

## Clinical relevance of a new pre-treatment laboratory prognostic index in patients with oral squamous cell carcinoma

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**Abstract** The purpose of this retrospective study was to develop a pre-treatment laboratory prognostic index (LPI) based on laboratory results that might serve as an extension to clinicopathological parameters for prognosis and treatment in patients with oral squamous cell carcinoma (OSCC). Pre-treatment LPI was calculated from C-reactive protein (CRP), hemoglobin (Hb) levels, and count of white blood cells (WBCs) due to significant ( $P < 0.05$ ) association with locoregional recurrence measured for each parameter by receiver operating characteristic (ROC) curves in 187 patients with OSCC. Positive predictive values (+PV, precision rate) and negative predictive values (−PV) of LPI were measured. Likelihood ratios (LRs) were used to assess how good the pre-treatment LPI diagnostic test is to determine locoregional recurrence of the disease. CRP expression by cancer cells was confirmed by immunocytochemistry and FACS analysis. ROC analysis determined cutoff values for CRP levels, Hb levels, and WBC count and showed significant differences between nonrecurrent and recurrent group of OSCC. On univariate analysis, patients with high pre-treatment LPI ( $LPI \geq 2$ , hazard ratio (HR) = 3.8670, 95% confidence interval (CI) = 2.2518–6.6407,  $P < 0.0001$ ) had a signifi-

cant poorer prognosis. Multivariate analysis showed that the most important independent prognostic factor was high pre-treatment LPI ( $LPI \geq 2$ , HR = 3.6450, 95% CI = 2.3964–5.5441,  $P < 0.0001$ ). Moreover, pre-treatment  $LPI \geq 2$  showed high probability that locoregional recurrence will be present later (+PV,  $LPI \geq 2$ , 86.4%, 95% CI = 65.1–97.1). High +LR gave an excellent indication for a good quality of the test (LR+,  $LPI \geq 2$ , 12.77, 95% CI = 8.8–18.6). Immunohistochemistry and FACS analysis confirmed inflammatory CRP expression by cancer cells. This study highlights the combination of inflammatory CRP levels, Hb levels, and WBC count as the most important independent prognostic factor in predicting disease recurrence of patients with OSCC. LPI can be used as a pre-treatment inflammatory biomarker that may identify OSCC with a more aggressive biological phenotype of the disease and might be helpful for guiding further post-operative treatment in OSCC.

**Keywords** Prognostic factors · Clinicopathological parameters · Oral squamous cell carcinoma · Pre-treatment laboratory prognostic index (LPI)

### Abbreviations

OSCC	Oral squamous cell carcinoma
LPI	Pre-treatment laboratory prognostic index
CP	Clinicopathological parameters
CRP	C-reactive protein
Hb	Hemoglobin
WBCs	White blood cells
ROC	Receiver operating characteristic
+PV	Positive predictive value
−PV	Negative predictive value
+LR	Positive likelihood ratio
−LR	Negative likelihood ratio

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## Introduction

Several clinicopathological parameters (CP) have been implicated in prognosis, recurrence, and survival following oral squamous cell carcinoma (OSCC). The purpose of this retrospective study was to develop a pre-treatment laboratory prognostic index (LPI) based on laboratory results that might serve as an extension to CP for prognosis and treatment in patients with OSCC.

The presence of a systemic inflammatory response has been thought to indicate poor prognosis in OSCC, based on the assumption that the processes underlying such a response play important roles in the progression of OSCC. In 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance cell proliferation [1]. Basic scientists have clearly demonstrated the importance of molecular and cellular pathways linking cancer and inflammation [1–3].

OSCC frequently metastasizes to the lymph nodes, which represents the most important predictor of patient survival rates. For about 50% of patients with OSCC have detectable lymph node involvement at presentation. Less than 40% of patients with lymph node metastasis at presentation survive 5 years, compared to 90% of patients without metastasis [4–6], meaning that the survival rate decreases by approximately 50% when nodal metastasis is present. Therefore, identification of poor prognostic factors in association with inflammation has become an important issue in the management of OSCC [7]. Poor prognosis is well known in advanced International Union Against Cancer (UICC) stages of the disease [8]. Other prognostic factors have also been proposed in the literature. In the pathogenesis of OSCC, pre-treatment measurement of elevated serum C-reactive protein (CRP) levels [9–14] and low hemoglobin (Hb) levels [15–20] has been identified to be promising for adverse prognosis. Increased count of white blood cells (WBCs) is associated with adverse prognosis in other cancer entities [21–24] but has not been identified for OSCC [25]. However, in the pathogenesis of OSCC, there are no studies regarding these risk factors together in a clinical context. Moreover, cutoff values for each parameter have not been determined for predicting survival of OSCC as yet. Therefore, the aims of this study were to measure pre-treatment cut-off values for laboratory parameters, to investigate relevant prognostic factors and to analyze survival rates in patients with OSCC in a larger patient cohort. Moreover, we elucidated a parameter that identifies a more aggressive biological phenotype of the disease, which might be helpful for guiding further post-operative treatment in OSCC.

## Materials and methods

### Patients and tumor specimen

Out of 484 patients with OSCC, we retrospectively reviewed the records of 187 patients after primary radical R0 tumor resection in our department between 1997 and 2010. Patients with nonresectable disease, inadequate follow-up data, and patients with preoperative antineoplastic therapies (chemoradiation/chemotherapy) were excluded from the study. The material was archival formalin-fixed, paraffin-embedded tissue from routine histopathologic work-up. The material had been stored with permission of the local ethics committee, after informed consent obtained from the patients prior to surgical resection.

Follow-up data were obtained from our local tumor registry of Middle Frankonia/Germany and was complete (100%) for all patients. The last follow-up was recorded from the last outpatient visit or the date of death. Tumor and patient characteristics are summarized in Table 1.

### Histopathologic analysis and tumor staging

Tumor blocks of paraffin-embedded tissue were selected by experienced pathologists, evaluating the routine H.E.-stained sections. Sections from all available tumors underwent intensive histopathologic assessment, blinded to the prior histopathology report. Serial tissue sections (2  $\mu$ m thickness) were cut from formalin-fixed paraffin-embedded (FFPE) blocks on a microtome and mounted from warm water onto adhesive microscope slides. Tumor staging was performed according to the 6th edition of the TNM staging system by the UICC/AJCC of 2002 [26]. Grading was performed according to WHO criteria [27]. Tumor characteristics (UICC stage, pT-categories, pN-categories, cM-categories, infiltrated lymph nodes, residual tumor status, tumor size, site distribution, grading, microvascular invasion, and lymphatic vessel involvement), treatment characteristics (surgery alone, surgery and radiotherapy with/without chemotherapy, or brachytherapy) patient characteristics (gender, age, personal history, habitual history) were collected in a database (EXCEL, Microsoft). Surgical margin status was determined on final histopathologic evaluation. Close margins were deemed positive in all analyses, whereas negative margins were considered greater than or equal to 10 mm from resection margin after tissue fixation.

