# Serum IL10, IL12 and circulating CD4+CD25high T regulatory cells in relation to long-term clinical outcome in head and neck squamous cell carcinoma patients

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**Abstract.** IL10, but not IL12 or T regulatory cells in the circulation of newly presenting, pre-treatment head and neck squamous cell carcinoma (HNSCC) patients, has been shown previously to be related to survival over a mean follow-up period of 15 months. Here, we followed the same patients for a longer period to determine whether these associations change. Pre- and post-treatment serum IL10/IL12 and circulating T regs were measured using ELISA and flow cytometry respectively and were correlated with survival after a 33 month average follow-up in a cohort of newly presenting HNSCC patients (n=107), with cancers of the hypopharynx (n=16), larynx (n=36), oral cavity (n=21), oropharynx (n=25), sinonasal (n=4) or unknown origin (n=5). Although the mean survival time of patients with detectable levels of IL10 pre-treatment was lower (40.6 months) than that of those without detectable levels of IL10 (45.6 months), the difference was no longer significant, in contrast to earlier follow-up data. In conclusion, although serum levels of IL10 may be a prognostic indicator for HNSCC patients over the short-term, they become less significant as follow-up time increases.

## Introduction

Head and neck cancer is the sixth most common solid tumour in the world, the majority of which are histologically squamous cell carcinoma (HNSCC). Recent reports have indicated a better prognosis for patients with human papillomavirus (HPV) positive oropharyngeal tumour (82.4% 3 year overall survival rate), however the survival rate for those patients with HPV negative tumours is still much lower (57.1% 3 year overall survival) despite advances in surgical, chemo- and radiotherapeutic treatment strategies (1).

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Immunosuppression in cancer patients is a well established phenomenon, particularly in patients with HNSCC (2,3) and can include changes in cytokines (4,5) and the balance of immune cell populations. T helper cells play a key role in controlling the immune response and can be subdivided further into T-helper 1 (Th1) and T-helper 2 (Th2) like cells. These cells are defined by the cytokine repertoire they produce and responses they induce; IL12 and IL10 are commonly used as representatives of the Th1 and Th2 responses, respectively. Th1-like cells are principally involved in promoting cell-mediated immunity, and are generally considered as the host's main anti-cancer mechanism, whereas Th2-like cells stimulate a humoral or antibody-mediated response, principally against extracellular pathogens. The Th1 and Th2 responses are reciprocally balanced and a shift towards a Th2-like response has been observed in cancer patients, including those with HNSCC, by measuring serum cytokines (6,7).

Many attempts have been made to enhance the anti-tumour immunity of cancer patients with limited success, and even increasing the levels of T cells containing tumour specific antigens by vaccination does not guarantee tumour regression. T regulatory cells (Tregs) are thought to be largely responsible for the ineffective T cell response, as their removal during immunotherapy of cancer leads to enhanced immune effects (8). Tregs act to suppress the activity of peripheral T cells, particularly the cytotoxic subset, and also decrease antigen presentation and promote the immunosuppressive functions of dendritic cells, monocytes and macrophages together resulting in reduced anti-tumour immunity (9). Naturally occurring Tregs (nTregs) represent approximately 5-10% of peripheral $CD4^+$ T cells. To date there is no single marker to identify nTregs, however they express high levels of the surface marker CD25 (α chain of IL2R) along with the forkhead box transcription factor (Foxp3) which are essential for nTreg maintenance, development and function respectively (10). Tregs are elevated in the peripheral blood of HNSCC patients where they have been related to early recurrence (11,12).

An initial study by our group on this cohort of patients determined the serum levels of the cytokines IL10 and IL12, along with the circulating levels of Treg cells both pre- and post-treatment and investigated the relationship between the results and patient outcome over an average 15 month follow-up and found that: IL10 detectability was significantly higher in patients vs.

