo specifico di alcune citochine in caso di metastasi linfonodali. Tali dati aprono prospettive interessanti soprattutto ai fini di una miglior definizione del rischio di metastasi/recidive linfonodali con implicazioni sia in termini di impostazione terapeutica che di follow up.*

PAROLE CHIAVE: Carcinomi squamosi della testa e collo • Metastasi linfonodali • Fattori predittivi • Citochine • Infiammazione • Interferon-gamma

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## Introduction

Head and neck squamous cell carcinoma (HNSCC) represents approximately 3% of all malignant tumours in the United States <sup>1</sup> and Europe <sup>2</sup>, but is much more prevalent in other parts of the world <sup>3</sup>. The standard therapeutic approach, which includes surgery, irradiation and chemotherapy, either alone or in combination, has been somewhat modified over the last 30 years; however, the overall survival rate has not drastically improved <sup>1,3</sup>.

The persistently high rate of advanced lesions is among the potential reasons for the failure to improve the HNSCC survival rate. In general, screening attempts have been completely unsuccessful, such that for the larynx, the most frequently affected primary site in European patients <sup>4</sup>, where less than 1% of SCC patients are asymptomatic at presentation. The definition of high-risk groups that should be submitted to screening would likely increase this rate. Oncogenic viruses, particularly Epstein-Barr and high-risk papillomavirus, have been shown to

play causative roles in subsets of nasopharyngeal<sup>5</sup> and oropharyngeal<sup>6</sup> cancers, respectively, and therefore might be a good starting point. Nevertheless, the most frequent site of origin for HNSCC are the oral cavity and larynx, for which the main proven risk factors remain cigarette smoking and alcohol consumption<sup>3</sup>; however, these factors do not define a sufficiently restricted population to monitor for effective prevention and/or screening.

Another issue in head and neck oncology is that the prognostic stratification is inadequate because patients who are affected by tumours with similar clinicopathological parameters and are undergoing the same treatment can differ widely in their prognoses. This is likely due to the extreme biological heterogeneity of HNSCCs, and contributes to the known controversies concerning therapeutic algorithms (on management of cN0 necks)<sup>7</sup>.

Many clinical and epidemiologic studies have suggested a strong association between inflammation, with its mediators, and cancer<sup>8-11</sup>, including cancers of the head and neck<sup>12-14</sup>.

In the present study, we investigated the serum concentrations of various cytokines in patients with laryngeal and oral SCC, and compared these with the concentrations in healthy subjects, to identify alterations in inflammatory pathways associated with carcinogenesis.

We tested a panel of 26 cytokines that are important in the inflammatory response and immune system regulation and associated with different immune pathways, giving specific information, and in particular concerning Th2 and Th1 immune responses.

Cytokine levels were also correlated with tumour characteristics (e.g., site of origin and T and N classification) and oncologic outcomes to identify parameters that might supplement TNM and other information currently available to clinicians to assess prognoses, treatment responses and clinical behaviours (e.g., tendency to regional metastasis) of HNSCC patients.

## Materials and methods

### *Patients*

From February 2010 to March 2012, we enrolled 31 patients (5 women, 26 men; age range, 32-82 years; mean, 57 years) who were affected by primary, previously untreated and biopsy proven SCC arising from oral cavity or larynx, as evaluated by the multidisciplinary head and neck tumour board of the Policlinico Agostino Gemelli, Università Cattolica del Sacro Cuore, Rome, Italy.

Staging according to the TNM AJCC classification 7<sup>th</sup> edition, and treatment recommendation were defined, patients signed informed consent for their participation in

the present study, which had been previously approved by the ethical committee.

The circulating levels of interleukin-1b (IL-1b), interleukin-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-17, eotaxin, basic fibroblast growth factor (FGF-b), granulocyte colony-stimulating factor (G-CSF), interferon-gamma (IFN- $\gamma$ ), IFN- $\gamma$ -inducible protein 10 (IP-10), human monocyte chemoattractant protein 1 (HuMCP-1), macrophage inflammatory proteins-1-alpha and 1-beta (MIP-1 $\alpha$  and 1 $\beta$ ), platelet-derived growth factor-BB (PDGF-BB), regulated upon activation, normal T-cell expressed secreted (RANTES), tumour necrosis factor-alpha (TNF- $\alpha$ ) and vascular endothelial growth factor (VEGF) were investigated in sera collected before primary treatment. All SCC originated from the oral cavity (n = 16) and larynx (n = 15), which are the most frequent sites of HNSCC<sup>1</sup>. All patients had a history of cigarette smoking.

