Prognostic Significance of Tumour-Associated Macrophages in Head and Neck Cancers: a Systematic Literature Review and a Study into their Effects in Nasopharyngeal Carcinoma

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Foreword
This bachelor thesis consists of two articles, a systematic literature review that assesses the diverse roles of macrophages in head and neck cancers and a scientific research article identifying the prognostic role of macrophages in nasopharyngeal carcinoma.

Article 1: Prognostic Significance of Tumour-Associated Macrophages in Cancers of the Head and Neck: a Systematic Literature Review

ABSTRACT

Background: Head and neck cancers account for a global incidence of more than 650,000 cases annually. Since head and neck cancers are characterized by poor survival rates it is essential to identify prognostic factors. Tumour-associated macrophages (TAMs) have a diverse set of functions in tumours and can be both pro- and anti-tumourogenic.

Objective: To assess the prognostic role of TAMs for survival in HNSCCs with a focus on identifying diverse roles of the subpopulations of TAM in a systematic literature review.

Methods: A systematic search of PubMed and Embase was performed for publications matching the domain of "head and neck cancer," determinate of "macrophages" up to the date of 7th of July, 2016. All the articles were screened for prognostic studies that matched with the domain and the determinate. Using Quality in Prognostic Studies (QUIPS) relevant studies were evaluated for risk of bias. Information on immunomarkers, macrophage subtypes and other relevant clinicopathologic characterteristics was extracted from the data and compared.

Results: The initial search generated 281 studies. Eight studies were finally selected on the inclusion criterion. This included 6 studies focused on oral squamous cell carcinoma (OSCC), 1 study on oropharyngeal squamous cell carcinoma (OPSCC) and 1 study on supraglottic laryngeal carcinoma. All 8 studies looked at pan-macrophage marker CD68, while 3 studies looked at M2 macrophage-specific marker CD163. Four of the studies reported that elevated CD68 count was related to poor survival outcome, while the other four reported non-significant findings regarding CD68 association with survival. The 3 studies looking at CD163, all reported significant correlation with poor prognosis.

Conclusions: Although some bias cannot be excluded, high density of TAM seems to be associated with worse overall survival in head and neck squamous cell carcinoma. M2 macrophage plays an active role in tumour development and progression.

Keywords: tumour-associated macrophages, M1, M2, head and neck cancer, CD68, CD163, prognosis

Article 2: The Prognostic Significance of Tumour-Associated Macrophages in Nasopharyngeal Carcinoma

ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is characterized by considerable amount of immune infiltrate. There is controversy among studies about whether tumour-associated macrophages (TAMs) density positively or negatively affect prognosis in tumours. Since NPC differs so significantly from other cancers of the head and neck region it is imperative to identify suitable prognostic factors.

Objective: To assess the prognostic significance of TAMs and T regulatory cells (Tregs) in NPC.

Materials and methods: This study included 92 patients with NPC. CD68, CD163 and FoxP3 antibodies were used to identify and assess the expression levels of TAMs and T regulatory cells using immunohistochemistry. A tissue microarray was used for the high throughput analysis of the samples. Samples were semi-quantitatively scored and divided into groups of high and low expression. Correlations between clinicopathologic characteristics were assessed using the Pearson X^2 test and ANOVA for continues variables. Survival analyses were performed using the Kaplan-Meier method and the Cox proportional hazards model.

Results: The mean immunomarker counts of CD68 and FoxP3 were significantly higher in EBV positive cells compared to EBV negative cells and elevated CD68 and FoxP3 expression was correlated with EBV-positive NPCs. Both CD68 and CD163 were positively correlated with high FoxP3 expression. Elevated FoxP3 expression was strongly associated with increased overall survival (HR 3.283 (95% CI: 1.139-9.464)). Macrophage markers, CD68 andCD163, did not show significant relation to survival.

Conclusions: Elevated levels of Tregs, immune cells that have previously been associated with tumour progression, are in fact beneficial for survival in patients with NPC. This may however be related to the EBV status in NPCs. Despite significant differences in TAM density in the different NPC subsets, neither macrophage marker was significantly correlated with survival rate.

Keywords: tumour-associated macrophages, nasopharyngeal carcinoma, EBV, CD68, CD163, FoxP3, Tregs, prognosis

Abbreviations

DFS	Disease-free survival	OPSCC	Oropharyngeal squamous cell carcinoma
EBV	Epstein-Barr virus		
HNSCC	Head and neck squamous cell carcinoma	OSCC	Oral squamous cell carcinoma
IMC	Infiltrating macrophage count	TAMs	Tumour-associated macrophages
M1	Classically activated type 1 macrophages	Tregs	FoxP3-positive T regulatory cells
M2	Alternatively activated type 2 macrophages	TGF-β	Transforming growth factor-β
NPC	Nasopharyngeal carcinoma	VEGF	Vascular endothelial growth factor
OS	Overall survival		

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Prognostic Significance of Tumour-Associated Macrophages in Cancers of the Head and Neck: a Systematic Literature Review

INTRODUCTION

Head and neck cancers account for a global incidence of more than 650,000 cases annually (Lambert et al., 2011). Subtypes of these cancers include cancers of the oral cavity, the larynx, pharynx and nasal passageways. In Europe it was estimated that head and neck cancers accounted for 250,000 incidence cases (4% of the cancer incidence) and was responsible for 63,500 deaths in 2012 (Gatta et al., 2015). Most (90%) of these cancers are to an extent biologically similar and fall under the category of head and neck squamous cell carcinoma (HNSCC). Within this category, oral squamous cell carcinoma (OSCC) is the most frequent, accounting for 90% of HNSCC cases (He et al., 2014). Head and neck cancers are strongly associated with environmental factors such as smoking, alcohol consumption and strains of viruses. Human papillomavirus (HPV) is strongly associated with oropharyngeal carcinomas and Epstein-Barr virus (EBV) is closely associated with nasopharyngeal carcinoma (NPC) (Sankaranavanan et al., 1998). In general, males are more affected than females with ratios ranging up to 4:1. The different HNSCC often show curious racial and geographical distribution. Laryngeal and oropahryngeal cancers, but not oral cancers, for example are much more common in African American men, with mortality also being significantly higher when compared to Caucasian Americans (DeSantis et al., 2013). OSCC is characterized by high rates of lymph node metastasis, and as a sub-category itself it is estimated to account for over 2.5% of all malignancies (He et al., 2014; Lu et al., 2009). OSCC has a poor prognosis with 5-year survivals having only improved marginally in the last decades (Hu et al., 2016). It is thus imperative to identify molecular and histologic markers that will help identify aggressive tumours that can be used as indicators for prognosis, and also possibly as therapeutic targets.

Macrophages are a heterogeneous population of tissue-resident myeloid cells involved in health and disease. Macrophages are derived from bone marrow progenitors that continuously proliferate and release immature monocytic precursors into the circulation (Lewis and Pollard, 2006). Monocytes then extravasate into tissues where they undergo specific differentiation depending on local cues developing into a specific type of resident tissue macrophage including Kupffer cells in the liver, alveolar macrophages in the lung and osteoclasts in the bone. Macrophages are potent antigen presenting cells and are therefore an integral part of the immune system, initiating immune responses against pathogens or tumour antigens (Weber et al., 2015). They perform a variety of functions and are essential for tissue remodeling, in inflammation and immunity (Bingle et al., 2002). The phenotype of the differentiated macrophages can differ both between and within tissues. In response to environmental cues macrophages usually take up one of two recognizable phenotypes; (1) the classically activated type 1 macrophages (M1) and (2) the alternatively activated type 2 macrophages (M2) (Zhang et al., 2012; Jablonski et al., 2015; Heusinkveld & van der Burg, 2011).

Leukocyte infiltration in human tumours was first described in 1863 by Virchow and was thought to reflect the onset of cancer at a site of previous chronic inflammation. It is

now been established that macrophages are prominent in nearly all types of malignancies. In fact, tumour-associated macrophages (TAMs) are the most prevalent inflammatory cells in the tumour and can account for 30%-50% of the total host leukocyte infiltrate, and can in some instances comprise up to 70% of the cell tumour mass (Lin et al., 2011; Kelly et al., 1988). Increasing amounts of studies suggest that the tumour microenvironment is critical for cancer development and metastasis and many studies have been conducted to assess the prognostic value of TAMs in tumours (Bingle et al., 2002, Hu et al., 2016, He et al., 2014). In humans, the detection of TAMs is predominantly based on the use of antibodies to the glycoprotein CD68 (Heusinkveld & van der Burg, 2011). However, the CD68 antibody recognizes both M1 and M2 macrophages. M2 macrophages however solely express the glycoprotein CD163, a heptaglobin scavenger receptor which can be used to discriminate between M1 and M2 macrophages (Costa et al., 2014; Heusinkveld & van der Burg, 2011).

A number of studies have tried to identify the prognostic value of TAM in solid tumours and in the literature conflicting data exists about whether TAMs are negatively or positively associated with survival outcomes. However, to our knowledge, no systematic reviews have studied the relationships between TAM expression and prognosis in HNSCC, by comparing expression of different immunomarkers. In the present study we assess the prognostic role of TAMs for survival in HNSCCs with a focus on identifying diverse roles of the subpopulations of TAM in a systematic literature review.

METHODOLOGY

Search strategy and study selection

A search for publications up to 7th of July 2016 was performed in PubMed/MEDLINE and EMBASE. Initially, a search was performed for peer-reviewed studies relating to the domain ("Head and Neck Neoplasms") and for the determinant ("Macrophages") or synonyms of these terms in the title or abstract or as Medical Subject Headings (MeSH) terms. MeSH term database and Emtree database were used to identify synonyms of the terminology in the research question. Broad filters for "Prognosis" were then applied to the searches results. PubMed and Embase searches were combined, with all duplicates being removed, resulting in 281 unique articles.