Table I. Clinicopathological features of HNSCC patients.

| Sex and age | No. | Tumour site | No. | Overall stage[b] | No. |
|---|---|---|---|---|---|
| Female | 22 | Hypopharynx | 16 | 1 | 27 |
| Male | 85 | Larynx | 36 | 2 | 10 |
| Total | 107 | Oral cavity | 21 | 3 | 16 |
| Median age (year) | 64 | Oropharynx | 26[c] | 4a | 47 |
| Age range (year) | 26-90 | Sinonasal/nasal | 4 | 4b | 4 |
| | | unknown | 5 | 4c | 4 |
| Tumour stage | No. | Nodal stage | No. | Metastasis | No. |
| T1 | 35 | N0 | 54 | M0 | 41 |
| T2 | 30 | N1 | 15 | M1 | 5 |
| T3 | 16 | N2 | 34 | Mx | 61 |
| T4 | 21 | N3 | 4 | | |
| Tx[a] | 5 | Missing | 1 | | |

[a]Five patients had metastatic SCC deposits in their cervical lymph nodes with an unknown primary site. [b]According to TNM classification of malignant tumours, 6th edition. [c]One oropharynx was found to have a brachial cyst and was excluded from the current analysis.

controls; pre-treatment IL10 levels were significantly elevated in samples from late stage, node positive patients in all sub-sites apart from the oral cavity; post-treatment IL10 levels dropped significantly. In addition the detectability of IL10 (but not IL12) had a significant negative influence on survival. Conversely, Treg cell levels were significantly higher in pre-treatment patients vs. controls, and post-treatment vs. pre-treatment levels, but did not correlate with any clinical parameters (13). Here we re-examined the same patient outcome with an average follow-up period of 33 months (maximum 67 months) to determine whether the circulating levels of specific Th1 (IL12) and Th2 (IL10) cytokines or Treg cell levels either before or following treatment are indicators of long-term survival in HNSCC patients.

## Materials and methods

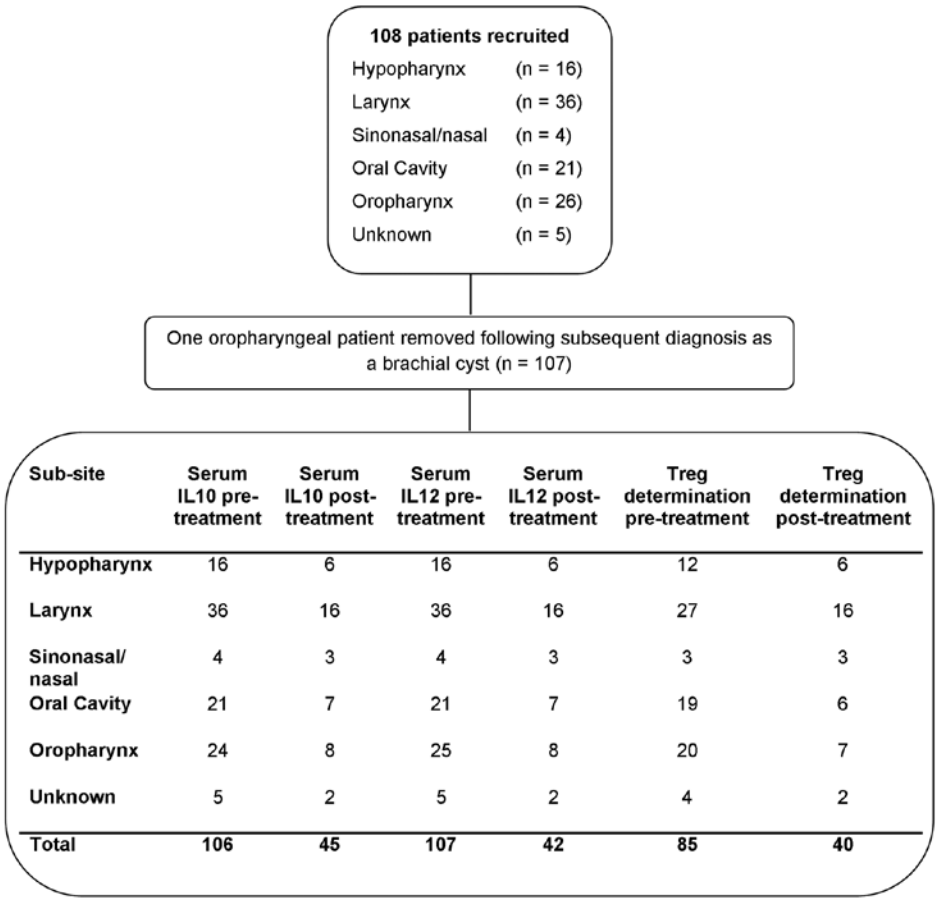
*Patients and controls*. As described in detail by Alhamarneh *et al* (13), following ethical approval (South Humber local research ethics committee; LREC-05/Q1105/55) newly-presenting patients with histologically proven HNSCC (n=108; Table I) were recruited into the study between 2004 and 2006. The exclusion criteria included previous diagnosis and treatment for any other form of cancer, active autoimmune or co-existing infectious disease. A single patient was found since the study by Alhamarneh *et al* (13) to have a brachial cyst and was therefore excluded from analysis (n=107). HNSCC patients were followed up from time of diagnosis for a maximum of 67 months (however data were censored at 60 months as this is generally considered as cured) until September 2009 to ascertain survival and presence of disease recurrence (mean follow-up 33 months). Patients of similar age and sex, undergoing non-cancer related operations (n=40) and without history of cancer (of any type), were recruited as controls.