Selection of primary treatment modality was always shared in the institutional multidisciplinary head and neck tumour board. All oral cavity cases were treated primarily by surgery. As for laryngeal SCCs, in our tumour board treatment choice is based mainly on cT, and on the patient's general condition and preference. Namely, we primarily irradiated cT1 and cT2 and we recommended primary surgery in cT4 cases; cT3 cases were discussed in the tumour board and underwent surgery or radiotherapy depending on the patient's general conditions and feasibility of function preservation surgery (supracricoid operations). Among patients who underwent primarily radiochemotherapy, there were also cT4 patients who refused the recommended total laryngectomy and preferred to try to keep their larynx, after having been informed about the lower survival rates reported in literature. Postoperative radiation (mostly associated with cisplatin) was administered for pN2-3 disease, extracapsular spread and adverse pathologic primary site features including pT4 disease, perineural invasion, involvement of surgical margins, or lymphovascular invasion.

The follow-up time was calculated from the treatment date (surgery or final session of chemo/radiotherapy). For controls, sera were collected from 6 healthy sex and age-matched volunteers who were current smokers with personal histories negative for malignancy, without recent trauma or surgery, and who were not pregnant. Personal data for cases and controls and clinical data for HNSCC patients are summarised in Table I. Peripheral venous blood was collected into Vacuette (Greiner bio-one, Kremsmünster, Austria) tubes and was immediately centrifuged at 4°C and 4000 rpm for 5 minutes. Serum samples were collected and multiple aliquots were stored

at -80°C until analysis, at which time the serum concentrations of cytokines were determined in a multiplex cytokine analysis.

#### *Multiplex cytokine analysis*

A Luminex xMAP system (Bio-Plex 200 System, BioRad Laboratories, Hercules, CA, USA), which is a multiplex biometric enzyme-linked immunosorbent assay (ELISA)-based immunoassay that contains dyed microspheres conjugated to monoclonal antibodies specific for target proteins, was used according to the manufacturer's instructions. The cytokines evaluated in the panel (BioPlex Human Cytokine Assay; BioRad) were IL-1 $\beta$ , IL1Ra, IL-2, IL-4, IL-5, IL-6, IL-7, CXCL8 (IL-8), IL-9, IL-10, IL-12 (p70), IL-13, IL-17, basic FGF, eotaxin, G-CSF, IFN- $\gamma$ , IP-10, MCP-1, MIP-1a and  $\beta$ , PDGF-BB, RANTESTNF- $\alpha$  and VEGF.

The serum samples were diluted 1:4 and incubated with antibody-coupled beads. The resulting complexes were washed and incubated first with a biotinylated detection antibody and then with streptavidin-phycoerythrin prior to assessing the cytokine concentration titres. Concentrated human recombinant cytokines were provided by the vendor (BioRad, Inc.). Broad ranges of standards (1.95-32,000 pg/ml) were used to establish standard curves with which to maximise the sensitivity and dynamic range of the assay. Cytokine levels were determined on a Bio-Plex array reader, an automated flow-based microfluidics device that uses a dual-laser fluorescent detector with real-time digital signal processing for quantification. This instrument quantifies multiplex immunoassays in 96-well plates. The concentrations in pg/ml were calculated from standard curves with software provided by the manufacturer. A regression analysis was performed to derive an equation that was then used to predict the concentrations of cytokines in the serum samples.

