The number and localization of CD68+ and CD163+ macrophages in different stages of cutaneous melanoma

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The role of tumor-associated macrophages (TAMs) in cutaneous melanoma is controversial. TAMs include immunogenic and immunosuppressive subtypes, and have distinct functions according to their microanatomical localization. Our aim was to investigate TAMs in benign, premalignant, and malignant melanocytic lesions to determine possible associations with tumor progression and clinicopathological characteristics. In total, 184 tissue samples, including benign and dysplastic nevi, in-situ melanomas, superficial (Breslow's depth <1 mm), and deep (Breslow's depth >4 mm) invasive melanomas and lymph node metastases, were analyzed for macrophage content. Samples were stained immunohistochemically for CD68 and CD163, representing all TAMs and M2-macrophages, respectively. Macrophages were counted by hotspot analysis, and assessed semiquantitatively from the tumor cell nests and stromal component of malignant cases. CD68+ and CD163+ TAMs were more abundant in invasive melanomas compared with benign nevi. The proportion of TAMs in the tumor nests was higher in deep melanomas and lymph node metastases compared with superficially invasive melanomas. High amounts of CD68+ macrophages in tumor cell nests were associated with recurrence, whereas low CD163+ macrophage proportion in tumor stroma was associated with recurrence and in primary melanomas also with poor overall survival. TAMs seem to promote tumor progression in cutaneous melanoma. In particular, CD68+ TAMs and their abundance in tumor nests were associated with poor prognostic factors. However, the correlation of low stromal CD163+ TAM proportion with a poor prognosis indicates that the role of TAMs depends on their subtype and microanatomical localization. *Melanoma Res* **29:237–247**

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Introduction

The incidence of cutaneous melanoma (CM) has increased during the past few decades in the Caucasian population and represents one of the most rapidly increasing cancers worldwide [1]. CM mortality is associated with local invasion and metastasis formation, and accounts for up to 80% of skin cancer-related deaths [2]. Recent studies have highlighted a significant role for the immune system in tumor control, and anticancer therapies activating the immune system have been promising. However, some tumors can resist immune cell attack by means of immunosuppression [3]. The interactions between malignant and stromal cells modify the tumor microenvironment (TME) to become anti-inflammatory and tumor promoting [4]. Thus, to improve the outcomes of immunotherapies, it is essential to gain a greater understanding of the formation of an immunosuppressive TME in melanoma.

Tumor-associated macrophages (TAMs) are part of the TME in many cancers. They are often classified into two main subsets [5]. Proinflammatory M1 macrophages are involved in the initiation steps of cancer by creating a mutagenic microenvironment, for example by producing free radicals [6]. In the more advanced stages of cancer, TAMs often differentiate into anti-inflammatory M2 macrophages, which enhance tumor growth by creating an immunosuppressive TME. The differentiation is mediated by hypoxia and various signaling molecules produced by the tumor and stromal cells. M2 macrophages lack antigen-presenting capacity and suppress T cell function by secreting immunosuppressive factors, for

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example interleukin-10. They recruit regulatory T cells into the tumor and directly inhibit T-cell function by expressing the immune checkpoint molecule PD-L1. They also promote angiogenesis, tumor invasion, and metastasis by producing proangiogenic molecules and proteolytic enzymes [5]. However, the division of TAMs into two distinct populations is an oversimplification as TAMs include several phenotypes that share properties from both extremities [6].

CD68 is a scavenger receptor expressed highly by tissue macrophages and generally considered a pan-macrophage marker, whereas the scavenger receptor CD163 is a highly specific marker for the M2 subpopulation. In most tumors, TAMs are often of the M2 phenotype [7]. However, the high amounts of CD68-positive and/or CD163-positive macrophages are found to correlate with advanced tumor stage and poor prognosis in several cancers, for example in breast [8] and ovarian [9] cancers.

The role of TAMs in CM is not established [10]. In some studies, TAMs have correlated with tumor stage and poor survival. Thus, dense CD163+ macrophage infiltration in tumor stroma and high CD68+ macrophage number in the invasive front predicted poor survival in stage I and II CMs [11]. Other studies [12–14] failed to find any association of TAMs with Breslow's depth, tumor stage, or unfavorable disease outcome.

In the present study, immunohistochemical staining of CD68 and CD163 in benign, premalignant, and malignant melanocytic lesions was analyzed to obtain a broader view of the immunological role of TAMs in melanoma tumorigenesis and TME. Melanomas of Breslow's depth lower than 1 mm and thicker than 4 mm were included in the study as these tumors have a clinically distinct prognosis. The aim was to further investigate the association of TAMs with tumor stage, clinicopathological parameters, and patient prognosis, and to study the significance of the macrophage localization in the tumor cell nests and stromal component.

Materials and methods

Histological specimens

Macrophages were analyzed from 184 cutaneous tissue specimens collected in Kuopio University Hospital (Finland) during the years 1980–2010. From a total of 224 tissue samples, highly pigmented samples and samples with large necrotic areas or destroyed tissue structure were excluded, as well as those melanin-containing samples from which the CD68+ macrophages could not be analyzed reliably on the basis of the cellular morphology (see the Materials and methods section), resulting in 184 adequate samples (Supplementary digital content 1, *http://links.lww.com/MR/A82*: Table showing the number of cases analyzed by hotspot analysis). Samples included 29 benign nevi (14 intradermal, 10 compound, and five junctional nevi), 27 dysplastic nevi, 16 in-situ melanomas, 78 invasive melanomas [36 superficial (Breslow's depth <1 mm) and 42 deep (Breslow's depth >4 mm) melanomas], and 34 lymph node metastases (LNM). Clinicopathological data were available from 88% (98/112) malignant cases. Standard histopathological parameters of the specimens were analyzed by an expert pathologist (R.S.). This study was approved by the research ethics committee of the Northern Savo Hospital District and by the Finnish National Supervisory Authority for Welfare and Health (VALVIRA, 6187/05.01.00.06/2010).