Following written informed consent two sets of blood samples were obtained from patients; the first was prior to any treatment intervention and the second was collected 4-6 weeks after allocated treatment (surgery, radiotherapy or chemo-radiotherapy). Individuals within the control group provided a blood sample on a single occasion prior to surgery.

*Serum separation*. Venous blood was collected from each patient into two 5 ml serum separator vacutainers (SST™ II, BD Biosciences, Oxford, UK) and incubated for 30 min at 4˚C before centrifugation (400 x g for 10 min). The resulting upper layer of serum was aliquoted and stored at -80˚C prior to ELISA analysis.

*Isolation of peripheral blood mononuclear cells (PBMC)*. A 50 ml venous blood sample was taken from each patient and control subject into a heparin coated syringe, and peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation, as described previously (13). Isolated PBMC were counted using trypan blue exclusion, the concentration of PBMC was adjusted to $2x10^7$ cells/ml by re-suspension in an appropriate volume of freeze media [foetal bovine serum plus 10% (v/v) dimethyl sulphoxide] and cryo-preserved in 1 ml aliquots for subsequent analysis of circulating Treg cells.

*Enzyme linked immunosorbent assay (ELISA)*. Commercial ELISA kits were used to determine the serum levels of IL10 and IL12 (human IL10 Ultra Sensitive and human IL12, Biosource, CA, USA). The minimum levels of detection for IL10 and IL12 (p40 homodimers and p70 heterodimers) were 0.2 pg/ml and 1.5 pg/ml, respectively and anything lower than this was classed as non-detectable. ELISAs were performed as directed: all samples were run in duplicate and the average values used for analysis when these did not differ by >20%. Both the detect-




| Sub-site | Serum IL10 pre-treatment | Serum IL10 post-treatment | Serum IL12 pre-treatment | Serum IL12 post-treatment | Treg determination pre-treatment | Treg determination post-treatment |
|---|---|---|---|---|---|---|
| Hypopharynx | 16 | 6 | 16 | 6 | 12 | 6 |
| Larynx | 36 | 16 | 36 | 16 | 27 | 16 |
| Sinonasal/ nasal | 4 | 3 | 4 | 3 | 3 | 3 |
| Oral Cavity | 21 | 7 | 21 | 7 | 19 | 6 |
| Oropharynx | 24 | 8 | 25 | 8 | 20 | 7 |
| Unknown | 5 | 2 | 5 | 2 | 4 | 2 |
| **Total** | **106** | **45** | **107** | **42** | **85** | **40** |

Figure 1. Flow chart of patient recruitment and samples used for each parameter investigated.

ability of IL10 and the median IL10 concentration were used for analysis.

*Treg cell characterisation and enumeration*. Treg cells were isolated from PBMC using magnetic microbeads (MACS® $CD4^+CD25^+$ regulatory T cell isolation kit, Miltenyi Biotec, CA, USA) as previously described (13). Briefly, stored PBMC were thawed at room temperature, washed twice in RPMI-1640 supplemented with L-glutamine, penicillin and streptomycin, and then enumerated using Trypan blue. The manufacturer's protocol was followed to isolate Treg cells, i.e. non-$CD4^+$ T cells, were first negatively selected by magnetic labelling of unwanted cells with MACS microbeads. The cell population was washed (500 ml PBS/2.5 g BSA/0.37 g EDTA) and applied to a MACS column whilst the unlabelled cells ($CD4^+$), passed through and were collected. This solution was then centrifuged, supernatant discarded and cells mixed with anti-CD25 microbeads. After incubation and washing, target cells ($CD4^+CD25^+$ T cells) were retained on a second column, while unlabelled cells passed through. The column was removed from the separator and the retained cells were eluted as the enriched, positively selected, fraction.