Titles and abstracts were screened using pre-determined inclusion and exclusion criteria by two independent researchers (MS, MO) (Fig. 1). This screening process yielded 54 potentially relevant and interesting publications. These were then further screened in a full-text analysis to determine a final selection. Studies were eligible for inclusion if they were original articles and addressed the prognostic value of macrophages carcinomas of the head and neck (all other tumors were excluded). In summary they addressed one or more of the following prognostic factors; overall survival, disease-specific survival, disease-free survival, recurrence-free survival or progression-free survival. The definition of original articles was based on a definition used by Ipenburg et al. (2016) as "primary research studies with new, unpublished results and were written by the researchers who performed the study." Studies were not included in the selection if they did not contain a prognostic study design, were animal studies, were repetitive studies on the same samples and if they were not written in English. Conference reports, case studies and meta-analysis were also

rejected. Six of these articles met the inclusion criteria and were selected for further analysis and data extraction. The selection was based on consensus and any differences were resolved by discussion by the two researchers. Finally, review articles on the topic of interest and references of selected articles were manually screened for titles not identified by the initial search. This led to the inclusion of 2 more papers, totaling 8 articles into the final selection.

Quality assessment

A quality assessment was performed of the relevant full text articles. The papers were appraised for risk of bias using the Quality in Prognosis Studies (QUIPS) tool (Hayden et al., 2013). Using this tool a risk of bias is determined based on the study design and the reported results (Swartz et al., 2015). Fourteen domains within QUIPS analysis were judged for risk of bias, being either positive or negative, with positive approval being associated with low bias. In this analysis, a positive approval got a score of 1 and a negative approval a score of 0. The higher the total score, the less chance of bias. Total scores were calculated by tallying the individual scores. Note that in this case, a higher score is associated with less chance of bias. The potential sources of bias are described in Appendix 1.

Data extraction

Data was extracted from the selected studies using a standardized data extraction form (Table 1). Extracted data included: first author's name, year of publication, the number of patients, gender and age range of the patients, head and neck site, the type of survival outcome, statistical analysis, cut-off value if applicable, tumour-associated macrophage density, correlations with survival outcome and the length of the follow-up. Results were not quantitatively pooled due to the methodological heterogeneity of the studies, especially the lack of equality in cut-off values used to determine TAM counts.

RESULTS

Critical appraisal

Using the QUIPS criteria (described more extensively in Appendix 1), the final selection of 8 papers was critically appraised (Table 1). Many studies did not describe if the cohort was consecutive with the reader often having limited access to the clinicopathological data of the sample groups. All the papers defined inclusion criteria and comprehensively described the selection processes. The methodologies of the research were always recounted clearly and standardizations were well defined. The follow-up lengths and characteristics lost to follow-up were often not well documented. A score was calculated to describe the risk of bias. The relavance cut-off was determined to be less than 1 and the risk of bias cut-off was less than 6. All studies scored higher than this and are thus included in this review. The articles are not homogenous in the type of carcinoma studies and in addition most studies used data-dependent cut-offs for their prognostic factor assessment, thus it was decided that it was not possible to make a clinically relevant meta-analysis.

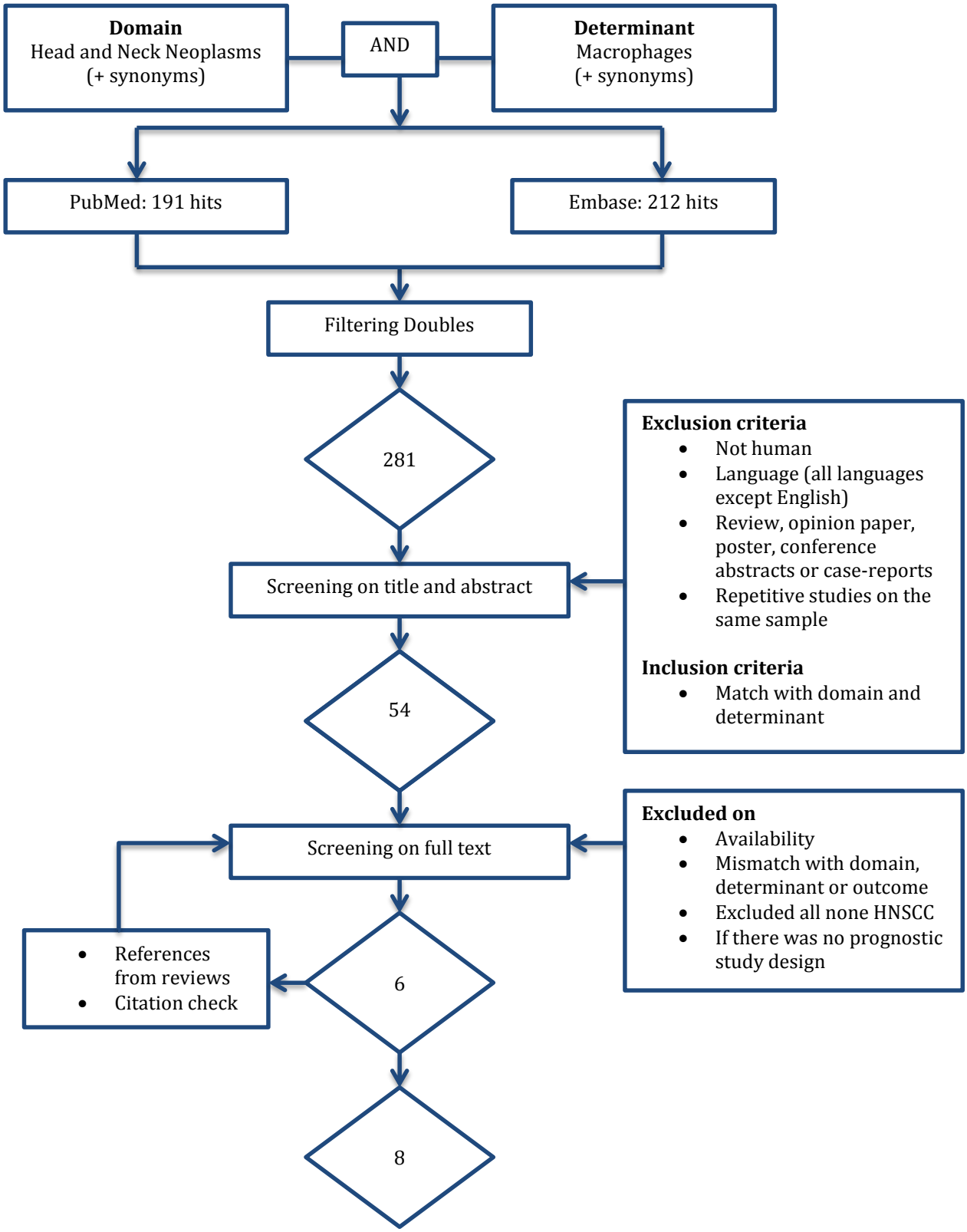

Figure 1. Flowchart of the study selection process (date of search 7th of July, 2016).

Table 1. Critical appraisal of the included studies

Study	Study design	Relevance					Risk of bias													
		Outcome																		
		DFS	OS	3/5 year survival	Chance of metastases	Relevance total	In/exclusion criteria	Selection process	Inception cohort	Outcome precise and valid	Estimation of prognosis	Follow-up duration	Complete follow-up	Difference follow-up	Other relevant prognostic	Standardization	Blinding	Kappa	Selection bias	Evaluation of immunostain	Risk of bias total
Hu 2016	RSC	-	+	+	+	3	+	+	+	+	+	+	+	-	+	+	-	-	+	-	10
Fujii 2012	RSC	-	+	-	+	2	+	+	+	+	+	+	-	-	+	+	-	-	+	-	9
Costa 2013	RSC	-	+	-	+	2	+	+	+	+	+	+	-	-	+	+	-	-	+	-	9
Lu 2009	RSC	+	+	-	-	2	+	+	-	+	+	-	-	-	+	+	+	+	+	-	9
He 2014	RSC	-	+	+	-	2	+	+	+	+	+	+	+	-	+	+	-	-	+	-	10
Liu 2007	RSC	-	+	-	+	2	+	+	-	+	+	-	-	-	+	+	-	-	+	+	8
Marc-us 2004	PSC	+	+	-	+	3	+	+	+	+	+	+	-	-	+	+	-	-	+	-	9
Lin 2011	RSC	+	+	+	+	4	+	+	+	+	+	-	-	-	+	+	-	-	+	+	9

Relevance cut-off: < 1 Risk of bias cut-off: < 6. + = 1 point, - = 0 points, RSC = retrospective cohort, PSC = prospective cohort, IHC = immunohistochemistry, (NB: See appendix 1 for the descriptions of the criteria used to score the risk of bias).

Study characteristics

A total of 8 articles were selected for analysis and discussion from the combined searches of PubMed and Embase. A summary of the study characteristics can be found in table 2. Three of the included articles looked at the prognostic value of both immunomarkers; CD68 and CD163, while in contrast, the other 5 articles only assessed the prognostic value of CD68. The immunomarkers were evaluated through the counting of cells after immunohistochemical staining. All 8 studies dichotomized their macrophage cell counting into groups of low and high macrophage infiltrate (or into

groups with similar meanings). The cut-off values, as mentioned briefly above, were data dependent and determined by the respective researchers. The size of the population studies ranged from 43 patients in the cohort of He et al. (2014) to 127 patients in the cohort of Hu et al. (2016). Seven of the 8 studies were retrospective studies, with the other (Marcus et al., 2004) being a prospective study. All studies performed survival analyses using statistical methods, including Kaplan-Meier and log-rank tests, or cox proportional hazards models. The average follow-up times of the included studies ranged from 51 months to 67 months, with three papers omitting information about follow-up lengths. Repetitive studies were not found. Of the 8 articles, 6 looked at the prognostic value of macrophages in OSCC, 1 at the prognostic value in oropharyngeal squamous cell carcinomas (OPSCC) and finally 1 supraglottic laryngeal carcinomas. No studies looked at the prognostic value of the immunomarkers in nasopharyngeal carcinoma (NPC).