#### *Statistical analysis*

Statistical analysis was performed with JMP software, release 7.0.1 (SAS Institute). The level was fixed at 0.05 for all statistical tests. Correlations between nominal variables were evaluated with a  $\chi^2$  test. Differences in the concentrations of different cytokines between controls (c) and patients (p) and between the cN0 and cN+ cases were evaluated with a t-test. Differences among tumours that originated in the two sites (oral cavity vs. larynx), controls and among different the cT and cN classifications were evaluated by ANOVA variance analysis, and pairs were then compared with the Tukey-Kramer test. For a more thorough evaluation of the variations in serum cytokine levels along with increasing tumour staging, we trans-

**Table I.** Characteristics of patients in the entire series according to the primary treatment group.

Characteristic	Patients (n = 31)		Controls (n = 6)	
<b>Age</b>				
Median	63		61	
Range	32-82		47-72	
<b>Sex-no. (%)</b>				
Male	26	(84%)	5	(83%)
Female	5	(16%)	1	(17%)
<b>Smoking habits-no. (%)</b>				
Less than 10 pack/years	10	(32%)	2	(33%)
More than 10 pack/years	21	(68%)	4	(67%)
<b>Drinking habits-no. (%)</b>				
Non drinker	12	(39%)	2	(33%)
1-4 glasses/day	13	(42%)	3	(50%)
More than 4 glasses a day	6	(19%)	1	(17%)
<b>Follow up</b>				
Median	8.3		-	
Range	0.2-21		-	
<b>Site of primary-no. (%)</b>				
Oral cavity	16	(52%)	-	
Larynx	15	(48%)	-	
<b>Primary treatment-no. (%)</b>				
Surgery	22	(71%)	-	
Radio $\pm$ chemotherapy	9	(21%)	-	
<b>TNM staging-no. (%)</b>				
I	3	(10%)	-	
II	9	(29%)	-	
III	2	(6%)	-	
Iva	17	(55%)	-	
<b>T stage-no.(%)</b>				
T1	4	(13%)	-	
T2	12	(39%)	-	
T3	3	(9%)	-	
T4a	12	(39%)	-	
<b>N stage-no.(%)</b>				
N0	17	(55%)	-	
N1	3	(10%)	-	
N2a	2	(6%)	-	
N2b	4	(13%)	-	
N2c	5	(16%)	-	

formed the nominal cT and cN parameters to numerical variables (numeric T and N scores; cT4a = 4, cT4b = 5, cN2a = 2, cN2b = 3, cN2c = 4, cN3 = 5) and sought a linear fit by analysis of variance. The impact of different parameters on survival were evaluated by a Cox regres-

**Table II.** Mean values of cytokines in controls and cases (statistically significant differences are indicated with \*).

Cytokine	Value in controls (mean±SD)	Value in cases (mean±SD)
IL-1b	3.3133333 ± 0.8841418	3.674 ± 0.8323154
IL-1ra*	174.90667 ± 17.770011	237.06833 ± 75.716824
IL-2*	1.715 ± 2.9156251	8.0387097 ± 7.2977836
IL-4	9.425 ± 2.0147332	11.432258 ± 2.3363158
IL-5*	3.4616667 ± 0.412864	4.4770968 ± 1.8866005
IL-6*	9.1083333 ± 1.1434232	14.095806 ± 4.3274864
IL-7	13.881667 ± 2.6957776	16.143548 ± 4.848343
IL-8*	32.186667 ± 5.5094489	40.000323 ± 10.059768
IL-9	33.548333 ± 18.8509	46.597097 ± 25.720585
IL-10	30.395 ± 33.645025	22.915161 ± 12.610672
IL-12	95.153333 ± 37.067161	132.97935 ± 84.327459
IL-13	12.19512,195 ± 3.6285024	14.570645 ± 5.7731684
IL-17*	190.29667 ± 61.275395	253.8671 ± 67.58494
Eotaxin	233.67167 ± 97.855583	243.08645 ± 112.18061
FGF-basic	47.305 ± 23.345764	55.560645 ± 27.172748
G-CSF	72.231667 ± 18.809514	75.645161 ± 16.2412
IFN-g*	311.015 ± 51.866943	376.45903 ± 75.010629
IP-10*	1488.3367 ± 510.41972	2502.8471 ± 1098.5081
Hu MCP-1(MCAF)	60.086667 ± 21.832594	45.274839 ± 16.429516
Hu MIP-1a	8.8483333 ± 2.2179217	9.0803226 ± 2.1559568
PDGF-bb	14614.73 ± 5313.271	17966.356 ± 4937.8699
MIP-1b	113.71333 ± 34.277828	118.45355 ± 30.917572
RANTES	9447.305 ± 850.45661	9032.6235 ± 1481.9593
TNF-alpha	39.131667 ± 7.2147	43.99129 ± 11.821503
VEGF	132.37667 ± 65.794253	203.30032 ± 175.79658

sion analysis (only univariate because of the low numbers of cases and recorded events).