Immunohistochemistry

Tissue samples were separately stained immunohistochemically for CD68 and CD163 representing all TAMs and M2 macrophages, respectively. The staining was performed as described previously by Tiainen *et al.* [15] First, 5 μm thick formalin-fixed and paraffin-embedded samples were dewaxed and rehydrated. For antigen retrieval, specimens were microwaved four times (5 min each) in 0.01 mol/l citrate buffer and cooled for 15 min. Endogenous peroxidase was blocked with 1% hydrogen peroxide and unspecific staining with 1% bovine serum albumin in 0.1 mol/l phosphate buffer. Thereafter, the sections were incubated with the primary antibody in a +4°C humidity chamber overnight. The primary antibodies (mouse monoclonal antihuman CD68 and CD163 antibodies; Thermo Scientific, Rockford, Illinois, USA) were used at 1 : 1000 and 1 : 600 dilutions, respectively. Thereafter, the samples were incubated with biotinylated antimouse secondary antibody (1 : 200; Vector Laboratories, Burlingame, California, USA) at room temperature for 1 h, followed by incubation in the ABC reagent. 3,3'-diaminobenzidine (DAB) was used as a chromogen to visualize stainings and the nuclei were counterstained with Mayer's hematoxylin. Finally, the samples were dehydrated and mounted in Depex. The controls included sections treated in the same way, but with the primary antibodies omitted. Because some of the melanoma samples contained melanin, a red AEC chromogen was also used. However, this did not yield a staining pattern, which could better distinguish between immunopositivity and pigment.

Evaluation of CD68 and CD163 staining using a hotspot analysis

Macrophages were calculated using hotspot analysis [15]. CD68+ and CD163+ macrophages were analyzed from separate tissue sections. The areas of the highest densities of macrophages were first detected by scanning the sections at a low magnification (40–100×) using an Axio Lab.A1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Depending on the size of the lesion, macrophages were counted from either three or five hotspots in the tumor cell nests and the stromal connective tissue compartment, including the invasive front. Three hotspots were picked if the lesion area was small, as it was in

most cases of benign nevi. Otherwise, five hotspots were evaluated. The number of macrophages in each hotspot was calculated from the picture taken with the microscope camera (Axiocam ERc5s; Carl Zeiss) at 200× magnification. Finally, the average of CD68 and CD163 macrophage numbers was calculated for each sample. Macrophages near the necrotic areas were omitted. The investigators (S.S., B.H.G.) were blinded to the clinicopathological data during the sample evaluation.

In total, 35 samples (four dysplastic nevi, four in-situ melanomas, five <1 mm melanomas, 15 >4 mm melanomas, and seven LNMs) contained melanin pigment and these were evaluated together with an expert pathologist (R.S.). CD163 antibody stains a macrophage subpopulation and therefore CD163+ macrophages could not be assessed from the melanin-containing samples as immunopositivity could not be distinguished reliably from melanin. CD68+ macrophages were evaluated from the melanin-containing samples on the basis of their cellular and nuclear morphology. In more challenging cases, macrophages were evaluated from the stromal compartment only to ensure that melanin-containing malignant cells were ruled out. If the hot spots could not be found in the stromal compartment, the sample was discarded. The number of cases analyzed in each group by hotspot analysis is specified in supplementary material (Supplemental digital content 2, *http://links.lww.com/MR/A83*, Table showing numbers of CD68 and CD163 stainings evaluated by hotspot analysis).

Semiquantitative evaluation

The macrophage content in invasive melanomas and LNMs was also assessed semiquantitatively using a five-level scoring system from 0 to 4. CD68-immunostained slides were digitalized using a whole-slide digital scanner (NanoZoomer-XR; Hamamatsu Photonics, Hamamatsu, Japan). Score 0 was assigned if the macrophage content was 0–5% of all cells and scores 1, 2, 3, and 4 were assigned if the macrophages comprised 6–25%, 26–50%, 51–75%, and 76–100% of the cells, respectively. Macrophage proportions were assessed separately from tumor cell nests and stroma. The stromal compartment included the stromal area below the invasive front and the thick stromal strands separating the tumor nests. The whole lesion was evaluated.

As in the case of the hotspot analysis, CD163+ macrophages could not be assessed from melanin-containing samples. The number of cases in each group analyzed by the semiquantitative method is specified in supplementary material (Supplemental digital content 3, *http://links.lww.com/MR/A84*, Table showing numbers of CD68 and CD163 stainings evaluated by the semiquantitative estimation method).

Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics 24 (IBM Corporation, Armonk, New York, USA). We used the nonparametric Kruskal–Wallis test with pairwise comparisons to compare the different histological groups and the Pearson χ^2-test to study the associations with clinicopathological parameters. The Kaplan–Meier method and the log-rank test were used for univariate analyses and Cox's regression for multivariate analyses of survival. For the χ^2-test and survival estimations, the macrophage counts obtained by hotspot analysis were divided into two groups (low or high) on the basis of the median. A low or high macrophage number corresponds to macrophage counts less than the median or to counts equal to or greater than the median (median = 92.80 for CD68+ and 76.87 for CD163+ macrophages), respectively. In the case of semiquantitative estimation, categories $0-1$ and $2-4$ were merged. Fused categories 0–25% and 26–100% corresponded to low and high macrophage proportions, respectively. P values of less than 0.050 were considered statistically significant.

