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*research article*

# Influence of surgical treatment and radiotherapy of the advanced intraoral cancers on complete blood count, body mass index, liver enzymes and leukocyte CD64 expression

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***Background.*** *The aim of our study was to evaluate the influence of the surgery and radiotherapy of the advanced oral squamous cell carcinoma on the complete blood count, body mass index (BMI), acute inflammatory response, liver enzymes and expression of the CD64 index on leukocytes in the peripheral blood.*

***Patients and method.*** *Venous blood was obtained from 16 patients with advanced oral squamous cell carcinomas treated with radical surgery and external beam radiotherapy. Blood samples were collected prior to surgery (T1), after surgery (T2) and after radiotherapy (T3). Blood samples were analyzed for whole blood count, immunoglobulin G levels, liver enzymes (transaminases (ALT and AST) and gamma-glutamyl trasferase (γ-GT)), inflammatory response markers (C-reactive protein, erythrocyte sedimentation rate, albumin, white blood count, leukocyte count and CD64 expression on leukocytes). Assessment of nutrition was done by calculating the body mass index.*

***Results.*** *Surgery caused anaemia, trombocytosis, leukocytosis, lymphopenia, rise in acute phase proteins, elevation of CD64 expression on monocytes and neutrophyls, elevation of liver transaminases and lowering of γ-GT, albumin, protein and bilirubin levels. After radiotherapy haemoglobin, leukocytes, C-reactive protein, erythrocyte sedimentation rate, liver transferases, albumin, bilirubin and proteins returned almost to T1 levels, levels of lymphocytes, γ-GT and body mass index lowered. IgG levels remained almost unchanged at T2 and T3. Levels of the CD64 expression on monocytes and neutrophyls also elevated after radiotherapy.*

***Conclusions.*** *Surgery caused a significantly larger acute phase response than radiotherapy, while radiotherapy worsened the already present lymphopenia.*

*Key words: intraoral cancer; surgery; radiotherapy; blood parameters*

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## Introduction

Oral squamous cell carcinoma (OSCC) is the sixth most common neoplasm in the European Union.[1] Advanced OSCC is treated with surgery and then radiotherapy (RT) and/or chemotherapy.[2] Five year survival rates for patients with advanced OSCC are below 55%[3], so researchers are still searching for better treatment modalities. The single most important factor influencing the outcome of patients with squamous cell carcinomas (SCC) of the upper aerodigestive tract is like at the other malignancies the stage of the disease at the time of initial diagnosis and treatment.[4-6] Once the tumour is no longer localized but has disseminated to regional lymph nodes, the probability of 5 year survival reduces to nearly half.[7-9] The patient's long-time prognosis is also worsened by the fact that the new primary SCC of upper aerodigestive tract occurred in 20% of these patients.[10]

Surgical trauma produces alterations in the hemodynamic, metabolic and immune responses of patients in the postoperative period. This injury response is a dynamic process that follows a specific pattern that has been defined based on clinical and scientific observations.[11] The initial proinflammatory immune response or systemic inflammatory response syndrome (SIRS) is mediated primarily by the cells of innate immune system. This is followed by a compensatory anti-inflammatory or immunosuppressive phenotype that is mediated primarily by cells of the adaptive immune system, which predisposes the host to septic complications[9] and may be also promotes the tumour growth and metastases.[13,14] Cell mediated immunity is suppressed for several days after surgery and more invasive procedures lead to deeper and longer immunosuppression.[14,15]

The role of RT for OSCC treatment is well established; modern equipment and techniques have minimized morbidity.[16] Toxicity is related to site and dose; acute toxicity is related to the inflammatory process induced within the radiation field. These effects occur to some degree in the majority of patients, but they are self-limited in duration. The presence of late toxicity is determined by the total dose of radiation given and not by the daily dose or fraction size.[17-19] Although lymphocytes are highly sensitive to radiation damage[19,20] this toxicity is not usually assessed.