Isolated cells were characterised and enumerated using flow cytometry on a FACSCalibur with CellQuest® analysis software Version 3 (BD Biosciences), following labelling with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and a phycoerythrin (PE)-conjugated anti-CD25 (AbD Serotec, Oxford, UK) to verify Treg phenotype. IgG isotype-matched antibodies were used as negative controls where appropriate. The analysis parameters were set according to standard procedures using negative controls and patient cells stained in either single or double colours. After $CD4^+CD25^+$ isolation, the $CD25^{high}$ population was identified by applying a gate to only include the $CD4^+$ T cells co-expressing CD25 with a mean fluorescence intensity (MFI) ≥100 (13).

*Statistical analysis*. Fig. 1 identifies which samples were analysed for each immunological parameter. The results were analysed using SPSS version 17 (SPSS Inc., Chicago, USA). IL10 results were analyzed as dichotomous variables due to the large number of samples with levels below the minimum level of detection, whereas IL12 and Treg values were analysed as continuous variables. Missing data were excluded from each analysis.

Associations between tumour site and serum IL10 detectability, serum IL12 concentration and Treg percentages were determined using $\chi^2$ (IL10) and one way ANOVA (IL12, Treg) following removal of the patients with unknown tumour sub-site. One way ANOVA was also used to determine relationships between sub-site and survival. Kaplan-Meier survival analysis with appropriate log-rank Mantel-Cox applied (IL10 detectability) and Cox regression analysis (IL12 concentration

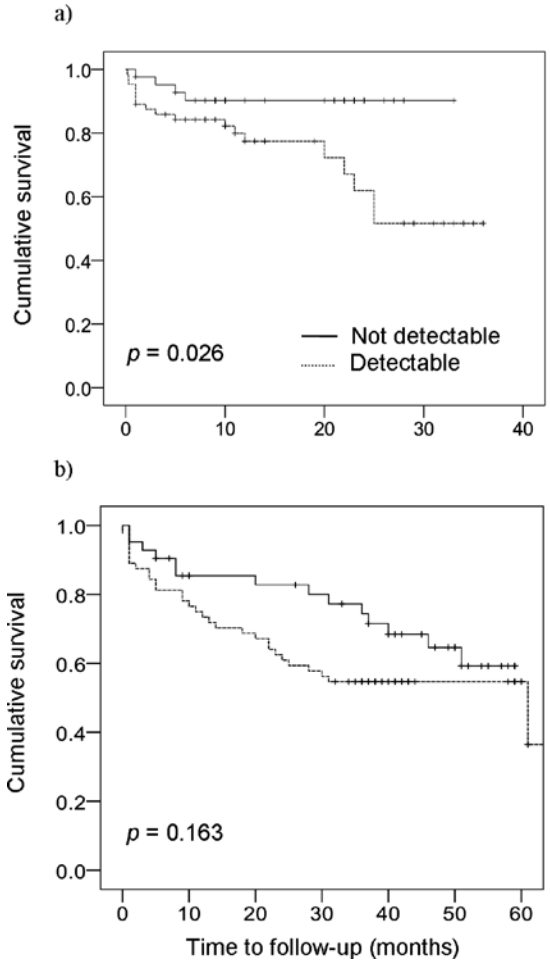



Figure 2. Kaplan-Meier survival curves showing the association between detectable pre-treatment serum IL10 levels in HNSCC patients (n=106) and survival after average follow-up times of (a) 15 months (range 1-36) and (b) 33 months (range 1-67, censored at 60 months).

and Treg percentage) were used to determine the relationship between the exploratory variable (pre- and post-treatment cytokine) and patient survival or recurrence.

## Results

The results from the initial study which determined the relationship between pre- and post-treatment serum levels of IL10, IL12 and circulating Treg cells with both survival and recurrence in a cohort of 108 HNSCC patients over an average 15 month (range 1-36 months) follow-up period have been reported by Alhamarneh *et al* (13). In summary the authors found that serum IL10 was more likely to be detected in patients compared with controls, and the pre-treatment samples had both more detectable and higher concentrations of IL10 compared with post-treatment samples. A significant association of IL10 detectability was found with tumour sub-site: tumours of the larynx, oropharynx and hypopharynx had a higher probability of having detectable levels of IL10 pre-treatment, whilst the converse was true for oral cavity tumours; IL10 levels in all anatomical sub-sites, apart from oral cavity, were significantly elevated in later stage, bulkier and node positive tumours. In the current extended follow-up study one of the original 26 oropharyngeal patients was found to have a non-malignant brachial cyst and so was removed from analysis. Repeat identical analyses were performed and no differences in the results to those described above were observed.