Results

Patient characteristics

Patient and clinicopathological characteristics are presented in Table 1. The mean follow-up duration was 8.63 ± 7.8 years (median: 5.2 years). 52 (54.2%) patients suffered relapse or had widely metastatic disease at the time of diagnosis. Of the patients with metastatic disease, 22 (43.1%) received interferon treatment, 25 (49.0%) received chemotherapy, and 33 (63.5%) received radiation therapy (data not shown).

Tumor-associated macrophage number is higher in invasive melanomas compared with benign melanocytic lesions

To detect M2 type macrophages and all TAMs, tissue sections were stained with CD163 and CD68 antibodies, respectively. Cells with CD163 or CD68 immunoreactivity and macrophage-like morphology were considered as M2-type or M1-type macrophages. Both CD163 and CD68 immunoreactivities localized mainly in the cytoplasm, and in some cases also on the plasma membrane. Staining patterns were often granular. CD68-positive cells contained both rounded and dendritic-like cells, whereas the morphology of CD163-positive cells was more often dendritic. In benign melanocytic lesions, TAMs were mainly located at the stromal compartment, whereas in invasive melanomas and LNMs, TAMs were also found inside the tumor (Figs 1 and 2). A significant correlation was found between CD68+ and CD163+ macrophage numbers analyzed by the hotspot method (Pearson's $r = 0.750$, $P < 0.001$).

CD68+ and CD163+ macrophages were significantly more abundant in malignant lesions compared with benign nevi ($P < 0.001$) (Fig. 3). CD68+ macrophage

Table 1 Clinicopathological parameters of the malignant cases

Characteristics	*n* (%)
Total number of patients	98
T and N classification	
pT1	33 (34)
pT4	36 (37)
pN1	29 (30)
Age	
Mean ± SD	58 ± 17
Range	5–92
Sex	
Female	44 (45)
Male	54 (55)
Breslow's depth (mm)	
Mean ± SD	4.8 ± 8.1
Range	0.3–60.0
Ulceration (pT1 and pT4 melanomas)	
Yes	23 (33)
No	45 (65)
Missing	1 (1)
Relapse	
Yes	49 (50)
No	44 (45)
Spread at diagnosis	3 (3)
Missing	2 (2)
Anatomic site of primary melanoma	
Head and neck	23 (23)
Trunk	13 (13)
Back	25 (26)
Upper limbs	11 (11)
Lower limbs	16 (16)
Feet	4 (4)
Hands	0 (0)
Fingers or toes	3 (3)
Not found	3 (3)
Cause of death	
Malignant melanoma	39 (40)
Other	11 (11)
Alive	37 (38)
Unknown	11 (11)

number was also higher in deep melanomas (Breslow's depth > 4 mm) and in LNMs compared with dysplastic nevi or in-situ melanomas ($P < 0.001$). Similarly, the number of CD163+ macrophages was higher in LNMs compared with dysplastic nevi ($P = 0.030$).

TAMs were also evaluated from invasive melanomas and LNMs separately from tumor nests and stroma using a semiquantitative method. The proportions of CD68+ and CD163+ macrophages in tumor nests were higher in deep melanomas and LNMs compared with thin melanomas ($P < 0.001$ for CD68 and 0.001 for CD163). There were no statistically significant differences in the stromal macrophage content between the malignant lesions (Fig. 4a – d).

Correlation of tumor-associated macrophages with clinicopathological parameters

Using hotspot analysis, high CD68+ macrophage number correlated positively with ulceration ($P = 0.006$), recurrence rate ($P = 0.040$), and distal recurrence ($P = 0.002$). Males had higher amounts of CD68+ TAMs more often than females ($P = 0.004$) (Table 2). Using semiquantitative analysis, high amounts of CD68+ macrophages in tumor nests correlated positively with the recurrence rate ($P = 0.006$) and also separately with locoregional ($P = 0.021$) and distal recurrence ($P = 0.001$) (Supplemental digital content 4, *http://links.lww.com/MR/A85*, Table showing associations of CD68+ macrophage proportions in tumor cell nests and stroma, analyzed by the semiquantitative method, to clinicopathological parameters).

Low CD163+ macrophage proportion in stroma, analyzed by the semiquantitative method, was associated with the aggressive nodular growth pattern ($P = 0.037$), the presence of microsatellites ($P = 0.038$), recurrence rate ($P = 0.005$), and locoregional recurrence ($P = 0.035$). Males had low stromal CD163+ macrophage proportions more often than females ($P = 0.034$) (Supplemental digital content 5, *http://links.lww.com/MR/A86*, Table showing associations of CD163+ macrophage proportions in tumor cell nests and stroma, analyzed by a semiquantitative method, to clinicopathological parameters).