Prognostic value of TAM

Clinicopathologic significance of TAM in OSCC

Of the 6 studies looking at the prognostic value of macrophages in OSCC, 3 looked at both immunomarkers, CD68 and CD163 while the other 3 only looked CD68. Lu et al. (2009) explored whether TAM count had a significant influence on the progression and prognosis of OSCCs, looking purely at the immunomarker CD68. They found that patients with an increased infiltrating macrophage count (IMC) have significantly shorter disease-free survival (DFS) and overall survival (OS). Furthermore, the study found that an increased TAM count was significantly associated with higher T status, N status, clinical stage, recurrence and mortality. In general, higher TAM density was an independent predictor for poor prognosis by Lu et al. (2009). Liu et al., (2007) examined TAM in OSCC using CD68 as immunohistochemistry (IHC) marker and looked at association with clinicopathologic factors and found similar results, showing correlation between higher infiltrating macrophage count and larger tumour size, positive lymph node metastasis and poor survival. Liu et al. (2007) also looked in more detail at the tumour microenvironment with particular attention paid to vascularization and the role that TAM might play in angiogenesis. The study found that elevated TAM count correlated with increased tumour angiogenesis implying that TAMs may change cancer to a more aggressive phenotype (Liu et al., 2007). Costa (2013) investigated macrophage populations, aiming to evaluate and characterize M1/M2 ratio in the tumour microenvironment of OSCC. Results of this study showed a predominance of M2 phenotype macrophages in the tumour microenvironment, with a general increase of macrophages in peripheral blood as well. In addition, the percentage of macrophages was shown to be higher in metastatic group than in the non- metastatic OSCC group. Significant correlations were found between CD68 expression and survival rates (Costa et al., 2013).

Fujii et al. (2012) examined the distribution of cancer-associated fibroblasts and the incidence of TAMs in OSCC. They looked at both CD68 and CD163 macrophage markers and found that the mean number of CD68 count was only slightly higher than the CD163 count. High number of CD68-positive macrophages correlated significantly with clinical stage and cancer invasion, whereas CD163 did not. Curiously however, significantly lower survival rates were noted in patients with higher levels with CD163 whereas there was no correlation between higher levels of CD68-positive macrophages and

survival outcome (Fujii et al., 2013). He et al., (2014) designed their study to investigate the expression of TAM markers (CD68 and CD163) in different tissue with pathological features and clinical outcomes. He et al. (2014) claim that their results indicate that CD68 and CD163 play important roles in carcinogenesis and progression of oral cancer. They demonstrated that there is significant difference between the TAM count between OSCC and normal oral mucosa and that positive expressions of both CD68 and CD163 was significantly associated with the aggressive behaviour of OSCC, but not with tumour stage or pathological grade. Expression of CD163 was significantly associated with OS, whereas CD68 was not. The sixth study looking at OSCC in this review, performed by Hu et al. (2016) examined the incidence of CD68 and CD163 and their relationships with clinicopathological features, and aimed to undercover some of the underlying mechanisms of action. They illustrated that high CD163 count, but not CD68, was significantly correlated with poor overall survival. Both were shown to significantly associate with lymph node metastasis while only CD163 correlated with recurrence and mortality (Hu et al., 2016). Hu et al. (2014) also suggested that CD163-positive macrophages may be more suitable to identify TAMs than CD68-positive macrophages.

Clinicopathologic significance of TAM in oropharyngeal carcinoma

Marcus et al. (2004) hypothesized that TAM contribute to HNSCC aggressiveness and in the study aim elucidated this claim by investigating whether primary macrophage content (measured by IHC of CD68) is related to clinical parameters. They found TAM density to be an independent predictor of lymph node extracapsular spread and lymph node metastasis, but did not significantly correlate with either DFS or OS.

Clinicopathologic significance of TAM in supraglottic laryngeal carcinoma

Lin et al. (2011) aimed to elucidate correlations between TAM infiltration with clinicopathologic characteristics and prognosis in patients with supraglottic laryngeal carcinoma. TAM infiltration, measured by means of CD68 count, did not have significant associations with most of the clinicopathological features. However, both high intratumoural and pertiumoural TAM infiltration was significantly correlated with poor survival.

Table 2. Characteristics of the studies included in the research analysis

	Country	Sample size (n) (M/F)	Age (years)	Tumour type	Follow-up (months)	Staining method	Immunomarker	Outcome	Significance	Result
Hu (2016)	China	127 (74/53)	34-88 Mean 61	OSCC	2-91 Mean 41.2 Median 39	IHC	CD68 CD163	OS OS	NS S	Indeterminate Poor prognosis
Fujii (2012)	Japan	108 (67/41)	29-93 Mean 66.4	OSCC	ND	IHC	CD68 CD163	OS OS	NS S	Indeterminate Poor prognosis
Costa (2013)	Brazil	45 (32/13)	42-90 Mean 61.7	OSCC	Mean 59	IHC	CD68	OS	S	Poor prognosis
Lu (2009)	Taiwan	92 (75/17)	21-76 Mean 51	OSCC	ND	IHC	CD68	OS DFS	S S	Poor prognosis Poor prognosis
He (2014)	China	43 (ND)	ND	OSCC	12-43 Mean 24	IHC	CD 68 CD163	OS OS	NS S	Indeterminate Poor prognosis
Liu (2007)	Taiwan	112 (93/19)	ND	OSCC	ND	IHC	CD68	OS	S	Poor prognosis
Marcus (2004)	USA	102 (76/26)	33-74 Median 56	OPSCC	Median 41	IHC	CD68	OS DFS	NS NS	Indeterminate Indeterminate
Lin (2011)	China	84 (77/7)	43-95 Median 67	Supra-glottic cancer	ND	IHC	CD68	OS DFS	S S	Poor prognosis Poor prognosis

(OSCC = oral squamous cell carcinoma, OPSCC = oropharyngeal squamous cell carcinoma, S = significant, NS = not significant, ND = not described in the article, IHC = immunohistochemistry, OS = overall survival, DFS = disease-free survival)

DISCUSSION

Evaluating TAM density in the tumour microenvironment and deciphering the roles of the macrophages and subpopulations of macrophages is essential for a better understanding of the clinical behaviour of carcinomas. The literature was systematically reviewed for studies assessing the association between macrophages and prognosis in cancers of the head and neck. As far as is known, this is the first systematic literature review to determine prognostic value of TAM in HNSCC.

Approximately two distinct polarization states are recognized; classically activated M1 macrophages and alternatively activated M2 macrophages (Heusinkveld & van der Burg, 2011). These macrophage subsets have often been implicated with either protective or pathogenic roles in cancer (Murray & Wynn, 2011). The highly versatile macrophage cells then react to the environmental cues presented to them in the tumour tissue with the release of

various growth factors, cytokines, chemokines and enzymes that can regulate tumour growth, angiogenesis, invasion and metastasis (Lewis and Pollard, 2006).

The classical framework dictates that differentiation to M1 macrophages is induced by pathogen or danger associated molecular patterns (PAMPs or DAMPs) and INFγ (interferon gamma) during infection or tissue damage (Biswas and Montavani, 2010; Jablonksi et al., 2015). M1 phenotype is designed to attract and activate cells of the adaptive immune system. M1 macrophages can express iNOS, ROS and produce IL-12 (the stimulating chemokine for NK and type 1 T-cells). These macrophages can phagocytose and kill target with efficient M1 macrophage response necessary to ensure tissue sterility and to control pathogen growth (Heusinkveld & van der Burg, 2011; Jablonski et al., 2015). M1 macrophages produce pro-inflammatory cytokines such as interleukin (IL)-12, IL-23 and IFNγ (Costa et al., 2013). Broadly, M1 macrophages are associated with protective roles in tumorigenesis by antagonizing pro-tumour activities of other members of the immune system while simultaneously indulging in activities such as the activation of T helper 1 responses and tumour-killing mechanisms throough the phagocytosis and production of iNOS and ROS (Costa et al., 2013; Heusinkveld & van der Burg; Murray & Wynn, 2011).

Contrastingly, M2 macrophages develop in response to IL-4 or IL-13. The M2 phenotype expresses ample scavenger receptors and is associated with the production of IL-1, IL-1β, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP). The M2 macrophages are necessary for wound healing, tissue repair, parasite clearance, angiogenesis and can also polarize T cells to T_h2 and dampen immune responses. (Biswas and Montavani, 2010; Heusinkveld & van der Burg, 2011; Jablonksi et al., 2015). M2 macrophages are associated with production of anti-inflammatory and immunosuppressive cytokines such as IL-10 and transforming growth factor-β (TGF-β) (Costa et al., 2013). These macrophages are implicated in contributing to tumour progression (Weber et al., 2015; Murray & Wynn, 2011). It is important to note that the extreme polarizations of the activation states of M1 and M2 is merely an oversimplification and that macrophages show an extremely diverse range of activation states ranging between the two described phenotypes dependent on the combination of the signals received in the tissue (Biswas and Montavani, 2010). The discussion in this systematic literature review shall however not tackle the intricacies of the spectrum of macrophage activation. It will therefore refer to M1 and M2 macrophages under the assumption that these two phenotypes are representative for the two poles of macrophage activation.