## Results

Statistically significant differences between controls and patients with regard to concentrations of the different cytokines are reported in Table II and schematically represented by box plots in Figure 1. The statistically significant differences observed among the two different tumour sites and controls are shown in Figure 2. Two cytokines were significantly overexpressed only in patients with specific SCC subsites, namely IL-6 in laryngeal SCC patients and IP-10 in oral cavity SCC patients.

We found no significant differences or correlations between the levels of various cytokines and the T classification, although a trend ( $p = 0.0615$  in the variance analysis) towards a positive correlation between the IP-10 levels and numeric T scores was observed.

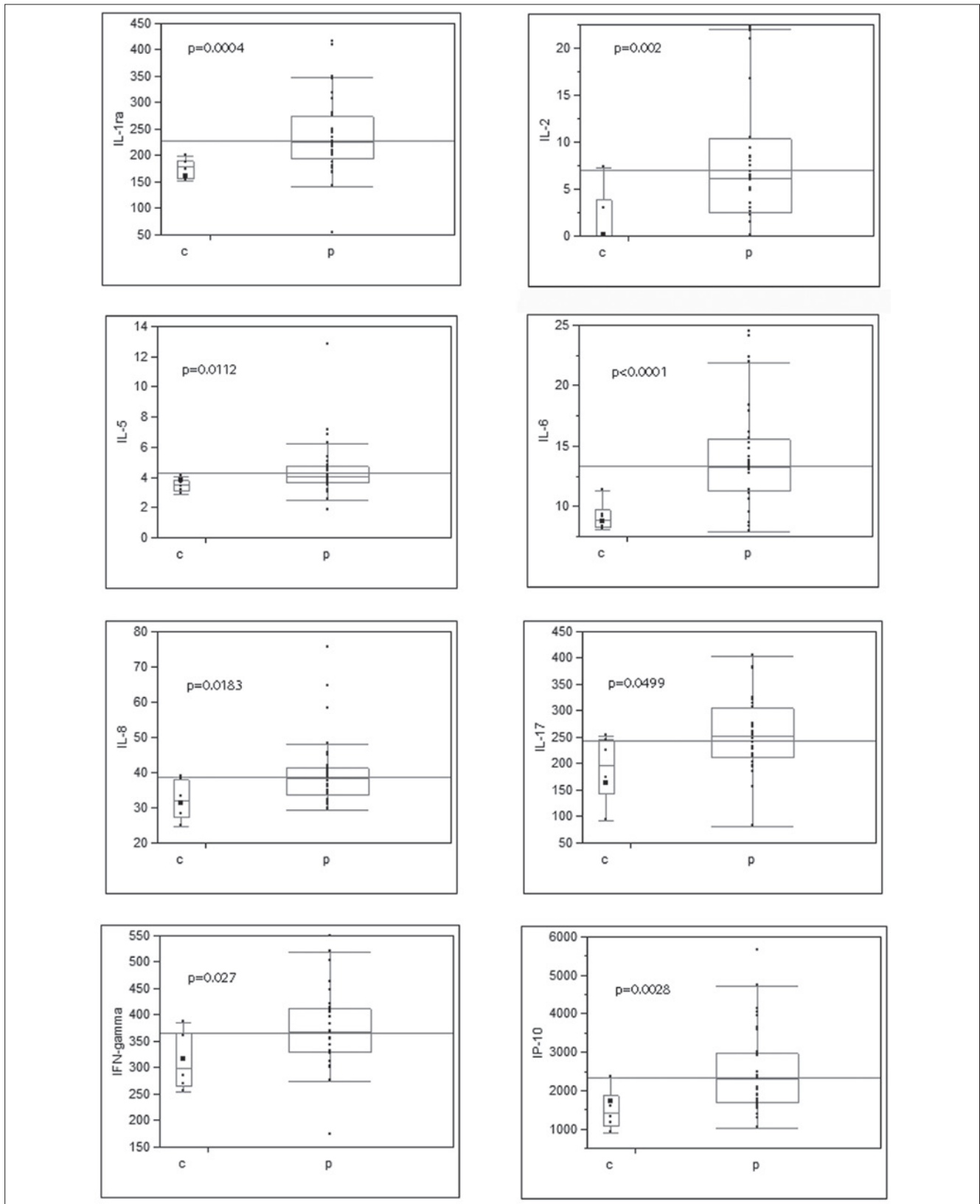
However, when we evaluated the association between cytokine levels and the N classification, a significant cor-

relation of lower levels of some with nodal involvement (IL-7, FGF-basic, IFN- $\gamma$ , HuMIP-1a, see figure 3 on the right) and with the numeric N score were seen (IL-7, eotaxin, IFN- $\gamma$ , see Figure 3 on the left).