After the initial mean follow-up period of 15 months no relationship between pre-treatment IL10 and recurrence was found although having detectable pre-treatment serum IL10 levels was shown to have a significant negative influence on survival time using Kaplan-Meier curves (log-rank, Mantel-Cox, p=0.026). The estimated mean of survival was shown to be 30.1 months (27.4-32.8, 95% CI) when pre-treatment levels of IL10 were not detected, compared with 25.9 months (22.2-29.6, 95% CI) when pre-treatment IL10 was detected (Fig. 2a). Although the mean survival time for patients with detectable levels of IL10 pre-treatment following an extended mean follow-up of 33 months (range 1-67) remained shorter (40.6 months; 34-47, 95% CI) compared with those without detectable levels of IL10 (45.6 months; 39-51, 95% CI) the significance was not maintained (p=0.163; Fig. 2b). The same was true for both overall survival (disease-free survival) and disease-specific survival (Table II). No relationship was found between pre- or post-treatment IL12

Table II. Relationship between serum IL10 detectability pre- and post-treatment and survival at 33 months average follow-up.

| Treatment stage | IL10 detectability | No. of patients (% died) | Mean survival time ± SEM | 95% CI | P-value |
|---|---|---|---|---|---|
| Disease-free survival | | | | | |
| Pre | Detectable | 64 (47%) | 40.6±3.5 | 34-47 | 0.16 |
| | Non-detectable | 42 (33%) | 45.6±3.2 | 39-52 | |
| Post | Detectable | 28 (36%) | 33.8±2.9 | 28-39 | 0.19 |
| | Non-detectable | 14 (14%) | 39.8±2.1 | 36-44 | |
| Disease-specific survival | | | | | |
| Pre | Detectable | 64 (30%) | 48.5±3.4 | 42-55 | 0.29 |
| | Non-detectable | 42 (21%) | 48.9±3.1 | 43-55 | |
| Post | Detectable | 28 (14%) | 38.9±2.4 | 34-44 | 0.49 |
| | Non-detectable | 14 (7%) | 41.6±1.3 | 39-44 | |

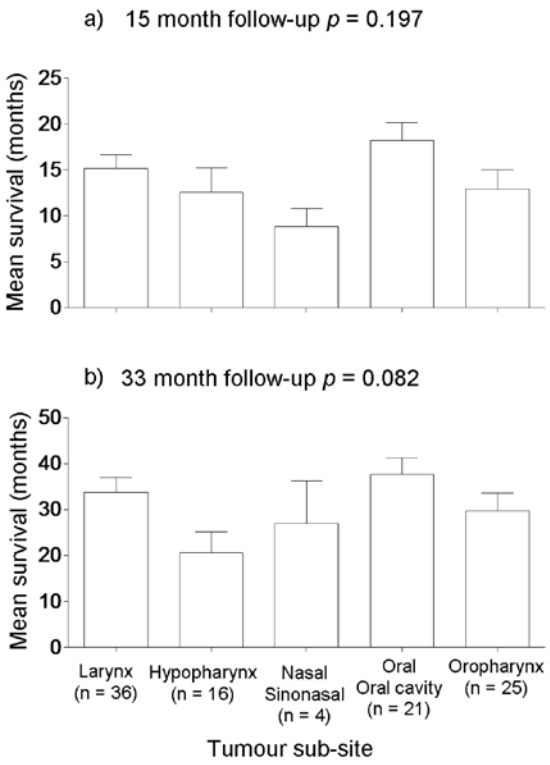



Figure 3. Bar charts showing the influence of tumour sub-site on survival (months) at average follow-up times of (a) 15 months; p=0.2 and (b) 33 months; p=0.08. P obtained from one way ANOVA analysis.

levels, recurrence or survival at either the 15 or 33 month follow-up.