The majority of the studies reviewed suggest that TAMs are advantageous for tumour growth and are correlated with poor prognosis. Lu et al. (2009) and Liu et al. (2007) both looked at CD68-postive macrophage expression and both found that elevated CD68 expression was an independent predictor of decreased survival rates in OSCC. CD68 is however a pan-macrophage marker and is used as a marker to identify all TAMs and does not distinguish between the two activation states, M1 and M2. On the other hand, CD163 is regarded as a highly specific macrophage marker for M2 macrophages (He et al, 2014). It is interesting to see that in the three studies (Hu et al., 2017; Fujii et al., 2012, He et al., 2014) that looked into both CD68 and CD163 expression, none found a significant association between elevated CD68 levels and survival. In contrast, all 3 of these studies found that CD163 was significantly correlated decreased survival rates (Hu et al., 2016; Fujii et al., 2012; He et al., 2014). On the basis of these studies, it seems that there is a significant association of elevated M2 macrophages and poorer survival. This would also align with the current dogma that M2

macrophages are beneficial for tumour progression and disadvantageous for survival (Weber et al., 2015; Murray & Wynn, Heusinkveld & van der Burg, 2011).

There is however an explanation for why studies by both Lu et al. (2009) and Liu et al. (2007) showed correlations between CD68 expression and survival outcome as CD68 antigen is expressed in both M1 and M2 phenotype. Fujii et al. (2012) showed that the mean CD68 count was only slightly higher than the mean CD163 count in OSCC. This suggests that the majority of the measured CD68-positive macrophages are of the M2 phenotype in tumours. This hypothesis is strengthened by the study performed by Costa et al. (2013), wherein a predominance of M2 phenotype is present in the tumour microenvironment. Although they did not look at expression of CD163, they innovatively circumvented this problem by looking at the expression levels of pro- and anti-inflammatory cytokines in the tumour microenvironment. They found that expression of anti-inflammatory cytokines (related to M2) was higher in OSCC than pro-inflammatory cytokines and thus suggested a predominance of M2 macrophages in OSCC. After having shown that the macrophages in the study group of Costa et al. (2013) were predominantly of the M2 phenotype, survival analysis showed poorer survival which was associated with elevated CD68 count, indicating that it is likely that it is the subgroup of TAMs, the M2 macrophages, that are associated with poor prognosis.

If CD68-positive macrophages were predominantly of the M2 phenotype, it would have been expected that the studies performed by Hu et al., Fujii et al., and He et al., 2014 would have also noted a correlation between CD68 count and survival. This was however not the case and raises another inconsistency. A possible explanation for this might be related to the cut-off value used to dichotomize the TAM count in groups of low and high expression. Marcus et al. (2004) researched IMC in OPSCC, did not find a significant correlation between CD68 count and survival but did note that the insignificance may be due to the cut-off value that was determined. The scientists determined cut-off values for TAMs themselves and may have been subject to bias, especially, if they had prior knowledge that specifically CD163 macrophages were correlated with poor prognosis.

From the reviewed literature it becomes clear that in HNSCC TAM density is generally correlated with poor prognosis and worse survival. Macrophages within tumour tissue are also predominantly of the M2 phenotype. It is thus interesting to briefly discuss the mechanisms through which carcinomas attract and activate the macrophage subsets, and what role the pro—tumourogenic macrophages play in in the multifactor process of cancer development. Chemokines provide the directional incentive for the movement into tumours and maturational stimulus for the development of monocytes into macrophages (Bingle et al., 2002). The major cytokines involved in this process that are produced by tumours are monocyte chemotactic protein-1 (MCP-1) and macrophage colongy stimulating factor (M-CSF) (Bingle et al., 2002). Mechanistically, tumour cells synthesize M-CSF, which in turn causes resident macrophages to produce epidermal growth factor (EGF). EGF recruits monocytes into the tumour area (Qian & Pollard, 2010). T helper cells in the tumour area produce IL-4, which as discussed above, induces type 2 macrophage activation, resulting in macrophages with an M2 pro-tumorgenic phenotype. M2 macrophages then produce a varitety of chemokines, in particular VEGF, promoting angiogenesis and allowing tumour growth (Biswas and Montavani, 2010). Once this motility is initiated, it continues in a EGF – M-CSF paracrine loop, with macrophages and tumour cells moving in lock step allowing growth of the tumour (Qian & Pollard, 2010). Monocyte chemotactic protein 1 (MCP-1) also plays a role. Chemokine receptor

CCR2, which binds MCP-1, is also associated with a pro-tumourogenic role (Sicca et al., 2000). Binding of this receptor further enhances M2 macrophage immunosuppressive activity.

Malignant tumours are unable to grow beyond the size of 2-3mm^3 without angiogenesis (Bingle et al., 2002; Marcus et al., 2004). The ability of cancer tissue to induce vascularization allows for the increased influx of nutrients and oxygen and efflux of waste products, as well as providing a route for tumour cells to metastasize. Angiogenesis is a complex multistep process reliant on local signals produced within the tumour microenvironment which comprises the degradation of extracellular matrix around a local venule, the propagation of capillary epithelial cells and their differentiation into functioning capillaries within the tumour (Bingle et al., 2002). As discussed earlier, macrophages are highly versatile cells and through secretion of matrix-degrading enzymes and cytokines can vascularize the tumour microenvironment. M2 macrophage expression of the cytokine VEGF, a key component of the angiogenic process, is vital for the creation of vasculature (Liu et al., 2007; El-Rouby 2004). What is intriguing however, is that tumour cells have also been reported to express VEGF, which in addition to inducing angiogenesis, is thought to have a chemotactic effect on TAMs, directing their migration into avascular areas. This creates a positive feedback loop, speeding up the process of angiogenesis, as well as strengthening the recruitment of M2 macrophages (Barleon et al., 1996; Bingle et al., 2002).

The ability of macrophages to produce anti-inflammatory cytokines contributes to the general the suppression of the immune system involved in inhibiting tumour development. M2 macrophage production of the chemokine TGF-β is particularly important for the inhibition of Th1 cells and cytotoxic T-lymphocytes, two major players in anti-tumour activity (Costa et al., 2013; Liu et al., 2007). TGF-β is also closely related with the generation of FoxP3-positive T regulatory cells (Tregs). The role of TGF-β mediated immunosuppression by Tregs is well documented in colorectal cancer (Zamarron & Chen, 2011; Somasundaram et al., 2002). These Tregs are a subpopulation of $CD4^+$ T cells and play an important role in tumour development by inhibiting the immune response against cancer cells. Normally, Tregs function to suppress the activation of effector immune cells that are specific for self-antigens, but in cancerous tissue may act counter-productively and suppress anti-cancer cell immunity. (He et al., 2014; Zamarron & Chen, 2011). Macrophages are clearly an integral part in tumour development and progression, with M1 macrophages in a protective role in tumourgensis and M2 macrophages in a pro-tumourogenic role. As has been demonstrated by several studies above, TAM are predominatly M2 phenotyped and TAM are thus implicated with poor prognosis. Taken together, these are reasons that explain why CD68 and CD163 count is often associated with tumour staging, metastasis, decreased survival and in general poor prognosis.

When comparing studies in this systematic literature review to studies of other solid tumours, largely similar results are found. The majority of studies suggest that TAM is advantageous for tumour growth and correlates with poor prognosis in many cancers including lymphoma, cervival cancer, lung cancer, gastric cancer, bladder cancer and breast cancer (Bingle et al., 2002, Lin et al., 2011, Weber et al., 2015, Zhang et al., 2012). TAM infiltration is also associated with more tumour metastasis. Contrastingly, other research studies present conflicting results showing that increased levels of TAM are associated with positive prognosis in melanomas, prostrate cancer, and colorectal cancer. Furthermore, there have also been studies that have not been able to find correlations between TAM infiltration and prognosis (Lin et al., 2011, 2015, Heusinkveld & van der Burg et al., 2011, Zhang et al., 2012). Importantly, not all of the studies above determined the polarizations of the macrophages, as most of the studies were

based on detecting macrophages according to the CD68 marker. It has been suggested that the ratios of M1 versus the M2 phenotype should be considered for prognosis, with improved survival associated with a high M1/M2 ratio (Heusinkveld & van der Burg, 2011).

In conclusion, even though there is controversy among studies, it is evident that TAMs can elicit diverse effects on the tumour microenvironment and could therefore be used as prognostic indicators for various types of cancer in the future. To date, very little is known about the exact roles of many of the subtypes of head and neck cancers, with this systematic literature review only looking at OSCC, OPSCC and supraglottic laryngeal carcinomas. This review has elucidated that in HNSCC, TAMs are predominantly of the M2 phenotype and are generally related to decreased survival outcome and poor prognosis. This paper delved into some of the underlying reasons why macrophages, especially M2 macrophages, are so prevalent in tumour mircroenvironments and how these macrophages can influence tumour development and progression. Future research would benefit from exploring the value of TAMs in other HNSCCs as well as looking more closely at the links between TAM populations and Treg cells with immunosuppressive behaviour. The second paper in this thesis will therefore investigate the prognostic value of macrophages in NPC, a topic on which there is currently very limited knowledge. In addition this article will also explore associations between TAMs and FoxP3-positive Tregs and see if this is related to clinicopathological characteristics and survival outcome. Since NPC differ so significantly from other HNSCC in occurrence, causes, behaviour and therapeutic considerations this is of particular importance.

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APPENDIX

Appendix 1. Descriptions of the criteria used to score the risk of bias in the critical appraisal (Ooft et al., 2016).