In Cox univariate survival analysis, we observed that higher IFN- $\gamma$  ( $p = 0.0218$ ) and lower IL-1ra ( $p = 0.0376$ ) levels were associated with longer disease-specific survival.

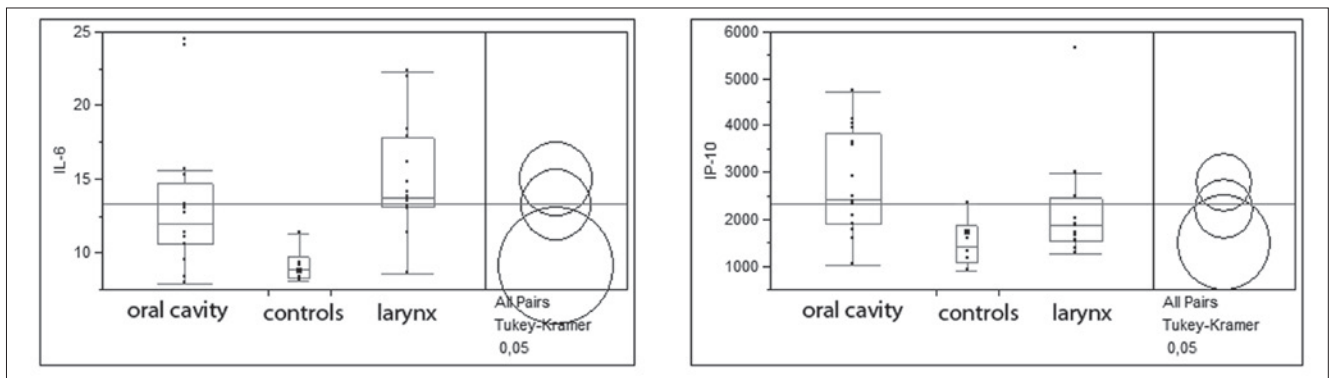
## Discussion

We observed increases in the levels of several cytokines in sera from HNSCC patients compared to sera from controls. This observation confirms the well-known link between inflammation and cancer that has already been observed in head-and-neck oncology. This observation could be linked to the presence of the tumour and its interactions with the host<sup>8 15-18</sup>, or could indicate an immunological predisposition to cancer development<sup>8 11 15</sup>. In the former scenario, inflammatory mediators should be evaluated as tumour markers that could be employed in



**Fig. 1.** Box plots of 8 cytokines (for which the levels were significantly higher in sera from patients (p), compared to controls (c). P-values were obtained with a two-sided t-test.