### Measurement of CRP levels, Hb levels, and WBC count

Standard laboratory techniques were used to determine pre-treatment CRP levels, Hb levels, and WBCs in 187 patients with OSCC.

**Table 1** Clinicopathological characteristics and prognostic factors in 187 patients treated for OSCC

Characteristics	Number of patients		P value	5-year DFS rate (%)	P value
	Total n = 187	Nonrecurrent group n = 125 (66.8%)			
Age (y)			0.6062		0.1051
<62	81 (43.3%)	52 (64%)		53	
≥62	106 (56.7%)	73 (73%)		40	
Gender			0.9268		0.9160
Male	123 (65.8%)	82 (67%)		48	
Female	64 (34.2%)	43 (67%)		50	
Site distribution of OSCC			0.9559		
Lips <sup>a</sup>	9 (4.8%)	8 (89%)		83	0.845 <sup>b</sup> ; 0.498 <sup>c</sup> ; 0.562 <sup>d</sup> ; 0.022 <sup>e</sup> ; 0.0009 <sup>f</sup>
Tongue <sup>b</sup>	37 (19.8%)	28 (76%)		79	0.236 <sup>c</sup> ; 0.472 <sup>d</sup> ; 0.001 <sup>e</sup> ; <0.0001 <sup>f</sup>
Floor of the mouth <sup>c</sup>	50 (26.7%)	39 (78%)		65	0.964 <sup>d</sup> ; 0.018 <sup>e</sup> ; <0.0001 <sup>f</sup>
Palate <sup>d</sup>	5 (2.7%)	3 (60%)		53	0.553 <sup>e</sup> ; 0.334 <sup>f</sup>
Buccal mucosa <sup>e</sup>	14 (7.5%)	10 (71%)		25	0.854 <sup>f</sup>
Alveolar ridge <sup>f</sup>	72 (38.5%)	37 (51%)		23	
Treatment modalities			0.0782		
Surgery alone <sup>g</sup>	99 (52.9%)	75 (76%)		64	0.036 <sup>h</sup> ; 0.0003 <sup>i</sup> ; 0.899 <sup>k</sup>
Surgery + RT <sup>h</sup>	12 (6.4%)	4 (33%)		28	0.881 <sup>i</sup> ; 0.071 <sup>k</sup>
Surgery + RT/CT <sup>i</sup>	48 (25.7%)	27 (56%)		31	0.008 <sup>k</sup>
Brachytherapy <sup>k</sup>	28 (15%)	19 (68%)		65	
Histological grading			<0.0001*		
G1 <sup>l</sup>	29 (15.5%)	28 (97%)		88	0.003 <sup>m</sup> ; <0.0001 <sup>n</sup> ; 0.317 <sup>o</sup>
G2 <sup>m</sup>	87 (46.5%)	62 (71%)		55	0.001 <sup>n</sup> ; 0.929 <sup>o</sup>
G3 <sup>n</sup>	69 (36.9%)	34 (49%)		28	0.613 <sup>o</sup>
G4 <sup>o</sup>	1 (2%)	1 (50%)		0	
Depth of invasion			<0.0001***		
pT1 <sup>p</sup>	84 (44.9%)	89 (90%)		80	0.002 <sup>q</sup> ; 0.001 <sup>r</sup> ; <0.0001 <sup>s</sup>
pT2 <sup>q</sup>	38 (20.3%)	24 (63%)		51	0.970 <sup>r</sup> ; 0.0002 <sup>s</sup>
pT3 <sup>r</sup>	16 (8.6%)	8 (50%)		46	0.010 <sup>s</sup>
pT4 <sup>s</sup>	49 (26.2%)	18 (37%)		12	
Cervical lymph node metastasis			0.0013		0.0029
pN0	135 (72.2%)	100 (74%)		56	
pN1-3	52 (27.8%)	25 (48%)		30	
Lymphatic vessel invasion (L)			0.0028		0.0001

**Table 1** continued

Characteristics	Number of patients		P value	5-year DFS rate (%)	P value
	Total n = 187	Nonrecurrent group n = 125 (66.8%)			
Negative	62 (33.2%)	51 (82%)	11 (18%)	74	
Positive	125 (66.8%)	74 (59%)	51 (41%)	43	0.0042
Microvascular invasion (V)					
Negative	165 (88.2%)	118 (72%)	47 (28%)	52	0.0005
Positive	22 (11.8%)	7 (32%)	15 (68%)	17	
UICC stage					<0.0001****
UICC I <sup>f</sup>	74 (39.6%)	66 (89%)	8 (11%)	80	0.023 <sup>h</sup> ; 0.013 <sup>v</sup> ; <0.0001 <sup>w</sup>
UICC II <sup>u</sup>	27 (14.4%)	18 (67%)	9 (33%)	55	0.998 <sup>s</sup> ; 0.0007 <sup>w</sup>
UICC III <sup>v</sup>	23 (12.3%)	15 (65%)	8 (35%)	51	<0.003 <sup>w</sup>
UICC IV <sup>w</sup>	63 (33.7%)	26 (41%)	37 (59%)	17	
Laboratory prognostic index (LPI)					<0.0001****
0 <sup>f</sup>	48 (25.7%)	44 (92%)	4 (8%)	76	0.067 <sup>††</sup> ; <0.0001 <sup>†††</sup> ; <0.0001 <sup>††††</sup>
1 <sup>††</sup>	71 (38%)	52 (73%)	19 (27%)	58	0.005 <sup>†††</sup> ; 0.0001 <sup>††††</sup>
2 <sup>†††</sup>	46 (24.5%)	26 (56%)	20 (44%)	30	0.069 <sup>††††</sup>
3 <sup>††††</sup>	22 (11.8%)	3 (14%)	19 (86%)	7	

y: years; G: grading; UICC: International Union against Cancer, RT/CT: radiotherapy with/without chemotherapy  
 Reference groups: <sup>a</sup> lips; <sup>b</sup> tongue; <sup>c</sup> floor of the mouth; <sup>d</sup> palate; <sup>e</sup> buccal mucosa; <sup>f</sup> alveolar ridge; <sup>g</sup> surgery alone; <sup>h</sup> surgery + RT; <sup>i</sup> surgery + RT/CT; <sup>k</sup> brachytherapy; <sup>l</sup> G1; <sup>m</sup> G2; <sup>n</sup> G3; <sup>o</sup> G4; <sup>p</sup> pT1; <sup>q</sup> pT2; <sup>r</sup> pT3; <sup>s</sup> pT4; <sup>t</sup> UICC I; <sup>u</sup> UICC II; <sup>v</sup> UICC III; <sup>w</sup> UICC IV; <sup>†</sup> LPI 0; <sup>††</sup> LPI 1; <sup>†††</sup> LPI 2; <sup>††††</sup> LPI 3. \* G1/2 versus G3/4; \*\* pT1/2 versus pT3/4; \*\*\* UICC I/II versus UICC III/IV; \*\*\*\* LPI 0/1 versus LPI 2/3