Risk of bias	Description
In/exclusion criteria	Were the in/exclusion criterion well defined. Was defined which patients were included in the study (e.g. co-morbidity, gender, age). Were the included patients adequately chosen?
Selection process	Did the study give adequate information about the setting in which the patient was chosen, and also about the selection process itself?
Inception cohort	Were the patients chosen at a similar time/stage of their disease?
Outcome precise and valid	How were the outcomes measured? How was the technique used to measure the outcome? What materials were used to measure the outcome?
Estimation of prognosis	Was there a p value given or a 95% confidence interval to adequately judge the significance of the measured results
Follow-up duration:	Was there mention of follow-up duration?
Complete follow-up	Was there reporting on the loss-to-follow-up? Was there reporting on the reasons of loss-to-follow-up? Was there mention of censured observations?
Difference follow-up duration	Was there in the statistical evaluation of the results consideration for the differences in follow-up duration between individual patients in the study?
Other relevant prognostic factors	Were other prognostic factors such as age, TNM stage, gender looked into as covariates?
Standardization	Was there proper mention of the materials and methods used in the study (including brand of the antibodies used)?
Blinding	Were the evaluator(s) of the results of the tests blinded to the patient/tumor characteristics?
Kappa	Was there a Kappa value measured for the evaluator(s)?
Selection bias	Was there any form of bias in selecting the patients for the study?
Evaluation of immunostain	Was the right type of staining pattern measured (e.g. cytoplasmic staining) and how was the staining scored?

The Prognostic Significance of Tumour-Associated Macrophages in Nasopharyngeal Carcinomas

INTRODUCTION

Nasopharyngeal carcinoma (NPC) as defined by the WHO is "a carcinoma arising in the nasopharyngeal mucosa that shows light microscopic or ultrastructual evidence of squamous differentiation" (Barnes, 2005). NPC is widely regarded as an Epstein-Barr virus (EBV) associated disease and differs significantly from other cancers of the head and neck region in occurrence, causes, natural behaviour and therapeutic considerations.

NPC shows a distinct racial and geographical distribution and multifactorial etiology with NPC being particularly more common in South-East Asia and North Africa (Barnes, 2005). Worldwide, there are 80,000 incident cases resulting in an estimated 50,000 deaths annually (Jain et al., 2016). NPC has highly malignant behaviour with extensive loco-regional infiltration, early lymphatic spread and very high incidence of haematogenous dissemination (Barnes, 2005). The anatomical proximity of the nasopharynx to critical areas in the head and neck add to the treatment difficulty. NPC is a chemo-sensitive disease and the 5-year survival rate in the disease stages 1 and 2 is high; over 80% but 5-year survival rates in stage 4 are very poor; less than 10% (Jain et al. 2016). NPC is most common in adults between the ages 40 and 60 years but the tumour does present in children as well. Interestingly, African children are more commonly affected than Chinese children. There is strong male to female incidence ratio of approximately 3:1, irrespective of the geographic location (Thompson, 2007).

Macroscopically this neoplasm is usually found on the lateral wall of the nasopharynx and is frequently seen at the pharyngeal recess (Rosenmüller's fossa) posteromedial to the medial crura of the eustachian tube opening in the nasopharynx (Wei & Sham, 2005). NPC is usually an exophytic tumour (approximately 75%), with less than 10% described as being ulcerated. The tumour often presents as a smooth, discrete raised nodule below the mucosa (Thompson, 2007).

Currently the TNM scoring system (**T**umour size, Lymph **N**odes affected, **M**etastasis) and present WHO histologic classification systems are used to assess patients presenting with NPC but neither of these methods has significant prognostic value (Wang et al., 2011). NPC patients within similar clinical stages frequently undergo considerably different clinical courses underlining the necessity to develop more precise methods for predicting NPC prognosis. The current WHO classification broadly divides NPCs into three major groups according to their histology; (1) keratinizing squamous cell carcinoma (WHO-1), (2) non-keratinizing carcinoma (WHO-2) and (3) basaloid squamous cell carcinoma (WHO-3) (Barnes, 2005). Type 1 NPC shows squamous differentiation with the presence of intercellular bridges and/or a large amount of keratinization. Type 2 NPC can be further subdivided into differentiated and undifferentiated carcinomas. These tumours are generally more radiosensitive than type 1 and have stronger associations with EBV (Wei & Sham, 2005). It should however be noted that squamous cell carcinoma and non-keratinizing carcinoma have been viewed by some researchers as variants of a fairly homogenous group of tumours (Barnes, 2005).

Evidence suggest that major etiological factors involved in influencing the development of NPC are both environment and genetic, including exposure to nitrosamines in salted foods, genetic polymorphisms, as well as certain human leukocyte antigen subtypes (Chou et al., 2011; Lo et al., 2004). NPC shows a very strong association with EBV infection, irrespective of the ethnic origin of the patients with EBV-associated disease positive serology of EBV found in close to 100% of patients with non-keratinizing NPC (Jain et al., 2016; Barnes, 2005). In contrast to other HNSCC, this is a unique feature of NPC. Latent EBV infection is identified in virtually all patients with NPC in endemic regions. (Lo et al., 2004). EBV latently infects around 90% of the world's adult population and its association with NPC is thought to be mediated through a variety of factors including environmental ones (with smoking and samples of salted fish thought to be EBV-activating) and genetic pre-dispostion (high risk HLA allotypes) (Yu & Yuan et al., 2002; Jain et al., 2016). Expression of viral antigens (EBV in particular) makes the disease an attractive target for immunotherapy strategies in the future (Jain et al., 2016).

NPC is characterized by considerable amount of tumour infiltrating lymphocytes (TIL) consisting of T cells, B cells, dendritic cells, macrophages and eosinophils (Jain et al., 2016). As discussed in the systematic review, tumour-associated-macrophages (TAMs) are the predominant leukocyte infiltrate in the tumour microenvironment. The detection of TAMs is predominantly based on the use of antibodies to the glycoprotein CD68 (Heusinkveld & van der Burg; 2011). CD68 is a pan-macrophage marker and identifies both M1 and M2 macrophages. CD163 is a hepatoglobin scavenger receptor and a more specific marker of the M2 macrophage phenotype. In the systematic review above, FoxP3-positive T regulatory (Treg) cells were briefly discussed and implicated in tumour progression as well. FoxP3, also referred to as scurfin, is a specific marker of the natural- and adaptive- T regulatory (Tregs) cells (Costa et al., 2014; Heusinkveld & van der Burg, 2011). This study used immunohistochemistry (IHC) to measure infiltrating leukocyte count in tissue specimens of patients that presented with NPC. As a first study of its kind, the prognostic role of TAMs and FoxP3-postive Treg cells was examined in different subsets of NPC.

METHODOLOGY

Patients and tissue specimens

This study looked at 92 tissue samples of patients with NPC. All samples were sequentially diagnosed formalin-fixed paraffin-embedded (FFPE) NPC specimens. For the purpose of this study, the clinicopathological records of the NPC patients collected at the University Medical Center Utrecht (UMCU) were retrospectively analyzed. The code of conduct for the use of human tissue in medical research as stipulated in "De Gedragscode Gezondheidsonderzoek," states that no ethical approval is required for the use of anonymous leftover tissue (www.federa.org). Note that this is also part of the standard treatment agreement with patients at the UMCU (source).

All hematoxylin and eosin (HE) histological slides were inspected by two head and neck pathologists as well as a senior pathology resident, all experienced in the evaluation of NPCs. Characterizations of prognostic terms are as follows; the date of diagnosis was defined as the date on which the tissue sample was extracted from the patient. Disease-free survival (DFS) was the survival time from extraction to the date that recurrence of the disease as was experienced by the patient, or at which recurrence was determined by a physician. Overall survival (OS) was the survival time from diagnosis to death due to any cause.

Tissue microarray construction

Tissue microarrays (TMAs) were used for the high throughput analysis of the samples. This technique is hugely advantageous in allowing large amounts of data to be obtained rapidly through the use of a single immunostaining protocol to avoid experimental variability (Kampf et al., 2012). The TMAs were created from the 92 FFPE tissue samples using the TMA Grand Master instrument (3D HISTECH, Budapest, Hungary). Tumour areas were marked by a head and neck pathologist (SW) and a senior pathology resident (MO), both experienced in the histological evaluation of NPCs. From each specimen three tissue cylinders (chores) with a 0.6mm diameter were punched out from the marked tumour areas and arrayed into a recipient paraffin blocks (Noorlag et al., 2014).

EBV status

EBV status was identified by applying EBV-encoded RNA (EBER) in situ hybridization (ISH) to the TMA. A BenchMark ULTRA automated staining instrument (Ventana Medical systems, Tuscon, AZ, USA) was used for ISH of the TMA using an EBV specific probe (INFORM EBER (EBV Early RNA) PROBE) (Ventana Medical systems) and ISH iVIEW Blue detection kit (Ventana Medical Systems) was used for staining according to the manufacturers' protocol.

Immunohistochemistry

A BenchMark ULTRA automated staining instrument (Ventana Medical systems, Tuscon, AZ, USA) was used for immunohistochemical staining of the 3 antibodies CD68 (Novacastra, CC1 24', mouse polyclonal, lot 6018783, 1:1600), CD163 (Novacastra, CCL 24', mouse polyclonal, lot 6006014, 1:400) and FoxP3 (Abcam, mouse polyclonal, lot GR108410-1, 1:2000). TMA sections of 4µm thickness were cut, and then heated to 75°C for 8 minutes and then rinsed by EZPrep solution to deparaffinate them. Next, the samples were pretreated with Long Cell Conditioner at 100 °C for 16 minutes and then a peroxidase inhibitor for 4 minutes. Subsequently the primary antibodies were added to the TMA and incubated for 32 minutes. Following application and incubation of the primary antibody, the slides were incubated with Optiview HQ Universal Linker and Optiview HRP multimer (Ventana Medical Systems) for 8 minutes. Next the samples were treated with hydrogen peroxide and DAB. Finally

counterstaining with haematoxylin was performed. This was followed by the counterstaining by adding haematoxylin and bluing reagent. Each consecutive step of the staining process was intermediated by the rinsing of the samples with a reaction buffer.