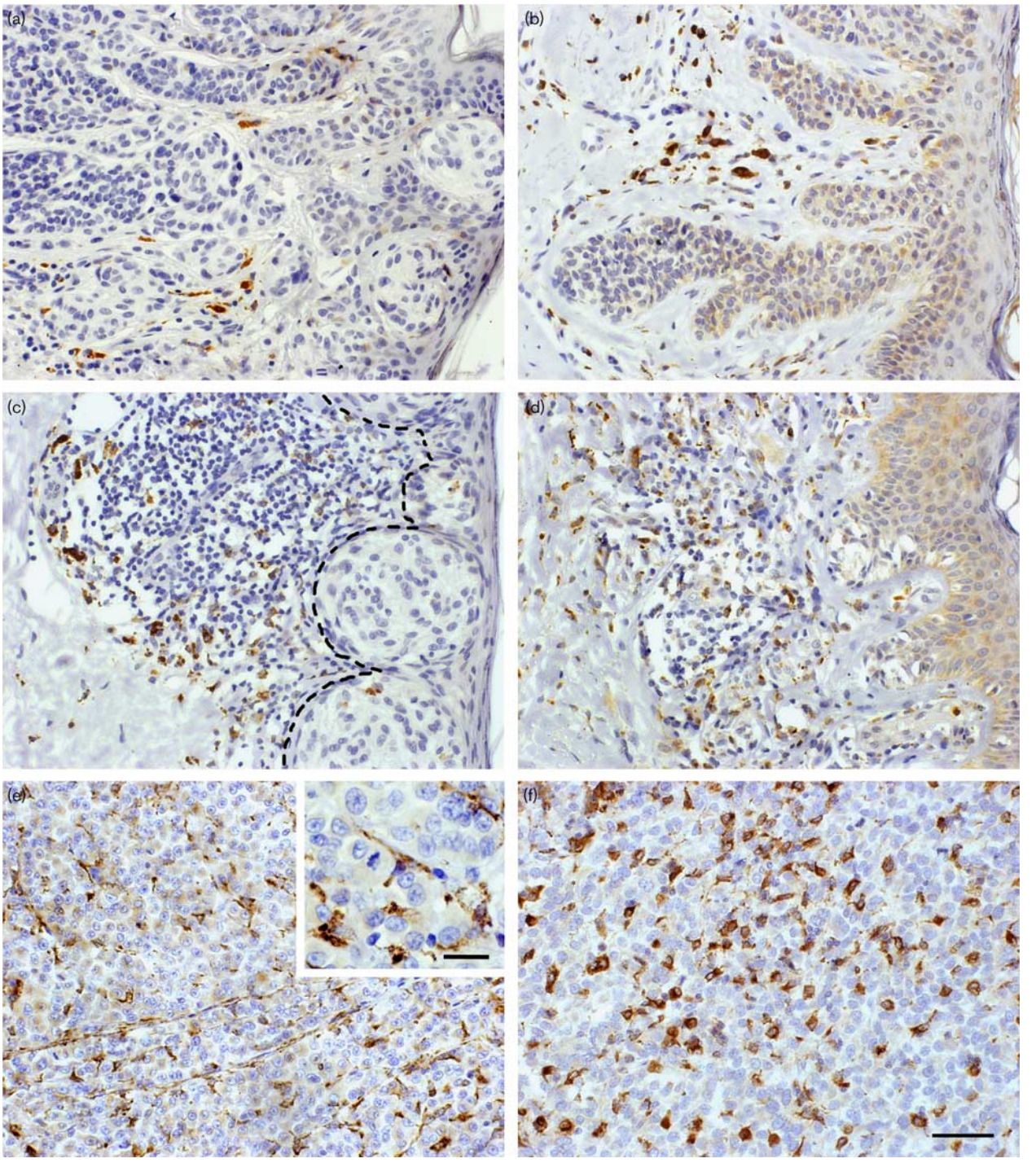
Low stromal CD163+ macrophage proportion correlates with decreased survival in primary melanomas

During the follow-up, 49 patients suffered a relapse. At the end of the follow-up, 37 patients were alive and 61 were deceased. In the univariate survival analysis carried out for the whole patient group, high CD68+ macrophage count analyzed by hotspot analysis correlated with decreased disease-specific survival (DSS) ($P = 0.032$). High CD68+ macrophage proportion in tumor nests was associated with poor DSS and poor **relapse-free survival (RFS) ($P = 0.016$ and 0.005, respectively), whereas low CD163+ macrophage proportion in stroma correlated with poor RFS ($P = 0.010$). TAMs did not correlate with prognosis in pT1 and pT4 groups only.

Multivariate survival analyses were carried out separately for all malignant lesions (pT1, pT4, and pN1) and for primary melanomas only (pT1 and pT4). For all malignant lesions, T and N classification (pT1, pT4, pN1) was used as a covariate. TAMs had no independent prognostic significance in the multivariate analysis carried out for the whole patient group.