The aim of our study was to evaluate the effect of major surgical procedures and RT of advanced OSCC on the complete blood count, body mass index (BMI), acute inflammatory response, liver enzymes and expression of the CD64 index on leukocytes in the peripheral blood by the prospective non-randomized study.

## Patients and methods

Our prospective non-randomized study was running from 2007 to 2009 on the Clinical Department for Oral and Maxillofacial Surgery, University Clinical Center in Ljubljana. Sixteen patients with advanced OSCC were selected. The study group included 12 men and 4 women with median age 61 years (range 42-80 years). All were Stage III and IV according to the American Joint Committee on Cancer staging.[21] Inclusion criteria were that surgery and radiotherapy were the only treatment modalities. The local Ethics Committee of the Republic of Slovenia ensured that research protocol and appropriate written consent was obtained from each patient.

Blood samples T1 were collected between 1 to 17 days before surgery (mean ± SD; 8.9 ± 5.9). All patients were surgically treated with *en bloc* excision of tumour and modified neck dissection (five of them with bilateral) and subsequent re-

construction with flaps (9 with free flaps: 6 radial forearm, 1 iliac crest, 1 anterior lateral thigh, 1 fibula; 3 with pedicled flaps: 2 pectoralis major muscle, 1 temporalis muscle). Median blood loss during operation was 430 ml (range 200-1000 ml) which was assessed by the anaesthesiologist and surgeon. In all but one patient, temporary tracheotomy was performed at the time of operation. All patients had confirmed clear margins with frozen sections at the time of operation. In one patient, revision of the operative field was required immediately after operation because of haemorrhage. All free flaps were viable and functioning. The tracheotomy tube was removed at median day 6 (range 3-15). All patients were fed by nasogastric tubes (NGTs) for the median time of 9 days (range 5-15). Six patients received postoperative transfusion of concentrated erythrocytes for the correction of haemoglobin levels bellow 90 mg/l. All patients were treated with antibiotics for the median time of 8 days (range 5-10). Blood samples T2 were taken between 8 and 26 days after surgery (mean ± SD: 15.4 ± 4.4).

Patients were irradiated with an external beam on the 6 MV linear accelerator. They received between 58 and 66 Gy (mean ± SD: 60.5 ± 1.9), divided on 2 Gy daily fractions, five times a week. This RT was applied within 6 weeks after the surgery. No patient received hyperfractionated RT or chemotherapy. Blood samples T3 have been collected from 28 days up to 128 days post-RT (mean ± SD: 56.6 ± 36.9).

At the time of blood sampling all patients were weighted and their height was measured at T1. These measures were used to calculate body mass index (BMI, kg/m$^2$).

### *Blood sampling*

The blood samples were taken from the cubital vein. 1 EDTA-containing test tube (5 ml) of peripheral blood was obtained for haematological, biochemical and cytometrical laboratory tests.

### *Expression of CD64 on neutrophils and monocytes*

Expression of CD64 on neutrophils, monocytes and lymphocytes was measured by quantitative flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson, NY, USA) and FACSCanto flow cytometer (Becton Dickinson, CD, USA) using the Leuko64TM assay (Trillium Diagnostics, LLC, Maine, USA). The assay is for research use only and is composed of three antibodies with specificities to CD64 (clones 22 and 32.2, both fluorescein isothiocyanate (FITC) conjugated) and a fluorescence bead suspension with three fluorescence signals (green fluorescence due to FITC, orange fluorescence similar to PE and red fluorescence of starfire red) for unique identification of beads, and used for instrument calibration and standardization of leukocyte CD64 expression in human blood. The sample preparation and flow cytometer setup were based on the manufacturer's instructions. Briefly, 50 μL of whole blood, or diluted whole blood to adjust leukocyte concentration to less than 25 x109 /L, was incubated for 15 minutes in the dark at room temperature with a mixture of murine monoclonal antibodies followed by red cell lyses with an ammonium-chloride-based solution (Trillium Lyse). Fluorescence beads were then added and flow cytometer analysis was performed on a minimum of 50,000 leukocytes. Data analysis for fluorescence intensity was performed by CellQuest software (Becton Dickinson, CA, USA). MFI was measured as a linearized value of log scale on monocytes (green, positive control, measuring CD64 expression), neutrophils (blue, measuring CD64 expression), and beads (aqua blue, measuring FITC and PE expres-