Cell culture and immunocytochemistry

We analyzed inflammatory CRP expression in cells ( $1 \times 10^4$ ) from the squamous cell carcinoma (SCC) cell line SCC-4 (American Type Culture Collection, ATCC) in cytopins as a positive control of CRP expression by cancer cells. Cytopins of the SCC-4 cell line were fixed in acetone and dried for 10 min. Slides were then incubated in normal serum (2%) and bovine serum albumin (BSA) (0.5%) at room temperature for 20 min to block nonspecific binding. Subsequently, slides were incubated with the horseradish peroxidase (HRP)-conjugated primary antibody (Ab) or control antibody overnight at 4°C in a humidified chamber, followed by 5 min of incubation with DAB (Biogenex) and counterstained with hematoxylin. Slides were analyzed using a Zeiss camera (Jena, Germany). The photographed images were imported into the Microsoft Office Picture Manager.

Flow cytometric analysis of CRP expression by SCC-4 cells

Cells ( $5 \times 10^6$ ) derived from SCC-4 cancer cell line were analyzed on a flow cytometer (Beckman Coulter, Krefeld, Germany) with an appropriate software package (Coulter, Epics XL-MCL, System II). In each sample, we analyzed a constant cluster of tumor cells. Fluorescein isothiocyanate (FITC)-conjugated CRP Ab was purchased from HyTest Ltd (Turku, Finland), and FITC-conjugated isotype control Ab was purchased from Beckman Coulter (Krefeld, Germany). The total suspension of cells was pelleted and resuspended in PBS. For intracellular staining, we used the Intraprep-Kit (Beckman Coulter, Krefeld). Cells were incubated with CRP or control Ab for 20 min. After washing, cells were analyzed by FACS, equipped with the FITC emission signal detector FL1 (488 nm, green).

Statistical analysis

Statistical analysis was performed with MedCalc Software, version 11.6.1 (Mariakerke, Belgium). Disease-free survival (DFS) was calculated from the time of tumor resection until obvious locoregional recurrence or tumor conditional death, respectively. The DFS times were estimated using the Kaplan–Meier method [28] and were compared by using the log-rank test [29]. Multivariate analyses were performed using the Cox proportional hazards model [30]. All parameters that were found significant on univariate analysis were included. Hazard ratios for variables that may influence survival status in univariate and multivariate analysis were provided with 95% CI. Chi-square test ( $\chi^2$ ) and Fisher’s exact test were used to investigate the relation between two categorical variables.

**Table 2** Definition of the prognostic index in 187 patients treated for OSCC (cutoff values for CRP, Hb, and WBC were determined by ROC analysis)

Laboratory prognostic index (LPI)	CRP (mg/dl)	Hb (g/dl)	WBC (count/nl)
0	<1.1	>12.7	<9.6
1	<b>≥1.1*</b>	>12.7	<9.6
1	<1.1	<b>≤12.7*</b>	<9.6
1	<1.1	>12.7	<b>≥9.6*</b>
2	<b>≥1.1*</b>	<b>≤12.7*</b>	<9.6
2	<b>≥1.1*</b>	>12.7	<b>≥9.6*</b>
2	<1.1	<b>≤12.7*</b>	<b>≥9.6*</b>
3	<b>≥1.1*</b>	<b>≤12.7*</b>	<b>≥9.6*</b>

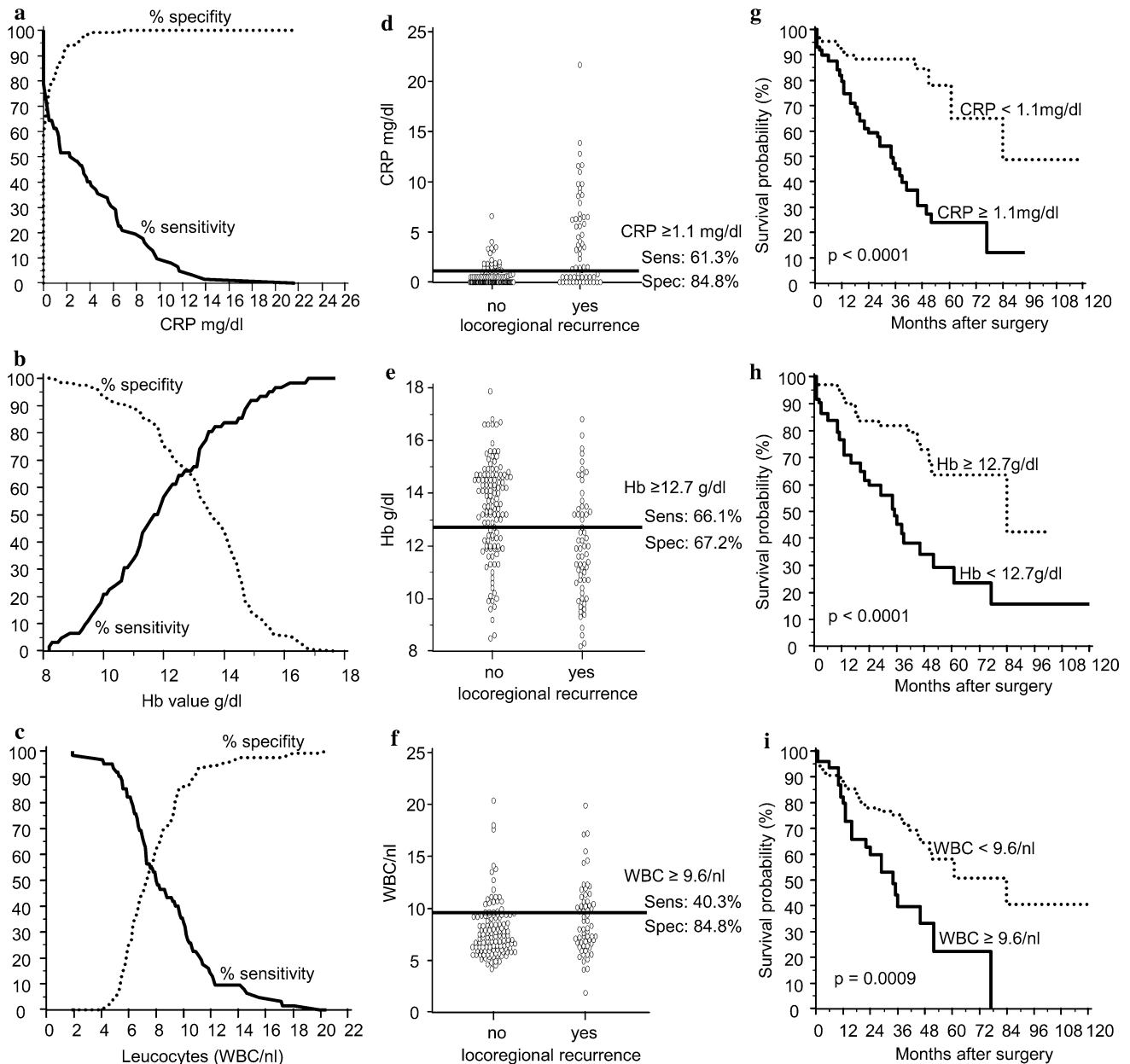
\* Unfavorable factor for recurrence of OSCC (in bold)