Quantification of tumor infiltrating lymphocytes

A head and neck pathologist (SW) and a researcher (MS) both blinded to the clinical characteristics of the patients, evaluated the leukocyte prevalence per core. For all the immunological markers, CD68, CD163 and FoxP3, the numbers of positive stained cells were manually counted at 20X magnification under a light microscope (type of microscope). Counting was carried out in a semi-quantitative method with leukocytes counted in groups of ten. Normalization of the results per area evaluated was unnecessary as each TMA has a diameter of 0.6 mm. If full core was available but still contained tissue, the data was extrapolated to represent a full core. If less than half a core was available data for that core was considered missing and excluded from the analysis. Cores that were clearly damaged/not viable were also entered as missing. To be included in the data analysis at least one core per patient needed to be scored. The average values for the counts of CD68, CS163 and FoxP3 were calculated as the mean count. A receiver operating characteristic (ROC) curve analysis, optimized for OS was utilized to determine a cut-off value per immunological marker, which allowed them to be dichotomized.

Statistical analysis

The relationship between the expression of the immunological markers CD68, CD16, FoxP3 and clinicopathological parameters was analyzed using the statistical program IBM SPSS Statistics software for Windows (version 22). The following covariates of interest were dichotomized for survival analysis: age (with 53 as the cut-off year), T status (T1/2 or T3/4), tobacco and alcohol usage, NPC histological subtype (keratinizing or non-keratanizing carcinoma) and EBV status (positive or negative). The likelihood of univariable independence between EBV-positive and EBV-negative groups was performed using the Pearson X^2 test (and the Fisher's exact test when appropriate) for categorical variables and the ANOVA for continues variables. Difference in mean counts of CD68, CD163 and FoxP3 between the EBV-positive and the EBV-negative NPCs were determined using a Pearson chi-square test and visualized using box-plots. Survival analyses were performed to establish the prognostic role of immunocytes in NPC. OS and DFS survival curves were calculated with the Kaplan-Meier method, using the log-rank test as a check for significance. Associations between immunomarkers, clinicopathologic parameters and survival outcome were examined by univatiate analyses using the Cox proportional hazards model for survival. Significance was based on a 2-tailed statistical analysis with $p < 0.05$ being considered statistically significant.

RESULTS

Patients/ demographic data

The assessment of 92 patients with NPC in this analysis revealed that 63 were men (68.5%) and 29 were women (31.5%). The age ranged from 10 to 87 years with a mean of 53.5. With regards to the last follow up, which ranged from 1 to 52 months, the mean survival time was 38.1 months (95% CI = 34.2–41.7). The patients showed either keratinizing (14.1%) or non-keratinizing (84.8%) NPC. Note that no cases of basaloid squamous cell carcinoma were seen. The majority (65.2%) of the patients were EBV-positive. EBV positive NPCs were associated with a non-keratinizing histological phenotype. Details concerning clinical and microscopic finding are summarized in table 1. T1/2 stage at diagnosis occurred more frequently in EBV-positive NPC, while contrastingly, T3/4 stage correlated with tumours that had a negative EBV status.

Table 1. Characteristics of the study population summarized (%) (n=45)

Clinical and microscopic features	%
Age	
<= 53.45	53.3
>53.5	46.7
Gender	
Male	68.5
Female	31.5
Tobacco usage	
Yes	35.9
No	19.6
Missing	44.6
Alcohol consumption	
Yes	26.1
No	27.2
Missing	46.7
T stage	
T1/2	50.0
T3/4	40.2
Missing	9.8
Clinical outcome	
Dead	73.9
Alive (OS)	20.7
EBV status	
Negative	31.5
Positive	65.2
Missing	3.3
NPC subtype	
Keratinizing	14.1
Non-keratinizing	84.8
Missing	1.1

Clinicopathological characteristics

Age was associated with EBV status, with higher age being related to negative EBV status. EBV positive NPC was associated with a non-keratanizing histological phenotype with virtually all EBV-positive NPC showing this phenotype. Tobacco usage, alcohol and gender were all not associated with EBV status. The AJCC staging system, utilizes the TNM scoring system (**T**umour size, Lymph **N**odes affected, **M**etastasis), was the classification system used to described cancer progression in the patient cohort. T status in patients presenting a carcinoma at T1/2 stage at diagnosis more frequently showed EBV-positive NPC phenotype. Neither of the N nor M scoring showed significant association with EBV status. A summary of the clinicopathological features of the cohort is shown in table 3.

Differences in expression of TAM between EBV-positive and EBV-negative NPCs

Boxplots were drawn to illustrate the differences of immunomarker counts between EBV-positive and EBV-negative NPCs (Fig. 1). Statistical significance was determined using the Pearson chi-square test. The mean immunomarker counts of CD68 and FoxP3 were significantly higher in EBV positive cells compared to EBV negative cells. There was no significant difference between CD163 counts between the two EBV groups.

The mean CD68, CD163 and FoxP3 counts were dichotomized into two groups of either high or low expression. ROC curves were used to determined cut-off points, and cut-off was determined at the point with the highest sensitivity and specificity. The optimal cutoffs points were 114 for CD68, 128.5 for CD163 and 41.5 for FoxP3. CD68, CD163 and FoxP3 were all more numerous in EBV-positive NPCs, however only CD68 and FoxP3 showed statistically significant differences between EBV groups (Table 2).

CD68 and FoxP3 expression correlated significantly with positive EBV status. High CD68 and FoxP3 expression groups were associated with EBV-positive NPCs, whereas low CD68 and FoxP3 expression was strongly associated with EBV-negative NPC.

Table 2. Differences between expression levels of immunomarkers between EBV groups

	Total known EBV (n)	**EBV positive NPC**	**EBV negative NPC**	**P value**
CD68				**0.001**
≤114	35	17	18	
>114	49	41	8	
	Total: 84	Total: 58	Total: 26	
CD163				0.552
≤128.5	56	37	19	
>128.5	29	21	8	
	Total: 85	Total: 58	Total: 27	
FoxP3				**0.006**
≤41,5	55	13	14	
>41,5	27	43	12	
	Total: 82	Total: 56	Total: 26	

Significant values are depicted in bold.

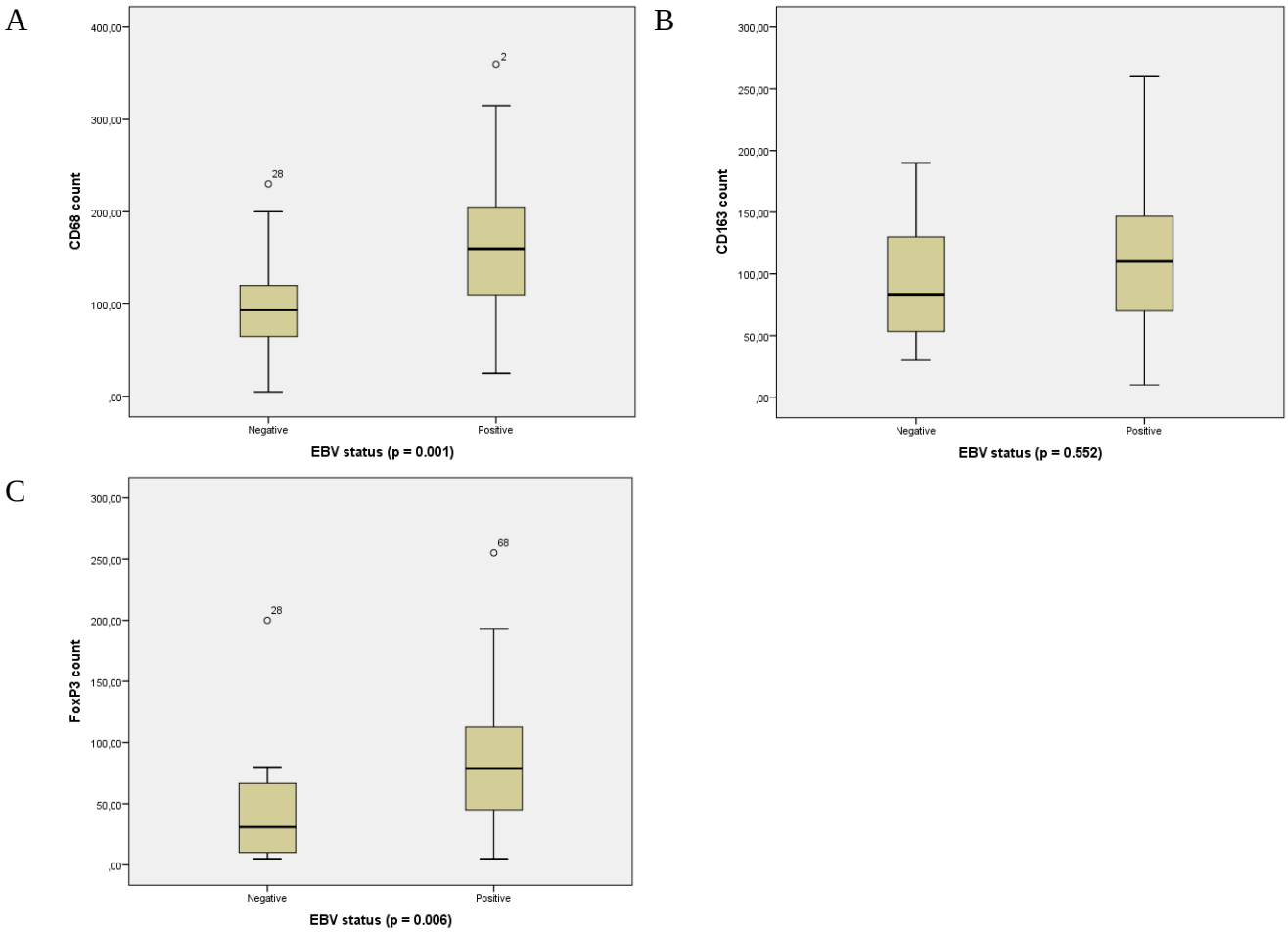

Figure 1. Boxplots illustrating the differences in CD68, CD163 and FoxP3 counts between EBV-positive and EBV-negative NPCs. CD68 and FoxP3 counts differed significantly between the two EBV statuses, whereas CD163 count did not.