**Table 1.** Median values; SD of leukocytes, platelets, neutrophils and lymphocytes (10*9/L), erythrocytes (10*12/L) and Hb levels (g/L) in observed times (before surgery (T1) and after surgery (T2); after RT – T3) and their normal values. The significant differences ($p<0.05$) according to the preceding value are marked by *

| Blood | T1 | T2 | T3 | Normal values |
|---|---|---|---|---|
| Leu (10*9/L) | 8.37 (3.7) | 9.06 (2.9) | 7.16 (2.3)* | 4.0-10.0 |
| Erci (10*12/L) | 4.16 (0.7) | 3.73 (0.5)* | 4.33 (0.4)* | 4.20-6.30 |
| Hb (g/L) | 138.38 (14,2) | 119.06 (10.9)* | 134.31 (13.8)* | 120-180 |
| Pt (10*9/L) | 248 (94) | 466 (180)* | 279 (79)* | 140-340 |
| Neutr (10/9/L) | 5.1 (2.7 ) | 6.2 (2.5)* | 5.1 (2.0)* | 1.6-7.5 |
| Lym (10*9/L) | 2.10 (0.8) | 1.96 (0.7) | 1.13 (0.6)* | 1.4-3.3 |

Legend: Leu - leukocytes; Erci - erythrocytes; Hb - haemoglobin; Pt - platelets; Neutr - neutrophils; Lym - lymphocytes

sion). Index calculation was performed by Leuko64 QuantiCalc software (Trillium Diagnostics, Main, USA). Index measurements were derived by the ratio of linearized MFI of the cell population to the FITC signal from the beads. An internal negative control of the assay was provided by the automated measurement of the lymphocyte CD64 index, which had to be less than 1.0, and an internal positive control of the assay was provided by automated measurement of the monocyte CD64 index, which had to be more than 3.0. Flow cytometry was performed up to 36 hours after blood sampling. Before the beginning of the study, the influence of delayed sample analysis was done and no significant difference in levels of CD64 expression was detected in the first 36 hours after blood sampling. Isotype-control antibodies were routinely used in each experiment to detect non-specific staining; however the calculation of CD64 MFI was done without subtracting isotype-control MFI in order to accurately compare the ratio (index) of linearized MFI to MFI alone.

### *Statistics*

Data were presented as the median and 95% confidence interval of the mean. Comparison between groups was made using the unpaired Mann-Whitney test and analysis of variance (ANOVA). Proportions of patients

**Table 2.** Median values, SD of BMI (kg/m2), levels of proteins, albumin, IgG (g/L), bilirubin (μmol/L), ALT, AST and γ-GT (μkat/L) in observed times. The significant differences ($p<0.05$) according to the preceding value are marked by *

| Nutrition&Liver | T1 | T2 | T3 | Normal values |
|---|---|---|---|---|
| BMI (kg/m2) | 23.03 (4.8) | 22.18 (4.8)* | 21.11 (4.6)* | 19-25 |
| Proteins (g/L) | 73.56 (5.5) | 71.94 (4.4) | 75.88 (4.6)* | 65-80 |
| Albumin (g/L) | 41.94 (4.8) | 38.81 (3.2)* | 42.06 (4.9)* | 32-55 |
| IgG (g/L) | 12.46 (3.7) | 12.15 (2.3) | 12.78 (2.6) | 6.90 – 14.00 |
| Bilirubin (μmol/L) | 13.19 (7.1) | 6.75 (2.7)* | 10.19 (5.2)* | <17 |
| AST (μkat/L) | 0.53 (0.3) | 0.58 (0.4) | 0.43 (0.3) | <0.58 |
| ALT (μkat/L) | 0.35 (0.1) | 0.69 (0.5)* | 0.42 (0.3)* | <0.74 |
| γ -GT (μkat/L) | 164 (2.7) | 1.34 (1.0) | 0.52 (0.3)* | <0.92 |