Receiver operating characteristic curves (ROC) were plotted to determine the best cutoff ranges for OSCC recurrence group screening for each value, and the relevant sensitivities and specificities were calculated [31]. Area under the curve (AUC) analysis was determined for quality measurement of the classifier (CRP, Hb, WBC, LPI). The cutoff point was determined as the value corresponding with the highest diagnostic average of sensitivity and specificity (highest diagnostic accuracy). These values were graphical displayed in a plot versus criterion values and in an interactive dot diagram to study the accuracy of each diagnostic test. Based on resulting sensitivity and specificity, the likelihood ratios (LRs) were calculated  $+LR = \text{sensitivity}/(1 - \text{specificity})$  and  $-LR = (1 - \text{sensitivity})/\text{specificity}$ . LRs were used to assess how good the single values and pre-treatment LPI diagnostic test are to determine locoregional recurrence of OSCC. LR is the ratio between the probability of a positive test (positive likelihood ratio, +LR) or a negative test (negative likelihood ratio, -LR) result given the presence of disease recurrence and the probability of a positive or negative test result given the absence of disease recurrence. The predictive value (PV) is the probability that disease recurrence is present (positive predictive value, +PV, precision rate) when the test is positive or absent (negative predictive value, -PV) when the test is negative (expressed as a percentage). For the calculation of predictive values, prevalence of disease recurrence has been stated to be 33.2% among subjects with OSCC (Table 1). All *P* values presented were 2-sided, and *P* < 0.05 was considered statistically significant.

Results

Demographic data of all patients

Out of 484 with OSCC, 187 patients were identified with complete pre-treatment laboratory parameters (CRP levels,

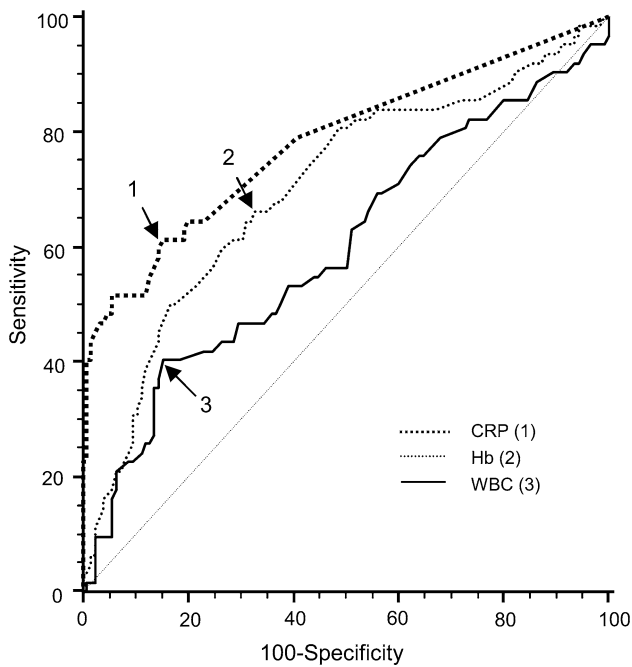


**Fig. 1** Plot versus criterion values (a–c), interactive dot diagrams (d–f), and univariate disease-free survival (DFS) Kaplan–Meier curves (g–i) for CRP, hemoglobin (Hb), and white blood cell count (WBC) in patients of nonrecurrent and recurrent group with OSCC. In Plot versus criterion values (part of ROC curve analysis), the sensitivity and specificity are plotted against the different criterion values for identifying best cutoff values: CRP (a), Hb (b), and WBC (c). In the interactive dot diagrams (part of ROC curve analysis), the data of the nonrecurrent and recurrent group are displayed as dots on

two vertical axes. The horizontal line indicates the cutoff points with the best separation/highest accuracy (minimal false negative and false positive results) between nonrecurrent and recurrent group. The corresponding test characteristics sensitivity and specificity are shown at the right side of the display: CRP (d), Hb (e), and WBC (f). Kaplan–Meier survival curves for DFS stratified by measured cutoff points with highest accuracy of CRP (g), Hb (h), and WBC (i) show significant differences in univariate analysis

Hb levels, and WBCs). There were 123 men (65.8%) and 64 women (34.2%). Mean age was 62 years (95% CI for the mean 60.5–63.9 years). The most common primary tumor site was floor of the mouth (26.7%) followed by alveolar ridge (38.5%), tongue (19.8%), buccal mucosa (7.5%), lips (4.8%), and palate (2.7%). Mean tumor thickness was

11.8 mm (95% CI for the mean 9.3–14.2 mm). Distant metastases were present in 1.6% of OSCC patients ( $n = 3$ ). Average follow-up period was  $26.4 \pm 21.3$  months. On histopathologic evaluation, tumors were regarded as well differentiated (15.5%, G1), moderately differentiated (46.5%, G2), poorly differentiated (36.9%, G3), or



**Fig. 2** Receiver operating characteristic curves (ROC) for CRP, hemoglobin (Hb), and white blood cell count (WBC) in patients with OSCC. The true positive rates (sensitivity) are plotted in function of the false positive rate (100-specificity) for different cutoff points: ROC analysis for the diagnosis of recurrent group shows calculated cutoff values with highest diagnostic accuracy (arrows) of CRP (1), Hb (2), and WBC (3)

undifferentiated (2%, G4). Comparing the depth of invasion, 84 (44.9%) tumors were pT1, 38 (20.3%) tumors were pT2, 16 (8.6%) tumors were pT3, and 49 (26.2%) tumors were pT4. Regarding cervical lymph node assessment, 52 patients (27.8%) had nodal metastases confirmed by pathologic examinations. Lymphatic vessel invasion (L+) was positive

in 125 patients (66.8%), and microvascular invasion (V+) was observed in 22 cases (11.8%). With regard to pathologic tumor stage, 74 patients (39.6%) had stage I disease, 27 patients (14.4%) had stage II disease, 23 patients (12.3%) had stage III disease, and 63 patients (33.7%) had stage IV disease. Table 1 presents the results in detail.