Differences between NPCs with high and low expression of FoxP3

There was a significant correlation between both CD68 and CD163 with high FoxP3 expression (table 3). High CD68/CD163 expression was associated with high FoxP3 expression and vice versa, low CD68 was associated with low FoxP3 expression. CD68 and CD163 were also more numerous in NPCs with high FoxP3 expression.

Table 3. Differences between expression levels of CD68 and CD163 between FoxP3 groups

	Total NPCs with FoxP3 expression (n)	**Association with FoxP3 count > 41.5**	**Association with FoxP3 count ≤ 41.5**	**P value**
CD68				**0.002**
≤114	32	14	18	
>114	50	39	11	
	Total: 82	Total: 53	Total: 29	
CD163				**0.006**
≤128.5	28	30	24	
>128.5	54	24	4	
	Total: 82	Total: 54	Total: 27	

Significant values are depicted in bold

Table 4. Summary of the clinicopathological features of the cohort

	Total known EBV (n)	**EBV-positive NPC**	**EBV-negative NPC**	**P-value**
Age	Mean 53.5 (SD 15.409) Total: 92	Mean (50.1) N = 60	Mean (59.3) N = 29	**0.007**
Sex Male Female	 63 26 Total: 89	 46 14 Total: 60	 17 12 Total: 29	0.079
NPC subtype Keratinizing Non-keratinizing	 12 76 Total: 88	 0 60 Total: 60	 12 16 Total: 28	**<0.001**
Smoking Yes No	 33 17 Total: 50	 21 13 Total: 34	 12 4 Total: 16	0.357
Alcohol Yes No	 24 24 Total: 48	 15 16 Total: 31	 9 8 Total: 17	
AJCC T-stage T1 T2 T3 T4	 25 21 12 23 Total: 81	 18 18 7 13 Total: 56	 7 3 5 10 Total: 25	0.160
T1/2 versus T3/4 T1/2 T3/4	 46 35 Total: 81	 36 20 Total: 56	 10 15 Total: 25	**0.042**
AJCC N-stage N0 N1 N2 N3	 23 21 34 2 Total: 80	 16 15 24 1 Total: 56	 7 6 10 1 Total: 24	0.954
N0 versus N1/N2/N3 N0 N1/N2/N3	 23 57 Total: 80	 16 40 Total: 56	 7 17 Total: 24	0.957
AJCC M-stage M0 M1	 45 3 Total: 48	 32 1 Total: 33	 13 2 Total: 15	0.227
Therapy Radiotherapy Chemotherapy Radio/chemotherapy Other	 20 57 1 4 Total: 82	 13 1 41 2 Total: 57	 7 0 16 2 Total: 25	0.440

Significant values are depicted in bold.

Survival analysis

To predict the prognostic value, survival analysis was carried out using Kaplan-Meier curves with log-rank test as well as univatiate Cox's proportional hazard regression models (table 5). Survival analysis was carried out in three groups, the whole NPC group (n=92), and both EBV-positive (n=60) and EBV-negative groups (n=29). Within the whole cohort the mean OS and DFS times were 52 and 45 months respectively. In the purely the EBV-positive group the OS and DFS times were 57 and 49 months respectively. In contrast, EBV-negative group showed shorter OS and DFS times of 43 and 36 months respectively.

NPC group as a whole.

Despite correlations with many poor prognostic factors, the macrophage count, measured by both CD68 and CD163, was not found to significantly correlate with OS (Fig. 3 e, f) nor with DFS. EBV status was however associated with OS ($p=0.042$), with the EBV-negative phenotype related to decreased OS. In addition tumour stage (T1/2 versus T3/4, $p=0.004$) and metastasis (M0 versus M1, $p<0.001$) were all significantly associated with OS, with later T staging (T3/4) and metastasis (M1) both being correlated with poorer survival (Fig. 3 a, b, c respectively). Age, gender, tobacco and alcohol usage, N stage and NPC histological subtypes on the other hand were not significantly associated with survival. FoxP3 count was significantly correlated with both OS and DFS, with higher FoxP3 count associated with improved prognosis. All hazard ratios are shown in table 4.

EBV-positive and EBV-negative NPC groups

Of all the studied markers, CD68, CD163 and FoxP3 none had any significant correlation with neither OS nor DFS. All clinicopathological covariates did not correlate with DFS in the EBV positive subgroup. In contrast, in the EBV negative subgroup, history of tobacco usage ($p=0.008$), T1/2 stage ($p=0.003$) and M0 ($p<0.001$) all predicted improved survival.

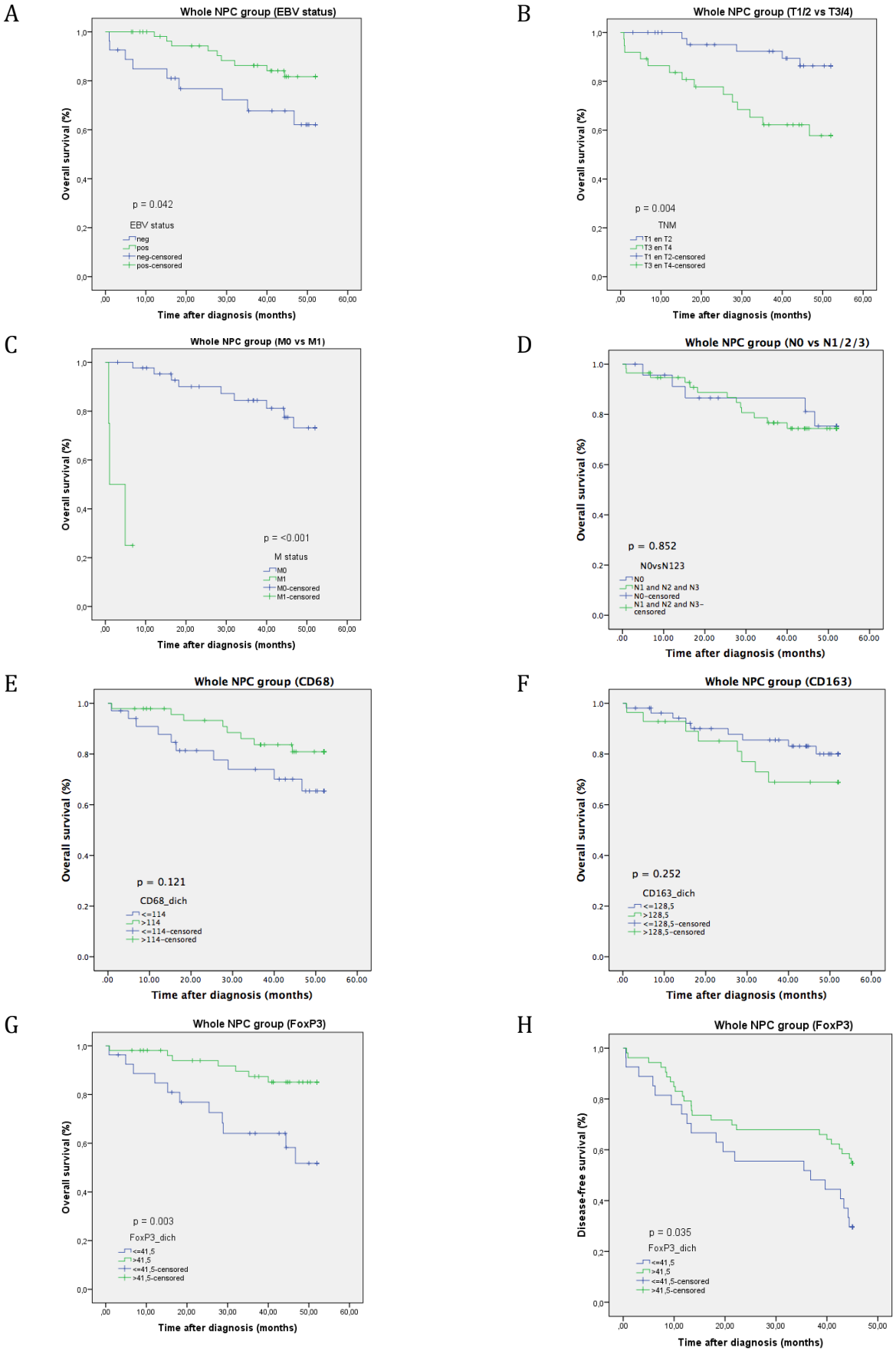

Figure 2. Kaplan-Meier survival curves showing relationships between clinicopathological factors and survival.