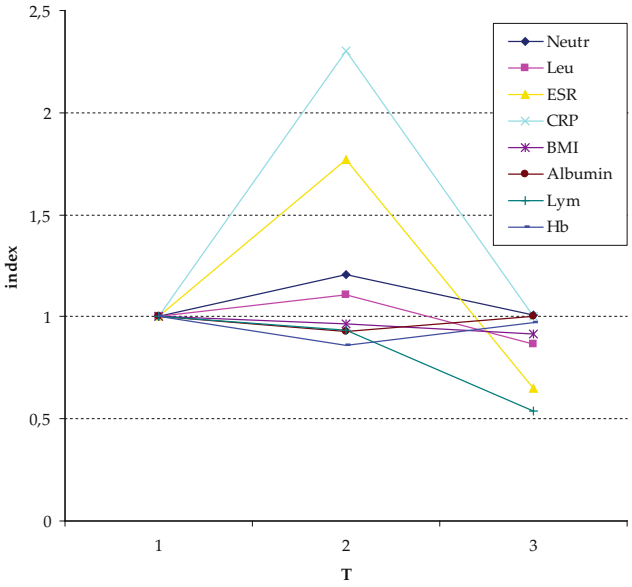


**Figure 1.** The average values of neutrophils, leukocytes, ESR, CRP, BMI, albumin, lymphocytes and haemoglobin are indexed on T1 value. Only the T2 values of leukocytes and lymphocytes did not reach the statistically significant level ($p<0.05$).

were compared by the $\chi 2$ test. The differences were considered to be statistically significant at the level of $p<0.05$. The statistical analysis was performed using Statistical Package for the Social Sciences for Windows, version 12.0 (SPSS Inc., Chicago, USA).

## Results

Blood cell counts and Hb results are presented in Table 1 at T1, T2 and T3 with the normal values of these parameters in our referential laboratory.

Values of markers of nutrition and liver enzymes at sampling times are presented in Table 2 with the normal values of these parameters in our referential laboratory.

Results of values at T1, T2 and T3 of the acute phase proteins together with neutrophils, leukocytes and indexes of CD64 expression on the monocytes and neutrophils are presented in Table 3.

The advanced surgical procedures of OSSC had caused anemia, thrombocytosis, leukocytosis, lymphopenia, rise in acute phase proteins (erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)), elevation of CD64 expression on monocytes and neutrophils, elevation of liver transaminases and lowering of γ-GT, albumin, protein and bilirubin levels. After RT, haemoglobin, leukocytes, ESR, CRP, liver transferases, albumin, bilirubin and proteins returned almost to T1 levels; levels of lymphocytes, γ-GT and BMI decreased (Figure 1). IgG levels remained almost unchanged at T2 and T3. Levels of the CD64 expression on monocytes and neutrophils also elevated after RT.

## Discussion

The purpose of our study was to establish the systemic influences of the surgical treatment and RT for the OSSC. Our results showed that advanced surgical procedures of OSSC caused a significantly larger acute phase response than radiotherapy, while radiotherapy worsened the already present lymphopenia. IgG levels remained almost unchanged at T2 and T3. Levels of the CD64 expression on monocytes and neutrophils also elevated after RT.

The postsurgical anaemia observed in our patients has been expected. During major surgery on the head and neck substantial blood loss may occur, varying between mean values of 500-1500 ml.[22,23] This results in a lower haemoglobin level after surgery and thus before radiotherapy, in particular when the haemoglobin level is