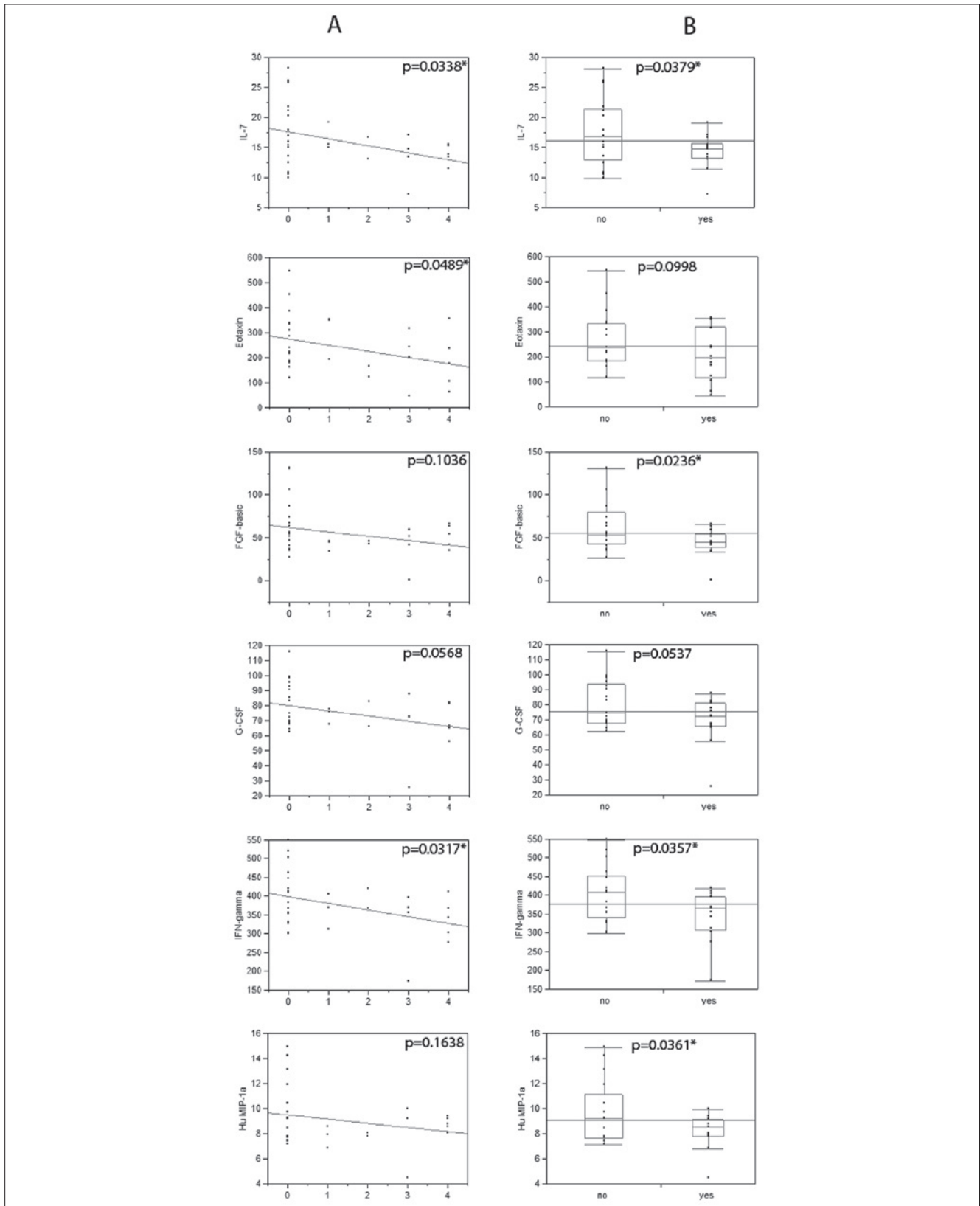
**Fig. 2.** When compared with controls, IL-6 serum levels were significantly higher in laryngeal but not in oral SCC patients; IP-10 levels were significantly higher only in oral SCC patients.

follow-up for early detection of recurrence; in the latter, certain immunological conditions might be risk factors for development of malignancy and could be taken into account when selecting subjects for screening.

Likely, both of these scenarios are true, and in our series, it was difficult to discriminate between the alterations that preceded and those that derived from cancer progression. In our patients, the cytokines with the most significant increases were IL-6 and IL-1Ra. Increased IL-6 levels have already been associated with HNSCC<sup>19-21</sup>, as well as with IL-8<sup>12,22</sup>, another upregulated cytokine among our patients. IL-6 might contribute to cancer progression through STAT-3-mediated paracrine or autocrine proliferative stimulation and through additional inhibitory effects on anti-tumour Th1 immune responses<sup>23,24</sup>. Nevertheless, in contrast to previous studies, in the present series, we failed to demonstrate any correlation between IL-6 itself and the disease stage<sup>25</sup> or prognosis<sup>19</sup>, although the limited number of patients and the short follow-up period might explain this result. Actually, in the present series, IL-6 was significantly increased only in laryngeal SCC patients, and the reasons for this observation remain to be clarified. We instead observed a correlation between increased levels of IL-1Ra and worse prognosis. IL-1Ra levels have been correlated with prognosis in Hodgkin lymphoma<sup>26</sup> and have been shown to be elevated in head and neck SCC tissues<sup>27</sup>. IP-10 and its corresponding receptor CXCR3 have been shown to be involved in carcinogenesis<sup>28</sup> including malignant melanoma<sup>29</sup>, ovarian carcinoma<sup>30</sup>, multiple myeloma<sup>31</sup>, B-cell lymphoma<sup>32</sup> and basal cell carcinoma<sup>33</sup>. In our series, the increased levels of IP-10 in patients with oral SCC, which are usually bulkier than laryngeal SCC, and in those with higher T scores (even if not significant), could suggest that IP-10 levels are influenced by tumour volume.