Table 5. Summary of univariate cox regression analysis for survival

Clinical Parameter	**p value**	**HR (95%CI)**
CORRELATION WITH OS		
Whole NPC group (n=92)		
CD68	0.300	0.596 (0.233-1.588)
CD163	0.300	1.711 (0.620-4.719)
FoxP3	**0.022**	0.313 (0.116-0.844)
T1/2 vs T3/4 - stage	**0.028**	3.283 (1.139-9.464)
Age	0.109	2.290 (0.832-6.308)
Gender	0.172	1.991 (0.741-5.349)
Smoking	0.774	1.225 (0.306-4.908)
Alcohol	0.728	0.767 (0.171-3.426)
NPC subtype	0.122	0.409 (0.132-1.270)
EBV	**0.050**	0.396 (0.157-0.999)
EBV-positive NPC (n=60)		
CD68	0.356	0.538 (0.144-2.006)
CD163	0.329	1.996 (0.499-7.989)
FoxP3	0.212	0.433 (0.116-1.613)
EBV-negative NPC (n=29)		
CD68	0.831	1.177 (0.262-5.292)
CD163	0.498	1.680 (0.375-7.524)
FoxP3	0.137	0.282 (0.053-1.493)
Smoking	**0.023**	0.149 (0.029-0.766)
CORRELATION WITH DFS	**p-value**	**HR (95%CI)**
Whole NPC group (n=92)		
CD68	0.347	0.744 (0.401-1.379)
CD163	0.574	0.824 (0.419-1.620)
FoxP3	0.090	0.538 (0.313-1.088)
EBV-positive NPC (n=60)		
CD68	0.723	0.865 (0.388-1.928)
CD163	0.795	0.895 (0.389-2.060)
FoxP3	0.966	0.981 (0.412-2.337)
EBV-negative NPC (n=29)		
CD68	0.481	0.658 (0.206-2.103)
CD163	0.375	0.560 (0.156-2.015)
FoxP3	**0.044**	0.301 (0.094-0.969)

Significant values are depicted in bold.

DISCUSSION

As discussed in the systematic literature review above, macrophages are highly versatile cells that react to the environmental cues presented to them in the tumour tissue with the release of various growth factors, cytokines, chemokines and enzymes that can regulate tumour growth, angiogenesis, invasion and metastasis (Lewis and Pollard, 2006). They exist as roughly two distinct polarization states, classically activated M1 macrophages and alternatively activated M2 macrophages (Heusinkveld & van der Burg, 2011). There is increasing amounts of evidence implicating the role of TAMs in tumour progression with many showing correlations between macrophage density and prognosis in a variety of cancers. Three types of HNSCC are discussed in the systematic literature review, but to date, no studies assessing the prognostic value of macrophages have been performed in NPC. NPC differs curiously from other cancers of the head and neck and is regarded as the prototype of a family of morphologically distinctive tumours, the lymphoepithelial carcinomas. What makes NPC so interesting is its close association with EBV. This current study identified clinical and histological features that are predictive of decreased survival, as well as looking at differences between EBV status groups.

There is however controversy about whether TAMs positively or negatively inhibit tumour progression. Initially, macrophages were associated with a cytotoxic role on tumour cells, engulfing necrotic tissue and present tumour-associated antigens to T cells (Liu et al., 2007). Contrastingly, TAMs also became implicated with immunosuppression and creating vasculature for tumours. Consequently, TAMs became associated with reduced survival in other tumours. With the knowledge of the discussion in the literature review, it was hypothesized that there would be significant correlations with both CD68 and CD163 count and poor survival (for this hypothesis it was assumed that the majority of CD68-positive cells, were M2 macrophages). It is striking that in the current study TAM levels were not correlated with OS or DFS. High IMC has previously been described as correlating with T stage and lymph node metastasis. This was not found in the studied cohort.

As discussed in the systematic literature review above, the lack of significant association between CD68 and survival is not necessarily very surprising (Hu et al., 2016, He et al., 2014; Marcus et al., 2004, Fujii et al., 2012). CD68 is a pan-macrophage marker and includes both M1 and M2 macrophage phenotypes, which as discussed in the systematic literature review above, are thought to have opposing effects on the tumour microenvironment. However, CD163 was associated with deceased survival in all the studies systematically reviewed. In addition, M2 macrophage is associated with a pro-tumourogenic role, producing anti-inflammatory cytokines and indirectly inhibiting Th1 and cytotoxic T-lymphocytes (both involved in counter-acting tumour development). This discrepancy in overlap may be a result of the relatively short follow-up times in some of the patients or that the cohort was too small to see a significant difference. Both short follow-up times and the small cohort are major limitations of this study. It is also possible that cut-off value, determining high and low expression groups of TAMs, was not completely reliable. This is particularly true for the CD163 marker. The ROC curve for CD163 count versus survival time was suspiciously close to the line of equality, making the determination of a reliable cut-off point difficult. For future research it would be interesting to more specifically determine the M1 and M2 macrophage populations and see if these groups would reflect the hypothesized differences.

Interestingly, positive EBV status correlated with better OS. In addition, T1/2 stage at diagnosis occurred more frequently in EBV-positive status. Contrastingly, T3/4 stage tumours

correlated with EBV-negative status. Although both high CD68 and FoxP3 counts were significantly associated EBV-positive NPC, neither immunomarker was predictor of OS or DFS in EBV-positive NPCs. To date, very little is know about the different mechanisms of action of immune cells between EBV-positive and EBV-negative infected NPCs and should be area for future research.

Treg cells are naturally present in the immune system and are vital for the maintenance of dominant self-tolerance and immune homeostasis (Sakaguchi et al., 2010). Tregs are a subset of $CD4^+$ T cells and are broadly characterized by their expression of the nuclear transcription factor forkhead box p 3 (FoxP3) (Bronkhorst et al., 2012). FoxP3+ Treg cells are described to be able to suppress activation, proliferation and effector functions such as cytokine production or a diverse group of immune cells including CD4 and CD8 T cells, NK cells, B cells and antigen-presenting cells (Sakaguchi et al., 2010; Zamarron & Chen, 2011). It is thus expected that FoxP3 expression leads to more prevalent tumour growth by inhibiting the immune response against cancer. This was shown to be true in uveal melanomas by Bronkhorst et al. (2012) elevated levels of FoxP3 expression were associated with poor prognosis in tumours. As discussed in the systematic literature review, Tregs are integral in the process of suppressing the activation of effector immune cells that recognize self-antigens. The expression of TGF-β by M2 macrophages can activate Treg cells in the tumour microenvironment. Findings in the current study agree that there is an interrelationship between M2 macrophages and Treg, showing significant correlations between both CD68 and CD163 with high FoxP3 expression. Activated Tregs can subsequently prevent the action of $CD4^+$ and $CD8^+$ T cells and accordingly suppress anti-cancer cell immunity. High Treg expression is thus expected to be positively associated with tumour progression and result in poorer prognosis. Studies assessing FoxP3-positive Tregs in non-small cell lung cancer, ovarian cancer and colorectal cancer, showed that the prevalence of Treg cells to be much higher than in healthy patients and linked FoxP3-positive Tregs to tumour progression (Woo et al., 2001; Somasundaram et al., 2002).

Therefore, it is surprising that in the current study elevated FoxP3 count was associated with significantly improved OS and DFS. A possible explanation for this could be that Treg cells are very heterogeneous in gene expression, phenotype and function and that a new basis for reliable delineation is required as is suggested by Sakguchi et al. (2010). Another explanation is that FoxP3-positive Treg cell expression is linked to EBV status. FoxP3 counts correlated significantly with EBV status. CD68 also correlated positively with EBV status, but this might be a reflection of the interrelationship of macrophages and Treg cells. Note that significant results could potentially have been seen for CD163 as well, if a reliable cut-off point for CD163 count had been determined. High CD68 and FoxP3 expression groups were associated with EBV-positive NPCs, whereas low CD68 and FoxP3 expression was strongly associated with EBV-negative NPC. Furthermore, EBV status was significantly associated with OS, with better survival associated with positive EBV status. Thus correlations between high FoxP3-positive Treg count and better survival could in fact be a reflection of positive EBV status. A limitation of this study is that a multivariate cox regression was not performed to properly look at the independent prognostic value of FoxP3-positive Tregs. This could however not be done due to the small number of events for DFS and OS, and could be rectified by repeating the study in a larger cohort. For future research it would also be interesting to look at the relative distributions of M1 and M2 macrophages and see if they are then still correlated to FoxP3 expression.

There are a few limitations that should be mentioned. The cohort was quite small and that significant results may be uncovered if a larger group is studied. This applied in particular for when the group was divided in EBV positive and negative groups. The EBV-negative group consisted of solely 29 patients and could have led to false negative results. The limited number of OS and DFS events made it unreasonable to perform a multivariate survival analysis to assess the independence of variables. Finally, there is a risk of bias due to tumour heterogeneity because a TMA was used for the assessment of the TAMs.

CONCLUSION

In conclusion, this is a novel study looking at the prognostic value of TAM macrophage in NPC, a unique form of HNSCC that is closely associated with EBV. Since NPC differs so significantly from other cancers of the head and neck region in occurrence, causes, natural behaviour and therapeutic considerations it is important to identify suitable prognostic factors. Interestingly, elevated levels of Tregs, immune cells that have previously been associated with tumour progression, are in fact beneficial for survival in patients with NPC. This may however be related to the EBV status in NPCs. The pan-macrophage marker, CD68, and the Treg marker, FoxP3, were found to be associated with EBV virus, with significant differences in TAM density between the EBV-positive and –negative groups. This studied aimed to elucidate the prognostic value of TAMs but found that neither macrophage marker was significantly correlated with survival. For future research it would be interesting to determine the M1/M2 ratios and assess if there are correlations between the specific subsets, survival and the activation of Tregs. Finally, very little is still known about immune cells in NPCs with different EBV status. It would therefore be advantageous to explore the different mechanisms of action of the immune cells in the different NPCs, which could also open up an avenue specifically designed for immunotherapeutic treatments.

Conflict of Interest

There is no conflict of interest to declare.

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