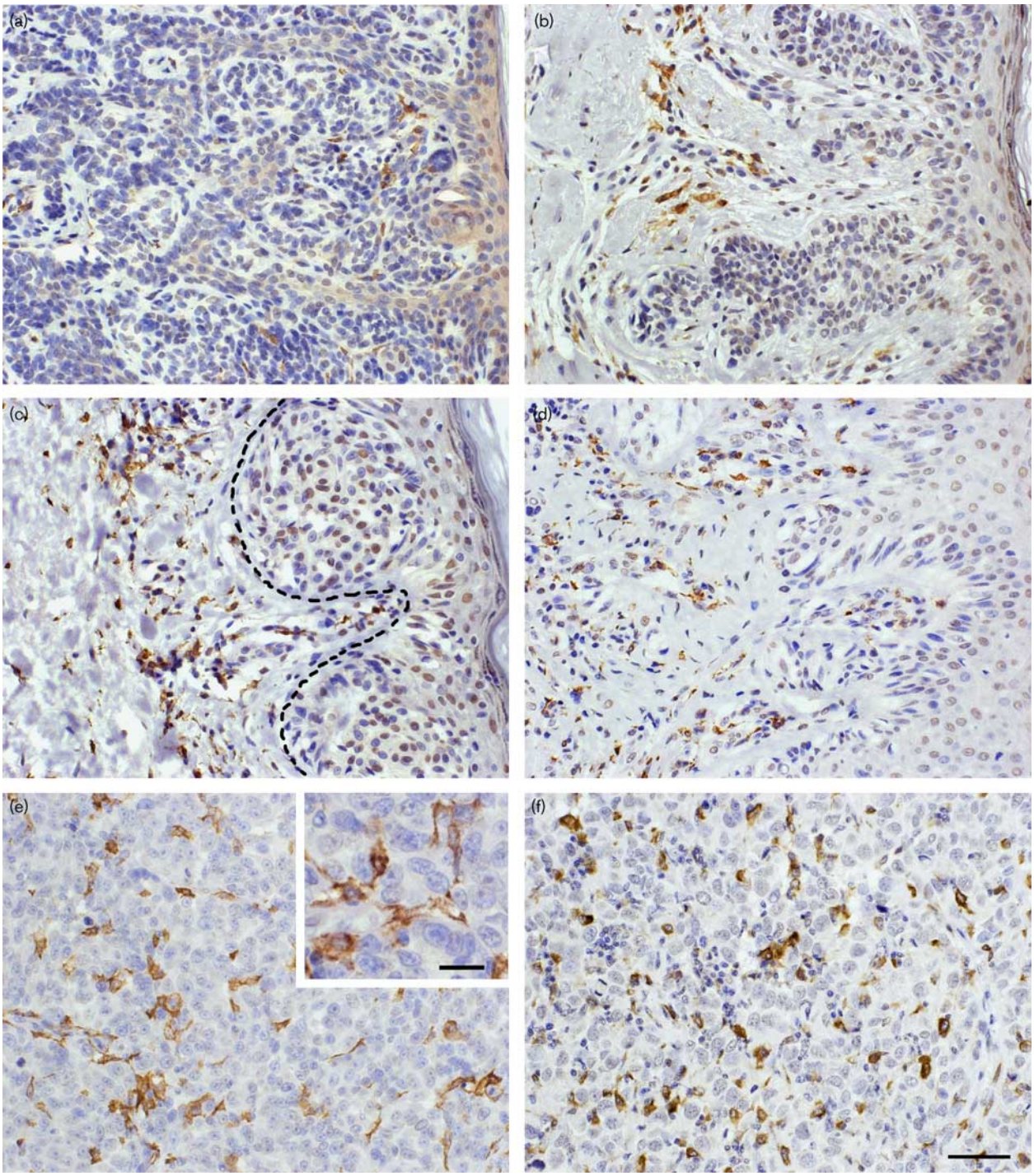
For primary melanomas, the covariates used were Breslow's depth, ulceration, and stromal CD163+ macrophage proportion. Significant factors correlating with OS were Breslow's depth [$P = 0.006$, hazard ratio (HR): 1.063, 95% confidence interval (CI): 1.018 – 1.111], ulceration ($P = 0.021$, HR: 3.020, 95% CI: 1.177 – 1.747), and low stromal CD163+ macrophage proportion ($P = 0.025$, HR: 0.392, 95% CI: 0.173 – 0.890). Factors associated with DSS were Breslow's depth ($P = 0.012$, HR: 1.068, 95% CI: 1.015 – 1.123) and low stromal CD163+ macrophage proportion ($P = 0.017$, HR: 0.153, 95% CI: 0.033 – 0.715), whereas ulceration ($P = 0.013$, HR: 5.143, 95% CI: 1.419 – 18.642) and low stromal CD163+ macrophage proportion ($P = 0.014$, HR: 0.171,

Fig. 1



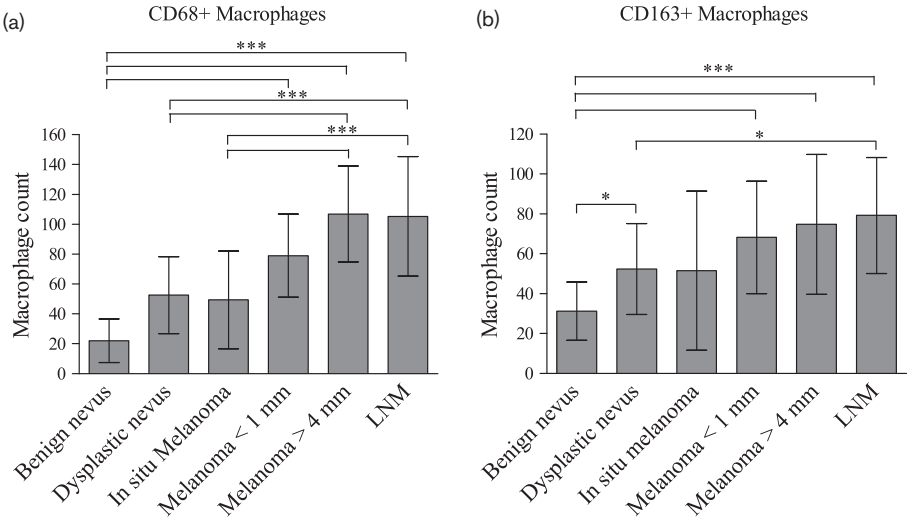
Immunohistochemical staining of CD68 in benign (a) and dysplastic nevi (b), in-situ melanoma (c), superficial (Breslow's depth <1 mm, d) and deep (Breslow's depth > 4 mm, e) melanomas and lymph node metastasis (f). The dashed line in c marks the border between tumor and stroma in in-situ melanoma. In benign lesions, in-situ melanomas (a–c), and thin melanomas (d), macrophages are mainly located in the stroma, whereas in more invasive lesions (e–f), macrophages are also located inside the tumor. Insert in e shows the granular staining pattern and typical morphology (round/dendritic) of CD68+ TAMs. Scale bar is 50 μm in panels a–f (×200 magnification) and 20 μm in panel e (×400 magnification).