Concerning the clinicopathological characteristics and prognostic factors that may influence survival status (Table 1), all patients were categorized into two groups based on whether they had locoregional recurrence or had no locoregional recurrence. Comparison of the clinicopathologic characteristics between the nonrecurrent group ( $n = 125$ ) and recurrent group ( $n = 62$ ) disclosed no significant difference in age ( $P = 0.6062$ ), gender ( $P = 0.9268$ ), site distribution ( $P = 0.9559$ ), and treatment modalities ( $P = 0.0782$ ). Statistically significant difference between the nonrecurrent group and recurrent group was observed in histological grading ( $P < 0.0001$ ), depth of invasion ( $P < 0.0001$ ), cervical lymph node metastasis ( $P = 0.0013$ ), lymphatic vessel invasion ( $P = 0.0028$ ), microvascular invasion ( $P = 0.005$ ), and tumor stage ( $P < 0.0001$ ). A comparison of variables between the two groups is presented in Table 1.

Evaluation of the prognostic impact of single laboratory tests (CRP/Hb/WBC) and calculated Pre-treatment laboratory prognostic index (LPI) for the screening of OSCC disease recurrence

Similar to a previous work, a prognostic index was calculated from elevated inflammatory CRP and WBC [32]. In addition, low Hb levels were integrated in the calculation due to the suggested inflammatory condition in cancer patients. Subsequently, patients were categorized into four

**Table 3** Comparison of calculated sensitivities, specificities, positive likelihood ratios, negative likelihood ratios, positive predictive values, negative predictive values between single laboratory tests (CRP/Hb/WBC), and calculated laboratory prognostic index ( $n = 187$ ) for the screening of OSCC disease recurrence

	CRP (mg/dl)	Hb (g/dl)	WBC (count/nl)	Laboratory prognostic index (LPI)
Cutoff value*	$\geq 1.1$	$\leq 12.7$	$\geq 9.6$	$\geq 2$
Sensitivity (95% CI)	61.29 (48.1–73.4)	66.13 (53.0–77.7)	40.32 (28.1–53.6)	30.65 (19.6–43.7)
Specificity (95% CI)	84.80 (77.3–90.6)	67.20 (58.2–75.3)	84.80 (77.3–90.6)	97.60 (93.1–99.5)
+PV % (95% CI)	66.7 (52.9–78.6)	50.0 (38.7–61.3)	56.8 (41.0–71.7)	86.4 (65.1–97.1)
–PV % (95% CI)	81.5 (73.8–87.8)	80.0 (71.1–87.2)	74.1 (66.1–81.1)	73.9 (66.5–80.4)
+LR (95% CI)	4.03 (3.3–5.0)	2.02 (1.6–2.5)	2.65 (1.9–3.6)	12.77 (8.8–18.6)
–LR (95% CI)	0.46 (0.3–0.8)	0.50 (0.3–0.8)	0.70 (0.4–1.1)	0.71 (0.2–2.2)
AUC (95% CI)	0.781 (0.715–0.838)	0.701 (0.630–0.766)	0.598 (0.524–0.669)	0.767 (0.700–0.826)
P value**	<0.0001	<0.0001	0.0335	<0.0001

\* Value with highest diagnostic accuracy, +LR positive likelihood ratio, –LR negative likelihood ratio, CI confidence interval, +PV positive predictive value, –PV negative predictive value, AUC area under the ROC curve, \*\* The P value is the probability that the observed sample area under the ROC curve is found when, in fact, the true (population) area under the ROC curve is 0.5 (null hypothesis: area = 0.5). If P is low ( $P < 0.05$ ), then it can be concluded that the area under the ROC curve is significantly different from 0.5 and that therefore there is evidence that the laboratory test does have an ability to distinguish between the two groups (nonrecurrent group vs. recurrent group)

**Table 4** Pre-treatment laboratory prognostic index (LPI) associated with clinicopathological characteristics and prognostic factors in patients ( $n = 187$ ) with OSCC

Characteristics	Number of patients Total $n = 187$	Pre-treatment laboratory prognostic index (LPI)				<i>P</i> value
		LPI 0 $n = 48$ (25.7%)	LPI 1 $n = 71$ (38%)	LPI 2 $n = 46$ (24.6%)	LPI 3 $n = 22$ (11.8%)	
Age (y)						0.7484 <sup>†</sup>
<60	81 (43.3%)	26 (32%)	24 (30%)	18 (22%)	13 (16%)	
≥60	106 (56.7%)	22 (21%)	47 (44%)	28 (27%)	9 (8%)	
Gender						0.8045 <sup>†</sup>
Male	123 (65.8%)	27 (22%)	50 (41%)	33 (27%)	13 (10%)	
Female	64 (34.2%)	21 (33%)	21 (33%)	13 (20%)	9 (14%)	
Site distribution of OSCC						0.9062 <sup>†</sup>
Lips	9 (4.8%)	1 (11%)	6 (67%)	1 (11%)	1 (11%)	
Tongue	37 (19.8%)	13 (35%)	15 (41%)	7 (19%)	2 (5%)	
Floor of the mouth	50 (26.7%)	16 (32%)	17 (34%)	12 (24%)	5 (10%)	
Palate	5 (2.7%)	3 (60%)	1 (20%)	1 (20%)	0 (0%)	
Buccal mucosa	14 (17.5%)	3 (21%)	7 (50%)	3 (21%)	1 (7%)	
Alveolar ridge	72 (38.5%)	12 (17%)	25 (35%)	22 (30%)	13 (18%)	
Histological grading*						0.0363 <sup>†</sup>
G1	29 (15.5%)	17 (59%)	8 (27%)	4 (14%)	0 (0%)	
G2	87 (46.5%)	24 (27%)	32 (37%)	19 (22%)	12 (14%)	
G3	69 (36.9%)	7 (10%)	30 (43%)	22 (32%)	20 (29%)	
G4	2 (1.1%)	0 (20%)	1 (50%)	1 (50%)	0 (0%)	
Depth of invasion**						0.0002 <sup>†</sup>
pT1	84 (44.9%)	37 (44%)	33 (39%)	11 (13%)	3 (4%)	
pT2	38 (20.3%)	6 (16%)	14 (37%)	11 (29%)	7 (18%)	
pT3	16 (8.6%)	1 (6%)	5 (31%)	8 (50%)	2 (13%)	
pT4	49 (26.2%)	4 (8%)	19 (39%)	16 (33%)	10 (20%)	
Cervical lymph node metastasis						0.2231 <sup>†</sup>
pN0	135 (72.2%)	43 (32%)	47 (35%)	32 (24%)	13 (10%)	
pN1-3	52 (27.8%)	5 (10%)	24 (46%)	14 (27%)	9 (17%)	
Lymphatic vessel invasion (L)						0.0509 <sup>†</sup>
Negative	62 (33.2%)	27 (43%)	19 (31%)	11 (18%)	5 (8%)	
Positive	125 (66.8%)	21 (17%)	52 (41%)	35 (28%)	17 (14%)	
Microvascular invasion (V)						0.2382 <sup>†</sup>
Negative	165 (88.2%)	46 (38%)	62 (41%)	37 (11%)	20 (37%)	
Positive	22 (11.8%)	2 (9%)	9 (41%)	9 (41%)	2 (9%)	
UICC stage***						0.0002 <sup>†</sup>
UICC I	74 (39.6%)	35 (48%)	27 (36%)	9 (12%)	3 (4%)	
UICC II	27 (14.4%)	5 (18%)	10 (37%)	8 (30%)	4 (15%)	
UICC III	23 (12.3%)	4 (17%)	8 (35%)	10 (44%)	1 (4%)	
UICC IV	63 (33.7%)	4 (6%)	26 (41%)	19 (31%)	14 (22%)	