**Table 3.** Median values and SD of the markers of inflammatory response (leukocytes, neutrophils, ESR, CRP, indexes of CD64 on monocytes and neutrophils) at T1, T2 and T3. The significant differences ($p<0.05$) according to the preceding value are marked by *

| Inflammation | T1 | T2 | T3 | Normal values |
|---|---|---|---|---|
| Leu (10*9/L) | 8.37 (3.7) | 9.06 (2.9) | 77.16 (2.3)* | 4.0-10.0 |
| Neutr (10/9/lL) | 5.1 (2.7) | 6.2 (2.5)* | 5.1 (2.0)* | 1.6-7.5 |
| ESR (mm/h) | 33 (24) | 58 (21)* | 37 (18)* | <15 |
| CRP (mg/L) | 5 (6) | 11.5 (18)* | 5 (52)* | <5 |
| iCD64 mono. | 6.67 (1.0) | 7.92 (1.9)* | 8.67 (5.7)* | 4.34-8.70 |
| iCD64 neutr. | 0.64 (0.1) | 0.75 (0.2)* | 0.95 (0.6)* | 0.45-2.16 |

Legend: Leu - leukocytes; Neutr - neutrophils; ESR - erythrocyte sedimentation rate; CRP - C-reactive protein; iCD64 mono-index of CD64 expression on monocytes; iCD64 neutr - index of CD64 expression on neutrophils

kept relatively low to prevent thrombosis in a microvascular free flap reconstruction.[24] Anaemia is considered to be associated with hypoxia[21] resulting in worse clinical outcome as tumour hypoxia decreases the efficacy of radiation therapy as a result of decreased radiosensitivity[26-28] and because hypoxia itself may induce genetic alterations that are associated with worse outcome.[29] The blood loss in our study was low but still moderate anaemia developed. The cut off point of haemoglobin for transfusion used in our department is 90 g/l and 6 of patients were therefore given blood transfusion postoperatively. Blood transfusion is known to have some immunomodulatory effect[30] that might predict outcome.[31,32] Studies regarding anaemia prior to RT have sown that anaemic patients have lower locoregional control and lower overall survival.[27,33-35] Proposed levels of Hb prior to RT is 120 g/l[36], which was also the level of haemoglobin in our study after surgery.

After surgery there was a statistically significant rise in the number of platelets that returned within normal limits after RT in our results. This reactive thrombocytosis is known to be triggered by tissue trauma and major surgical procedures.[37] In a recent study Lu *et al.* showed that preoperative thrombocytosis was an independent prognostic factor of shorter survival in OSCC.[38] In our study none of them had preoperative thrombocytosis.

Leukocytes and neutrophil levels at all observed times were within normal values, although neutrophil levels elevated postsurgically also indicating inflammatory response. IgG levels did not change during treatment and were within normal limits; it was reported that IgG levels are normal in OSCC[39] and treatment modalities do not affect IgG levels.[40]

Major surgery is also a cause of transient lymphocyte decline[41,42], that returns to normal levels 5-8 days after surgery.[43] Since all blood tests were taken 8 days after surgery, we did not observe any statistically significant changes in lymphocyte decline after surgery. A study done by Kuss *et al.* has shown that lymphopenia persisted for more than 2 years after surgical removal of the tumour (squamous cell carcinoma of the head and neck)[44], which is not consistent with our findings.

Irradiation of the areas with abandoned bone marrow causes severe lymphopenia.[45,46] But even irradiation of the limited anatomical areas such as neck can cause lymphopenia lasting over 2 years, especially regarding some subpopulations of T lymphocytes.[44,47,48] Also in our study, RT almost halved the levels of lymphocytes. With RT, vertebrae are mostly spared from irradiation; also, the thymus is not in the radiation field and most of the neck nodes are removed at the time of operation. Possible mechanisms to explain lymphopenia are damage to lymphocytes in large vessels, and lymphatics in the radiation field at the time of irradiation. Also in older patients, there

is a limited thymopoesis even more potentiated with liver insufficiency and poor nutrition.[48] A longer period of observation will be required to compare our results with the studies that have shown a long lasting decline of lymphocytes after RT.[48]