Fig. 2



Immunohistochemical staining of CD163 in benign (a) and dysplastic nevi (b), in-situ melanoma (c), superficial (Breslow's depth <1 mm, d) and deep (Breslow's depth > 4 mm, e) melanomas and lymph node metastasis (f). The dashed line in c marks the border between tumor and stroma in in-situ melanoma. In benign lesions, in-situ melanomas (a–c), and thin melanomas (d), macrophages are mainly located in the stroma, whereas in more invasive lesions (e–f), macrophages are also located inside the tumor. The insert in e shows the granular staining pattern and typical morphology (often dendritic) of CD163+ TAMs. The scale bar in a–f is 50 μm (×200 magnification) and the scale bar in e insert is 20 μm (×400 magnification).

Fig. 3



Mean counts of CD68+ (a) and CD163+ (b) macrophages in cutaneous melanocytic lesions and lymph node metastasis analyzed by hotspot analysis. Macrophages were analyzed from 177 CD68-stained and 144 CD163-stained specimens. The data represent mean ± SD. Statistically significant differences between the groups are shown in brackets (Kruskal–Wallis test). $^{*}P < 0.05$, $^{***}P < 0.001$.

95% CI: 0.042 – 0.696) were associated with RFS (Fig. 4f – h).

Discussion

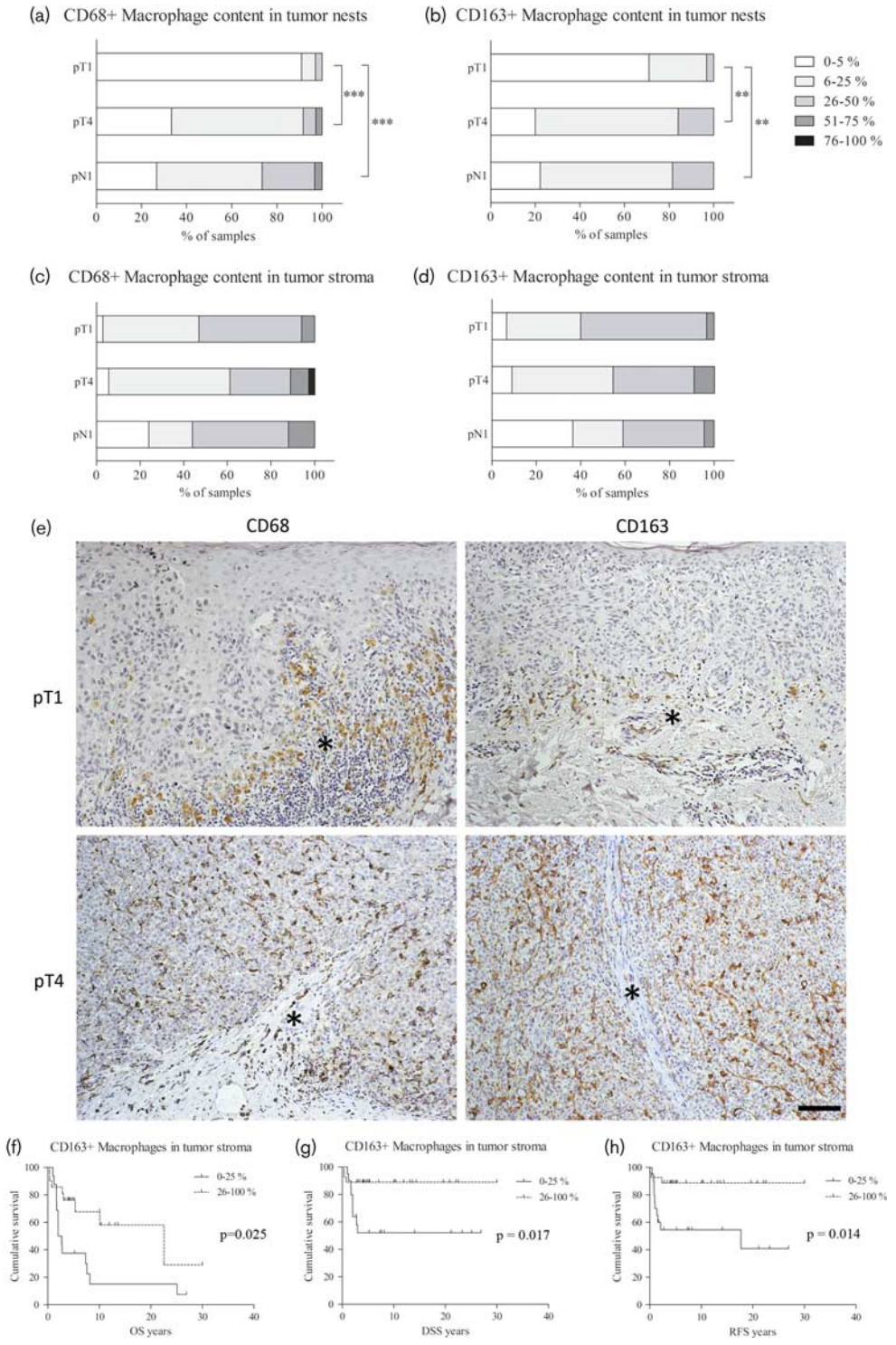
In the present study, both CD68+ and CD163+ TAMs were significantly more abundant in malignant melanocytic lesions compared with benign nevi. Assessment of TAMs separately from different tumor compartments showed that the number of TAMs were also higher in the tumor cell nests of deeply invasive melanomas compared with thin melanomas. High CD68+ macrophage content in the tumor cell nests and low CD163+ macrophage proportion in the stroma were associated with recurrence. These findings indicate that the presence of TAMs is associated with tumor progression in CM, but the role of TAMs seems to depend on their microanatomical localization in the tumor.