y: years, G: grading, UICC: International Union against Cancer, RT/CT: radiotherapy with/without chemotherapy

\* G1/2 versus G3/4; \*\* pT1/2 versus pT3/4; \*\*\* UICC I/II versus UICC III/IV; † LPI 0/1 versus LPI 2/3

different groups (Table 2). ROC analysis determined cutoff values for CRP, Hb, and WBC in OSCC recurrent versus nonrecurrent group (Figs. 1a–f, 2). LPI was calculated from pre-treatment CRP levels, Hb levels, and WBCs due to significant associations with locoregional recurrence

(Fig. 1g–i; Table 1) measured for each parameter by ROC analysis (Fig. 2). LPI was calculated because of low diagnostic efficiency of each value (CRP, Hb, and WBCs) alone (Table 3). Comparing Laboratory Prognostic index, 48 (25.7%) patients had LPI 0, 71 (38%) patients had LPI



**Table 5** Univariate analysis of prognostic factors of patients ( $n = 187$ ) with OSCC

Variable	Unfavorable factor	Hazard ratio (HR)	95% CI of HR	<i>P</i> value
LN (N)	Positive	2.2754	1.2836–4.0333	0.0008
Depth of invasion (pT)	pT3/4	4.3023	2.4754–7.4777	<0.0001
Grading (G)	High (G3/4)	2.8265	1.6639–4.8014	<0.0001
Microvascular invasion (V)	Positive	3.8538	1.5105–9.8325	<0.0001
Lymphatic vessel invasion (L)	Positive	2.4682	1.4633–4.1631	0.0045
Laboratory prognostic index (LPI)	High (LPI 2/3)	3.8670	2.2518–6.6407	<0.0001

LN Lymph nodes metastasis

1, 46 (24.5%) patients had LPI 2, and 22 (11.8%) patients had LPI 3. Table 1 presents the results in detail. Statistically significant difference between the nonrecurrent group and recurrent group was observed for LPI ( $P < 0.0001$ ).

The cutoff points of CRP level, Hb level, WBC count, as well as LPI that gave the best sensitivity and specificity for the diagnosis of disease recurrent group were evaluated using area AUC analysis (Fig. 2). Additionally, values were graphical displayed in a plot versus criterion value diagram and in an interactive dot diagram to show and to control the highest diagnostic accuracy of each diagnostic test (Fig. 1a–f). A comparison of variables, calculated sensitivities, and specificities between the parameters is presented in Table 3. The LRs and PVs of CRP (calculated cutoff value of 1.1 mg/dl) were +LR 4.03, –LR 0.46 and +PV 66.7%, –PV 81.5%, respectively. AUC was 0.781 ( $P < 0.0001$ , Table 3). Analysis of Hb (calculated cutoff value of 12.7 g/dl) showed +LR 2.02, –LR 0.50 and +PV 50.8%, –PV 80.0%, respectively. AUC was 0.701 ( $P < 0.0001$ , Table 3). Analysis of WBC (calculated cutoff value of 9.6 count/nl) showed +LR 2.65, –LR 0.70 and +PV 56.8%, –PV 74.1%, respectively. AUC was 0.598 ( $P = 0.0335$ , Table 3). Finally, LRs and PVs of LPI (calculated cutoff value of LPI 2) were identified as +LR 12.77, –LR 0.71 and +PV 86.4%, –PV 73.9%, respectively. AUC was 0.767 ( $P < 0.0001$ , Table 3).

Table 4 shows that LPI is associated with clinicopathological characteristics and prognostic factors in patients with OSCC. Statistically significant association between LPI and clinicopathological characteristics was observed in histological grading ( $P = 0.0363$ , Table 4), depth of invasion ( $P = 0.0002$ , Table 4), and tumor stage ( $P = 0.0002$ , Table 4).

#### Univariate analysis

Based on Kaplan–Meier survival analysis, the disease-free 1-, 3-, and 5-year survival rates in all surgically treated patients were 89, 76, and 65%, respectively. Kaplan–Meier survival curves for CRP, Hb, and WBCs are shown in Fig. 1g–i. Log-rank test analysis of Kaplan–Meier survival curves identified CRP ( $P < 0.0001$ , Fig. 1g), Hb

( $P < 0.0001$ , Fig. 1h), and WBCs ( $P < 0.0001$ , Fig. 1i), histological grading ( $P < 0.0001$ , Table 5), depth of invasion ( $P < 0.0001$ , Table 5), cervical lymph node metastasis ( $P < 0.0008$ , Table 5), lymphatic vessel invasion ( $P < 0.0045$ , Table 5), microvascular invasion ( $P < 0.0001$ , Table 5), and high LPI (LPI  $\geq 2$ ,  $P < 0.0001$ , Table 5; Fig. 3a) as significantly affecting survival. In addition, there was a significant difference in the 5-year DFS rate regarding these clinicopathologic characteristics and prognostic factors (Table 1). Conversely, there was no significant difference in the 5-year DFS rate in age ( $P = 0.1051$ , Table 1) and gender ( $P = 0.9160$ , Table 1).