Acute phase proteins are a family of inflammatory proteins synthesized by the liver whose levels change in response to injury, infection and neoplasia. ESR has been routinely used in the diagnosis of infections for decades. CRP levels rise approximately 4 to 12 hours after surgery and peak at 24 to 72 hours. Subsequently, CRP levels remain elevated for nearly 2 weeks.[49] All forms of significant tissue damage triggers acute phase response as well as different types of cancer, especially, when they are extensive and metastastatic.[50] High levels of ESR have also been found to be evidence of cancer progression in different malignancies and were correlated with poor prognosis.[51] Studies reported the prognostic value of the preoperative CRP in oesophageal, gastric, ovarian and colorectal carcinomas.[52-54] Recently a study from Khandavilli *et al.* reported that increased preoperative CRP was associated with worse overall survival in patients with OSCC.[55] In our study mean CRP levels at T1 were at the upper border of the normal range and in only three patients it was higher. By expanding the study and by enrolling more patients it would be interesting to compare patients with high CRP levels to their chances of overall survival. Results of levels of ESR and CRP showed that surgery caused a significant increase in the levels of ESR and CRP, and that at T3, levels returned almost to T1 levels. ESR levels were elevated even before surgery which is consistent with findings that acute and subacute changes of the ESR are most commonly due to malignancy, infection or inflammatory process.[56,57] Tang *et al.* also found no increase of CRP after RT in patients with cervical carcinoma[58] while some studies reported on considerably elevated levels after RT in patients with head and neck carcinomas and cervical cancer.[59,60] One of the reasons why levels of CRP and ESR levels returned to T1 levels after RT in our study is that blood samples at T3 were taken from 28 to 128 days after RT and in the above mentioned studies immediately after RT.

CD64 is a high-affinity and restricted isotype-specificity FcγRI receptor expressed on macrophages, monocytes, neutrophils, and eosinophils.[61] During bacterial infections, however, the neutrophil expression of CD64 is markedly increased.[63-65] Studies have confirmed that the level of CD64 expression is significantly higher in patients with infectious SIRS as compared to patients with noninfectious SIRS as well as adults and children.[66,67] All the indexes in our group were within the normal range, although surgery is known to elevate the levels of CD64 expression on monocytes and neutrophils.[68,69] The reason for this is due to the fast kinetics of these indexes making them useful for detecting bacterial infections and monitoring response to treatment. Levels declined 3 days after surgery[69] and all of our T2 blood samples were taken after day 3. However, there was a steady incline in indexes, which might be due to the increased microorganism invasion, most probably from the gut mucosa.

BMI in our patients steadily dropped throughout the course of the treatment (Table 2, Figure 1). Nieto *et al.* showed in their study that BMI lower than 22 for smokers and drinkers significantly increases odds ratio for developing oral cancer.[70] Since alcohol and tobacco abuse are the two most important etiological factors for OSSC[71], only 2 of our patients were nonsmokers and all admitted to drinking modestly. We decided to check BMI regularly in OSCC patients because of the known fact that cachexia-anorexia syndrome occurs in

30 to 80% of cancer patients.[72] Although BMI did not reach the malnutrition level it still points to the need of an even better nutritional support of these patients.

## Conclusions

Because of the low survival rate of patients with OSCC, we decided to evaluate many parameters of these patients in a prospective study. We studied the influence of surgery and radiotherapy on our patients. The major surgical procedure had caused anaemia, thrombocytosis, leukocytosis, lymphopenia, rise in acute phase proteins, elevation of CD64 expression on monocytes and neutrophils, elevation of liver transaminases and lowering of γ-GT, albumin, protein and bilirubin levels. After radiotherapy, haemoglobin, leukocytes, C-reactive protein, erythrocyte sedimentation rate, liver transferases, albumin, bilirubin and protein levels returned almost to T1 levels; levels of lymphocytes, γ-GT and BMI decreased. IgG levels remained almost unchanged during the therapies. Surgery caused a significantly larger acute phase response than RT, while RT worsened the already present lymphopenia. Levels of CD64 expression on monocytes and neutrophils increased after RT.

The major influences of surgical procedures were transient and reactive, while those after RT caused a more profound and long lasting effects. The results obtained from our study are the starting points for further investigations of OSCC patients.

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