Although only a few dysplastic nevi act as a precursor for melanoma, they are biological intermediates between common nevi and melanoma and can provide an insight into melanoma tumorigenesis [16]. In this study, we did not find a significant difference in the CD68+ macrophage content between the benign and dysplastic nevi and in-situ melanomas. However, the numbers of CD163+ TAMs were higher in dysplastic compared with benign nevi, which may indicate an association between immunosuppression and cell dysplasia. Significantly higher amounts of both CD68+ and CD163+ TAMs in malignant compared with benign melanocytic lesions suggest that TAMs promote tumorigenesis.

This study has shown that TAMs in tumor cell nests are more abundant in deeply invasive compared with superficially spreading melanomas. This is logical as TAMs are known to stimulate tumor invasion and angiogenesis by secreting factors such as matrix metalloproteinases, urokinase-type plasminogen activator, vascular endothelial growth factor, and adrenomedullin [5]. The present finding, that the number of TAMs are elevated in more advanced tumors, is in line with a previous study showing that melanomas in the vertical growth phase contain more peritumoral and intratumoral CD68+ TAMs compared with melanomas in the radial growth phase [17]. It has also been reported that intratumoral CD68+ and CD163+ macrophages are more abundant in metastatic compared with nonmetastatic melanomas [18], and some studies have shown a correlation between macrophage number and Breslow's depth, tumor stage, and poor prognosis [11,19–21]. Interestingly, there are also studies that failed to find a correlation between the macrophage content and tumor stage or survival [12–14]. These controversial findings may be partly because of variable compositions of the study materials in terms of the melanocytic lesions involved. In addition, the different assessment methods may have led to variations in the results. To reduce the source of error produced by the assessment method, we used both the hotspot analysis and a semiquantitative method, which yielded comparable results.

According to our results, high CD68+ macrophage number in tumor cell nests correlates positively with both locoregional and distal recurrence. In line with our finding, Tham *et al.* [22] reported that macrophage depletion reduced postsurgical tumor recurrence and metastatic growth in a murine melanoma model. They showed that

Fig. 4



Semiquantitatively assessed tumor-associated macrophage (TAM) content in tumor cell nests and stroma, and survival analysis of stromal TAMs in primary melanomas. CD68+ (a, c) and CD163+ (b, d) TAM content in thin (pT1) and deep (pT4) melanomas and lymph node metastases (pN1), analyzed separately from tumor cell nests and stroma. Statistically significant differences between the stages are shown with brackets (Kruskal–Wallis test). $^{**}P < 0.01$, $^{***}P < 0.001$. Immunohistochemical staining of CD68+ and CD163+ TAMs in thin (pT1) and deep (pT4) melanomas (e). The stromal compartment is marked with an asterisk. In thin melanomas, TAMs are mainly located at the stromal compartment adjacent to the lesion, whereas in deep melanomas, TAMs are also located inside the tumor. The scale bar in e is 100 μm (×100 magnification). In f–h, Kaplan–Meier curves show that a low CD163+ macrophage proportion in the stroma is associated with decreased overall survival (OS) (f), decreased disease-specific survival (DSS) (g), and decreased recurrence-free survival (RFS) (h) in primary (pT1 and pT4) melanomas ($P = 0.025$, 0.017 and 0.014, respectively). pT1 = Breslow's depth less than 1 mm; pT4 = Breslow's depth greater than 4 mm; pN1 = lymph node metastasis.

Table 2 Association of CD68+ and CD163+ macrophage numbers, analyzed by the hotspot method, with clinicopathological parameters

Variables	CD68 low [*n* (%)]	CD68 high [*n* (%)]	*P* value	CD163 low [*n* (%)]	CD163 high [*n* (%)]	*P* value
Sex			**0.004**			0.816
Male	20 (37)	34 (63)		19 (51)	18 (49)	
Female	28 (67)	14 (34)		18 (49)	19 (51)	
Ulceration			**0.006**			0.594
Yes	7 (30)	16 (70)		7 (50)	7 (50)	
No	29 (66)	15 (34)		21 (58)	15 (42)	
Growth pattern			0.096			0.723
Nodular	14 (44)	18 (56)		12 (52)	11 (48)	
Other	23 (64)	13 (36)		16 (57)	12 (43)	
Presence of microsatellites			0.519			**0.033**
Yes	4 (44)	5 (56)		5 (100)	0 (0)	
No	33 (56)	26 (44)		23 (50)	23 (50)	
Overall recurrence			**0.040**			0.237
Yes	20 (39)	31 (61)		17 (44)	22 (56)	
No	26 (61)	17 (40)		19 (58)	14 (42)	
Locoregional recurrence			0.053			0.339
Yes	14 (37)	24 (63)		13 (43)	17 (57)	
No	32 (57)	24 (43)		23 (55)	19 (45)	
Distal recurrence			**0.002**			0.479
Yes	14 (32)	30 (68)		16 (46)	19 (54)	
No	32 (64)	18 (36)		20 (54)	17 (46)	

Association of CD68+ and CD163+ macrophage numbers with clinicopathological parameters. Macrophage numbers, analyzed by hotspot analysis, were divided into two groups (low/high) according to the median (median = 92.80 for CD68+ and 76.87 for CD163+ macrophages).

macrophage density was associated with the growth of postsurgical tumor and metastases and suggested that surgical excision combined with macrophage depletion might be an effective approach to reduce postoperative tumor recurrence [22]. Our results support this finding as CD68+ TAMs seem to be associated positively with recurrence also in human CM.