#### Multivariate analysis

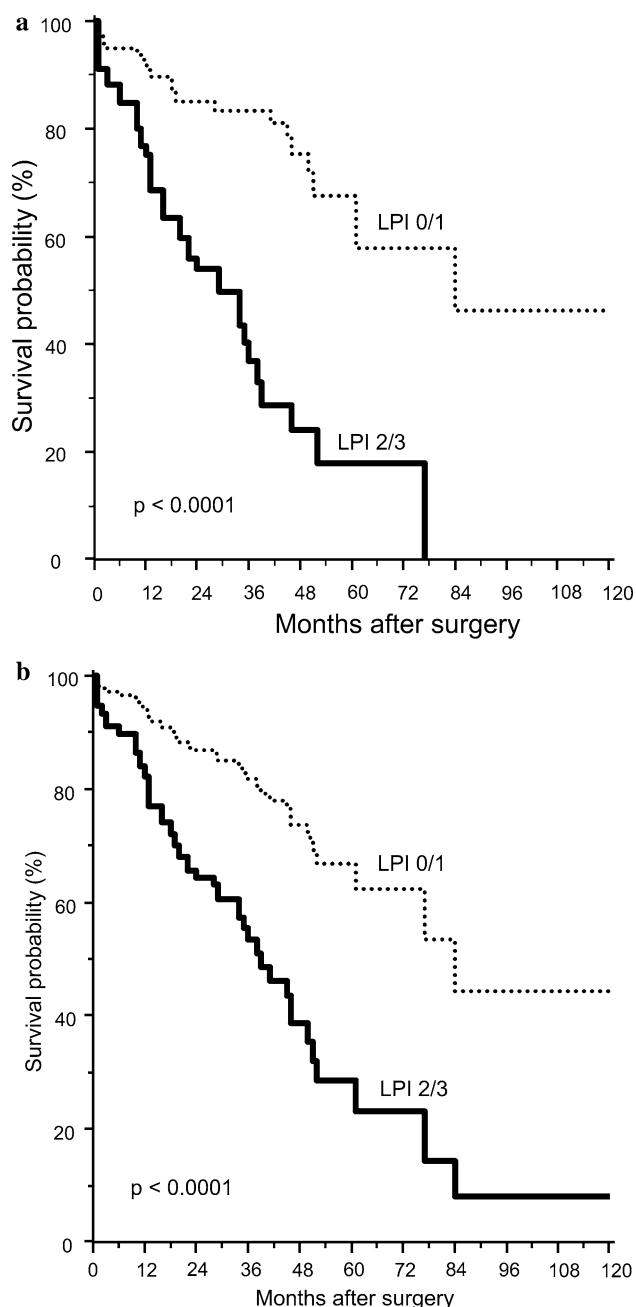
Multivariate analysis using the Cox proportional hazards model demonstrate depth of invasion (pT3/4,  $P = 0.0249$ , Table 6), microvascular invasion (V+,  $P = 0.0053$ , Table 6), and high LPI (LPI  $\geq 2$ ,  $P = 0.0001$ , Table 6; Fig. 3b) as independent prognostic factors in all ( $n = 187$ ) OSCCs. Cervical lymph node metastasis (LN+,  $P = 0.2289$ , Table 6), histological grading (G3/4,  $P = 0.3543$ , Table 6), and lymphatic vessel invasion (L+,  $P = 0.4918$ , Table 6) were not found to be independent prognostic factors.

#### Flow cytometric analysis and immunocytochemistry of CRP expression by SCC-4 cells

FACS analysis and immunocytochemistry were performed to show expression of inflammatory CRP by cancer cells. CRP expression of the cancer cell line SCC-4 measured by FACS analysis (Fig. 4a, b) was confirmed by immunocytochemistry (Fig. 4c, d).

#### Discussion

In this hospital-based study conducted in Middle Franconia/Germany, 187 patients with oral cavity squamous cell carcinoma treated at a single institution by primary surgical



**Fig. 3** Kaplan–Meier (a) and Cox-regression (b) survival curves for disease-free survival (DFS) stratified by low (LPI 0/1) and high (LPI 2/3) pre-treatment laboratory prognostic index (LPI). DFS 1-, 3-, and 5-year survival rates (LPI 0/1 vs. LPI 2/3) in univariate Kaplan–Meier survival curve (a) are 95% versus 77%, 83% versus 40%, and 67% versus 18%, respectively. DFS 1-, 3-, and 5-year survival rates (LPI 0/1 versus LPI 2/3) in multivariate Cox-regression (b) survival curve were 94% versus 84%, 83% versus 55%, and 67% versus 29%, respectively

resection and factors affecting survival were analyzed. This is the first study focusing on the development of a pre-treatment laboratory prognostic index (LPI) based on laboratory results that might serve as an extension to

clinicopathological parameters (CP) for prognosis and treatment in a representative patient cohort with oral squamous cell carcinoma (OSCC).

Although Khandavalli [10] has analyzed elevated CRP levels as an independent risk factor for OSCC, others do not support a positive association between pre-operative CRP levels and prognosis of OSCC [11]. Several possible mechanisms have been proposed for the relationship between CRP and cancer. First, tumor growth can cause tissue inflammation and hence increase CRP levels [33, 34]. Second, CRP could be an indicator of an immune response to tumor antigens [35]. Third, there is evidence that cancer cells can increase the production of inflammatory proteins, which could explain the high CRP concentrations in patients with cancer [35]. Some cancerous cells have been shown to express CRP [36, 37], and cancer cell lines have been shown to secrete Interleukin-6 (IL-6) and IL-8, which in turn induce the production of CRP [38, 39]. These mechanisms imply that increased CRP is a response to the neoplastic process and that CRP concentrations could thus provide a marker for identifying people with cancer at an early stage when treatment might be more effective. Our data support this suggestion although the cutoff value has been determined at a low level.

Similarly, anemia is associated with poor cancer control [20, 40]. Pre-treatment Hb values of <14.5 g/dl have already been identified with decreased local tumor control rate [41, 42]. Our data are well in line with these previous results. In anemic patients, hypoxia is more pronounced [43]. Tumor hypoxia, mostly resulting from poor perfusion and anemia, is one of the key factors in inducing the development of cell clones with an aggressive and treatment-resistant phenotype that leads to rapid progression and poor prognosis. Hypoxic conditions lead to the elaboration of proangiogenic and growth-stimulating proteins and provide some measure of therapeutic resistance [42].