After 15 months average follow-up the circulating Treg ($CD4^+CD25^{high}$) levels were found to be higher in the pre-treatment samples (median 4.6%, IQR 2.7-10.0) compared with controls (median 3.3%, IQR 0.01-5.6; p=0.002) and these levels increased significantly following treatment (median 7.2%, IQR 3.5-13.8; p=0.022), but again neither pre- or post-treatment levels influenced survival or recurrence at the 15 or 33 month average follow-up.

The relationship between the HNSCC sub-site and survival is shown in Fig. 3, after the 15 month follow-up period the group of patients with nasal/sinonasal tumours had the shortest mean survival time (8.8±2.0 months) whereas the oral/oral cavity had the longest mean survival time (18.2±1.9 months) however, the difference was not significant (one way ANOVA; p=0.197). The patient group with oral/oral cavity tumours maintained the longest survival time at 33 months average follow-up (37.6±3.6 months), but the group of patients with the shortest survival time was now the hypopharynx group (20.6±4.6 months) and this difference approached significance (one way ANOVA; p=0.08). When the survival of different sub-sites was stratified for pre-treatment IL10 detectability (Table III), it was observed that only patients with laryngeal and oral/oral cavity tumours having detectable pre-treatment serum IL10 had a shorter mean survival time than those without detectable IL10, however this difference was not significant (p=0.68, Kaplan Meier).

## Discussion

This study is the first to reveal that although pre-treatment serum IL10 may be a prognostic indicator over a short period of follow-up (average 15 months), over longer periods (average 33 months), the significance in relation to survival is lost, even though the length of survival for patients having detectable levels of IL10 is still less than those without detectable levels of IL10.

In order for a tumour to grow and metastasise it needs to evade attack by the host's immune system. Normally a balance of the Th1 (cytotoxic) and the Th2 (humoral) immune cells exists to maintain immune homeostasis, however, in many malignancies this balance is skewed towards a more suppressive Th2 response allowing uncontrolled growth and spread of the tumour (6,7). One of the reasons for this skewed response is an imbalance in the cytokines which influence the development and function of the Th1 and Th2 cells. IL10 is a Th2-like cytokine which has been shown to be both immunosuppressive and anti-inflammatory (14), with its main target cells being dendritic

Table III. Relationship between sub-site, pre-treatment IL10 detectability and survival.

| Sub-site | IL10 detectability | No. of patients | Mean survival time ± SEM (mths) | 95% CI |
|---|---|---|---|---|
| Larynx | Detectable | 23 | 39.7±5.5 | 29-51 |
| (n=36) | Non-detectable | 13 | 54.1±3.2 | 48-60 |
| Hypopharynx | Detectable | 15 | 27.6±6.2 | 16-40 |
| (n=16) | Non-detectable | 1 | 1.0±0.0 | 1-1 |
| Nasal/sinonasal | Detectable | 3 | 27.6±10.6 | 9-51 |
| (n=4) | Non-detectable | 1 | 20.0±0.0 | 20-20 |
| Oral/oral cavity | Detectable | 7 | 40.0±9.2 | 22-58 |
| (n=21) | Non-detectable | 14 | 47.0±4.1 | 40-56 |
| Oropharynx | Detectable | 14 | 45.8±6.1 | 34-58 |
| (n=24) | Non-Detectable | 10 | 32.5±7.5 | 18-47 |

Assessment at 33 month average follow-up (Kaplan Meier p=0.684).

cells (DC) and macrophages, where it inhibits antigen presentation, DC differentiation and maturation and Th1-like cytokine production. It was therefore no surprise that Alhamarneh *et al* (13) demonstrated, at an average follow-up time of 15 months, that having detectable levels of pre-treatment IL10 in serum, negatively influenced the survival of HNSCC patients. This is in agreement with other studies; for example in childhood soft tissue sarcomas (15) and gastric cancer (16) which found that higher pre-treatment serum and plasma levels of IL10 respectively were related to poorer 5-year survival. However, in the current study, using the same patient cohort when the follow-up period was extended to an average of 33 months (max 60 months for statistical purposes), even though patients with detectable levels of IL10 still had a shorter mean survival time than those without, the role of pre-treatment IL10 detectability in predicting survival was no longer significant. One possible reason for this could be that tumour elimination removes the source of systemic IL10, which was demonstrated by the reduced level of IL10 in the post-treatment serum samples, compared with the pre-treatment samples by Alhamarneh *et al* (13), however over a longer time period the effects of IL10 become less important in relation to other cytokines. It should be noted, however, that although the levels of IL10 have been found to be elevated in other cancers this has not always been associated with survival (17,18).