Recently, Falleni *et al.* [23] reported that CD68+/MRP18-4+ M1 macrophage and CD68+/CD163+ M2 macrophage density were associated inversely with poor prognostic factors and patient survival independent of their intratumoral distribution. These findings differ from our results showing that low stromal CD163+ macrophage proportions predict poor OS, DSS, and RFS in primary melanomas. This discrepancy may be partly explained by the different compositions of the study materials. Falleni *et al.* [23] used melanomas representing Breslow depths 1.01 to less than or equal to 4 mm (53 cases) in addition to melanomas with Breslow depths less than 1 mm (34 cases) and more than 4 mm (7 cases). Our material contained 36 cases of superficial melanomas (<1 mm invasive) and 42 cases of deep melanomas (>4 mm invasive). Thus, melanomas from Breslow depths 1.01 to less than or equal to 4 mm were absent in our material. At present, we can only speculate on the relevance of these differences, which warrants further studies.

In line with our findings, Algars *et al.* [24] have reported that the prognostic significance of TAMs depends on their microanatomical location and tumor stage in colorectal carcinoma. Although we found low stromal CD163+ macrophage numbers correlating with worse outcome in CM, it is not likely that the effect is direct. This may be related to diminutive immune responses against the tumor, or can indicate Th1-polarization of the TME and the dominance of pro-inflammatory M1 macrophages. Earlier reports show that a high density of stromal M1 macrophages in melanoma correlates positively with Breslow's thickness and Clark level [23]. Thus, it is possible that low CD163+ macrophage density in the stroma is associated with abundance of M1 macrophages, which correlates with advanced tumor stage in CM. This hypothesis could also offer an explanation for our results showing low stromal CD163+ macrophage numbers that correlate with decreased survival in CM.

Ulceration and male sex are well-known poor prognostic factors in CM, but the underlying biology is poorly understood [25,26]. According to this study, high CD68+ TAM number is associated with both ulceration and male sex. All ulcerated tumors (23 cases) were greater than 4 mm thick melanomas. Nonulcerated tumors included 33 cases of less than 1 mm thick and 12 cases of greater than 4 mm melanomas. Ulcerated tumors contained higher amounts of CD68+ TAMS than non-ulcerated tumors in both groups of all primary melanomas (pT1 and pT4) and of pT4 melanomas only (data not shown). Interestingly, it is known that cutaneous wounds heal slower in elderly men than women [27], and Lai *et al.* [28] found that androgen receptor (AR) expression on macrophages suppresses wound healing. Thus, mechanisms explaining the association of TAMs with ulceration and sex disparities may include macrophage AR expression. In CM, AR expression has been shown to promote metastasis and is associated positively with a poor prognosis [29].

The abundance of melanin pigment has been shown to correlate inversely with the OS and DFS in patients with

stage III and IV melanoma [30]. Melanogenesis can affect melanoma progression by regulating epidermal homeostasis, for example, by enhancing immunosuppression and creating mutagenic TME [31]. However, it has been shown that melanin granules inhibit melanoma cells migration *in vitro* [32]. Although the role of melanin pigmentation and melanogenesis in CM is not fully established, tumor melanin content should be considered while interpreting the present results. In our study, CD163+ TAMs were analyzed only from melanin-negative tumors, whereas analyses of CD68+ TAMs also included melanin-positive tumors. To ensure comparability between the results of CD68+ and CD163+ TAMs, the statistical analyses of CD68+ TAMs were repeated separately for melanin-negative tumors only (data not shown). All the reported results were found in this group also. In addition, some new correlations were discovered, and they were in line with the reported results, which indicates that the present results of CD68+ and CD163+ TAMs are comparable. The only exception in the results of the melanin-negative group, compared with the reported results, was the inverse correlation of high CD68+ TAM number with sentinel LNM.

In conclusion, high numbers of TAMs, especially in the tumor cell nests, seem to enhance CM progression. As a highly versatile cell type, TAMs are known to play distinct roles in different TMEs [33]. In particular, the role of anti-inflammatory M2 macrophages still remains controversial, and more detailed in-vivo and in-vitro studies are needed to clarify the effects of macrophage subpopulations on CM. However, because of the association of CD68+ TAMs with CM progression and tumor recurrence, TAMs seem to present a plausible target for therapies in the future.

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S.S. analyzed samples with the hot spot and the semi-quantitative method, carried out the statistical analyses, and wrote the manuscript. H.S. participated in designing the study protocol and revised and commented on manuscript. R.S. provided the study material, analyzed the standard histopathological parameters, and commented on the manuscript. K.T-K. commented on the manuscript. B.H-G. analyzed the samples with the hot spot method. M.V. collected the clinicopathological data. P.A. designed the study protocol, helped with statistical analyses, and commented on the manuscript. S.P-S. designed the study protocol and revised and commented on the manuscript.

Conflicts of interest

There are no conflicts of interest.

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