The effect of tumor development on circulating leukocyte number has not been clarified for OSCC. Elevated WBC count was found to be an independent prognostic factor, associated with reduced survival in human metastatic melanoma, pancreatic carcinoma, and renal carcinoma [21–24, 44–46]. Our results support these findings of other cancer entities. The infiltrating inflammatory cells within the tumor are associated with an increased circulating leukocyte number (WBC count) and have pleiotropic functions: inhibition of tumor growth by nonspecific cytotoxic mechanisms and induction of cell lysis, or conversely, promotion of tumor development through the release of inflammatory mediators [47]. Therefore, measurement of CRP level, Hb level, and WBC count is useful and cheap baseline inflammation markers regarding evaluation of prognosis in OSCC. Moreover, a cutoff value for

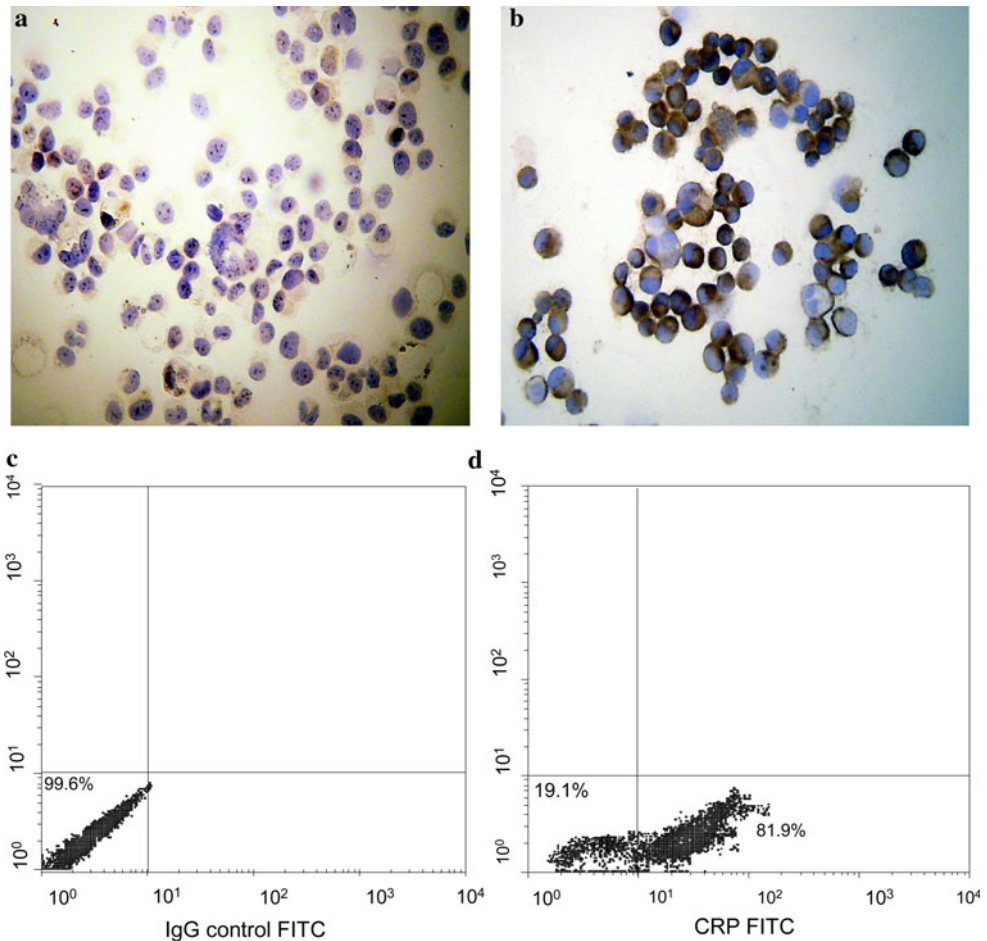
**Table 6** Independent prognostic factors identified by multivariate Cox proportional hazard model in patients ( $n = 187$ ) with OSCC

Variable	Unfavorable factor	Hazard ratio (HR)	95% CI of HR	<i>P</i> value
LN (N)	Positive	1.3989	0.8119–2.4103	0.2289
Depth of invasion (pT)	pT3/4	2.0582	1.0991–3.8542	0.0249
Grading (G)	High (G3/4)	1.3405	0.7233–2.4841	0.3543
Microvascular invasion (V)	Positive	2.5752	1.3283–4.9925	0.0053
Lymphatic vessel invasion (L)	Positive	1.2824	0.6333–2.5970	0.4918
Laboratory prognostic index (LPI)	High (LPI 2/3)	3.1028	1.7580–5.4766	0.0001

LN Lymph nodes metastasis

**Fig. 4** Immunocytochemistry staining and FACS analysis of CRP expression from the SCC-4 cancer cell line.

Immunocytochemistry IgG control (a). CRP staining (cytoplasmic staining pattern, brown) in cytopins serve as positive control of CRP expression by cancer cells and shows 80–90% positive cells. Original magnification:  $\times 200$ -fold. FACS IgG control (c). FACS analysis confirms immunocytochemistry CRP expression (d) by SCC-4 cancer cells



the association with locoregional recurrence measured for each parameter by receiver operating characteristic (ROC) curves has not been determined as yet. Indeed, single parameters were associated with significant reduced survival in univariate analysis. However, each value alone (CRP level, Hb level, and WBC count) failed to be relevant in predicting locoregional disease recurrence. Therefore, we measured a combination of these pre-treatment laboratory parameters (LPI). Multivariate analysis showed that the most important independent prognostic factor was high pre-treatment LPI ( $LPI \geq 2$ ). Moreover, high pre-treatment showed high probability that locoregional recurrence will

be present later. High +LR gave an excellent indication for a good quality of the test.

In the present study, we focused on the putative role of inflammatory parameters in the pathogenesis of OSCC. Others stated that the amount of extrahepatic synthesis of CRP in neurons, monocytes, lymphocytes, and tumor cells as a local inflammatory response is too little to influence serum CRP [37, 48]. However, our results of CRP expression by cancer cells measured by immunocytochemistry and FACS analysis might be in accordance with a ‘smouldering’ inflammation in OSCC suggested by the low CRP cutoff value.

Incorporated into a LPI, CRP levels, Hb levels, and WBC count identified 4 groups that were summarized into 2 practical subgroups (LPI 0/1 ‘low’ and LPI 2/3 ‘high’) of OSCC patients with distinct risks of disease progression and death. This information might be pertinent to treatment decision-making and might also influence a revised staging system. If the objective is prognostic stratification, then using anatomic features alone for staging is no longer sufficient.

## Conclusions

In conclusion, this is the first study that has analyzed a pre-treatment LPI by measuring a cutoff value for laboratory results by ROC analysis in patients with OSCC. It highlights the combination of inflammatory CRP, low Hb levels, and elevated WBCs as the most important independent prognostic factor in predicting disease recurrence of patients with OSCC. LPI can be used as a pre-treatment inflammatory biomarker that may identify recurrent group with a more aggressive biological phenotype of the disease. LPI might be helpful for guiding further post-operative treatment in OSCC. Therefore, high LPI is a new developed prognostic feature for OSCC patients with subsequent therapeutic implications.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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