Bien *et al* (15) not only demonstrated a negative relationship between pre-treatment serum IL10 and survival in childhood soft tissue sarcomas, but also found that higher levels of the Th1-like cytokine IL12 in these patients predicted a better survival and that it was the balance between the two cytokines, i.e. the Th1/Th2 response which was important rather than the individual cytokines. However in the current study the serum levels of IL12 in the patients both pre- and post-treatment were not reflective of the survival of patients. The change in serum IL10 without a concurrent change in serum IL12 has however, previously been demonstrated in head and neck cancer patients where it was found that, although levels of the Th2 cytokines IL4, IL6 and IL10 were elevated in cancer patients compared with controls, levels of Th1 cytokines IL2 and GMCSF were also increased and those of IL12 remained the same as controls (19,20). In addition, a trend towards better survival has been found with high expression of IL12 and IL7 within the tumour itself (21) and intratumoural administration of IL12 to HNSCC patients elicits a Th1 profile in the locoregional lymph nodes (22), indicating that the cytokines within the tumour microenvironment may contribute to survival and these need to be determined in addition to those in the circulation. It should also be noted that to gain a full understanding of a shift from a Th1 to a Th2 response a more extensive range of cytokines should possibly be examined. These observations are in agreement with Szaflarska *et al* (16) who found no relationship between pre-operative plasma levels of IL12 and survival in gastric cancers; thus differences may be tumour specific.

HNSCC are not a homogeneous group of tumours and differ in molecular profiles, risk factors, particularly with regard to HPV, pathogenesis and clinical behaviour (23). This is reflected in the results obtained from the survival data determined in relation to sub-site where, at both 15 and 33 months average follow-up it was found that patients with cancers of the oral cavity had a longer mean survival than any other forms of head and neck cancer, although this difference was not significant. This is in agreement with cancer statistics which show that patients with oral cancer have a better chance of survival compared with HNSCC from other sub-sites (24). This could arise from the fact that oral tumours are more obvious in their appearance and are generally spotted at an earlier stage, by the patient or by health professionals or that their location makes them more accessible to treatment than the other types of head and neck cancer, because in general prognosis worsens with increasing inaccessibility of the tumour. However, the study by Alhamarneh *et al* (13) clearly showed differences in the IL10 detectability between sub-sites in that the patients with oral cavity tumours were less likely to have detectable levels of serum IL10 than non detectable IL10, whereas the converse was true for the other sub-sites which could relate to survival. At 33 months of follow-up it was observed that both patients with oral/oral cavity and laryngeal tumours had a marginally longer survival if the patients did not exhibit detectable levels of IL10 in their serum pre-treatment, however this difference was not significant, possibly due to the variation in the numbers involved for each sub-site. In addition oral cavity tumours did not demonstrate higher levels of IL10 in later stage, bulkier and node positive tumours whereas samples from patients with tumours from other sub-sites did (13).

The exact source of the systemic cytokines in HNSCC still needs to be resolved; as it has been shown that not only are they produced by specific immune cells (6), but also by the tumour itself (25) and therefore removal of the tumour is highly likely to influence the type of response occurring.

Tregs have been found to be elevated in HNSCC patients compared with healthy controls (26,27), which is thought to be a further mechanism induced by the tumour to enhance immune evasion. However, neither pre- nor post-treatment Treg ($CD4^{+}CD25^{high}$) population levels correlated with survival or recurrence at 15 months or the extended 33 month average follow-up in the current study. This is in contrast to Boucek *et al* (11) who found that levels of Treg in the peripheral circulation correlate with a higher probability of recurrence over a 24 month period. The reasons for the lack of correlation of Treg levels with survival in the current study may be that survival is dependent on the balance of Tregs and other immune cells, including cytotoxic T cells, rather than an individual population (28), or as demonstrated by Watanabe *et al* (29) that it is the presence of Tregs within the HNSCC tumour microenvironment which may result in reduced survival. In this respect investigations into immune parameters within the tumour microenvironment are essential.

In conclusion, serum levels of IL10, IL12 and $CD4^{+}CD25^{high}$ regulatory T cells alone may not be relied upon as prognostic factors of long-term survival in patients with HNSCC, however IL10 may be useful over the short-term to identify patients which may benefit from immunotherapy in addition to conventional treatment.

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