The prognostic role of IL-1Ra deserves in-depth evaluation in larger perspective studies. Nevertheless, these results provide a perspective for the use of some cytokines as tumour markers in clinical practice, such as during follow-up of HNSCC patients, also on account of the clear lack of reliable serum follow-up markers, of which the SCC antigen is the most used, despite its known issues regarding sensitivity and specificity<sup>34</sup>.

Immune responses in head and neck SCC patients are reportedly unbalanced towards the secretion of Th2 cytokines, which would prevent effective antitumour Th1 immune responses<sup>35</sup>. In the present study, we observed the upregulation of both Th1 (IFN- $\gamma$ , IL-2 and IP-10) and Th2 (IL-4, IL-6 and IL-10) cytokines in HNSCC patients compared to healthy subjects, and the above-cited imbalance become evident only with regional progression, which is, however, the main clinical factor that affects survival in these patients. In fact, IFN- $\gamma$ , which is among the most important Th1 pathway cytokines, was clearly downregulated in cases with nodal involvement and such downregulation displayed a linear correlation with regional progression. IFN- $\gamma$  was also the only serum cytokine that displayed a protective effect when evaluated relative to prognosis; its crucial role in HNSCC has already been described at both a molecular<sup>36</sup> and clinical level<sup>22</sup>. In addition to IFN- $\gamma$ , other cytokines that did not have specific Th1 activity were significantly downregulated in cases with nodal involvement and/or showed linear correlations with numeric N scores in our series, and none of these differed significantly between the cases and controls (except IFN- $\gamma$ ). Among these downregulated cytokines, IL-7 was recently found to perform a relevant antitumour activity by inducing the expansion of tumour-specific CD4+ T cell effectors more efficiently than IL-2<sup>37</sup>. The serum eotaxin levels undergo significant modifications after the induc-



**Fig. 3.** Cytokine levels that correlated significantly with the numeric N score (on the left) and/or the nodal status (on the right). Statistical significance is indicated by asterisks.

tion of chemotherapy in head-and-neck cancer patients<sup>38</sup>. Basic fibroblast growth factor expression was associated with good outcomes in patients with squamous cell carcinoma of the oesophagus<sup>39</sup>.

These data might suggest that HNSCC development induces a non-specific immune response, along with increased serum levels of many cytokines, but tumour cells tend to overcome such responses by selectively down-regulating specific interleukins, thus promoting tumour progression, particularly lymphatic spreading. This phenomenon would be part of tumour-mediated immunosuppressive activity that, according to several authors, is associated with immune escape, rapid growth and frequent metastasis also observed in other cancers<sup>8 11 15</sup>.

## Conclusions

Regardless of the molecular/immunological basis of the findings of the present study, from a clinical point of view, the specific alterations associated with regional metastasis are very interesting for several reasons. The most immediate perspective is that downregulation of the above-cited cytokines, and IFN- $\gamma$  in particular, could be used as risk markers of neck metastasis and could serve to modify the therapeutic recommendations by indicating, for example, elective neck treatment in cN0 cases and comprehensive neck dissection followed by adjuvant radiochemotherapy in pN+ cases. The prognostic role of IFN- $\gamma$  could also facilitate prognostic assessments. Notwithstanding, further studies are necessary to confirm these findings on larger series and to determine the optimal cut-off value of serum IFN- $\gamma$  for risk prediction in clinical practice.

Moreover, the suppression of IFN- $\gamma$  and other cytokines, which we hypothesised to be tumour-dependent and to promote nodal metastasis, could be hindered pharmacologically in attempted immunotherapy for HNSCC, as previously proposed for other malignancies<sup>40</sup>.

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