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Prognostic Impact of CD68 and Kallikrein 6 in Human Glioma

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Abstract. *Aims: To evaluate the expression of CD68 and kallikrein 6 in human gliomas, and investigate their prognostic significance for survival of brain cancer patients in comparison to some known prognostic markers. Patients and Methods: Histological sections of 51 primary astrocytic tumours (11 benign, 40 malignant) were immunohistochemically stained for CD68, cathepsin B, kallikrein 6 and Ki-67. CD68 and kallikrein 6 expressions were also analyzed by real-time PCR in nine brain tumour biopsies. Results: CD 68 was expressed by both microglia and tumour cells. High CD68 and cathepsin B staining scores were significantly, more frequent in the malignant than in the benign tumours (p=0.036 and p=0.014, respectively). In contrast, the benign group presented a stronger immunoreactivity for kallikrein 6 compared with the malignant tumours (p=0.013). A CD68 staining score of tumour cells higher than 3 was a significant predictor of shorter overall survival (p<0.01) in all patients and of borderline significance in the malignant group (p=0.057). Strong CD68 staining was of greater predictive value in the subgroup of anaplastic astrocytomas (p=0.021). Furthermore, as expected on the basis of our previous studies, prognostic significance was confirmed for cathepsin B, but not for any of the other markers under evaluation. Conclusion: Kallikrein 6 was down-regulated in malignant glioma, but this differential expression did not have an impact on patient prognosis. In contrast, immunostaining of glioma tissue for CD68 and for cathepsin B may be used for prognosis of survival in these patients. This finding suggests that besides the known role of cathepsin B in invasion and angiogenesis, CD68 may be also associated with glioma progression.*

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Gliomas constitute approximately 68% of all primary brain tumours (1) and are derived from three basic types of glial cells: astrocytes, oligodendrocytes and ependymal cells. The most frequent are diffusely infiltrating astrocytomas, further classified into astrocytomas (A), anaplastic astrocytomas (AA) and glioblastoma multiforme (GBM), equivalent to World Health Organization (WHO) grade II, III and IV, respectively. GBM is the most common and lethal type of astrocyte-derived tumour (2), corresponding to 50% of adult primary brain tumour cases, followed by anaplastic astrocytomas (30%) and astrocytomas (20%). GBM may develop from astrocytomas or anaplastic astrocytomas (secondary GBM), but more frequently they manifest after a short clinical history *de novo*, without any evidence of a less malignant precursor lesion (primary GBM) (3). Although primary brain tumours are relatively rare compared with carcinomas, they are characterized by higher mortality rates and increased disability. The overall annual incidence rate of primary malignant and benign brain tumours in developed countries is approximately 15 per 100,000 individuals, and for primary malignant brain tumours it is 7 per 100,000 (4). Brain tumour incidence and mortality have increased by up to 300% over the past 3 decades, primarily in people aged over 75 years (5, 6). Successful treatment of malignant gliomas represents a challenge, as despite recent advances in neuro-imaging, neurosurgical resection techniques and the development of novel adjuvant therapies, the long-term survival of patients suffering from malignant glioma remains low. Although treatment with temozolomide and radiotherapy improved median survival after diagnosis of GBM from 12 months to 14 months (7), the survival rate still ranges from a few months to several years, which, together with the poor

prognosis, points to the need for new, independent prognostic factors that may enable individualized treatment modalities, including molecular-based therapies, of patients with unfavourable prognosis.

Various proteolytic enzymes have been demonstrated to be good prognostic factors in many types of tumours, due to their specific roles in individual steps of tumour progression (8). In gliomas, cysteine proteases, such as cathepsins B (Cat B), L and S, the urokinase-plasminogen serine protease system, as well as several metalloproteinases are up-regulated and some of these were demonstrated to have a prognostic impact for patient survival (9, 10). Our previous studies demonstrated increased levels of Cat B in tumour and endothelial cells in glioblastoma tumours, and that both had an impact on patient survival (11-13).

The human kallikrein-related peptidase (KLK) family consists of 15 homologous single-chain, secreted serine endopeptidases with trypsin- or chymotrypsin-like activity (14, 15). Kallikreins, also referred to as kallikrein-related peptidases (16), have been primarily associated with endocrine-related tumours (14, 15). Some of the kallikreins are known for their clinical application as cancer biomarkers: for example kallikrein 3, which is known as prostate-specific antigen (PSA), is one of the best available biomarkers for monitoring tumour burden in sera of prostate cancer patients and has also been evaluated as a marker for prostate cancer diagnosis and prognosis (17). *In vitro* data and animal experiments have indicated the role of kallikreins in several steps of tumour progression, such as tumour growth, invasion and metastasis, as well as increased angiogenesis (14-16). KLKs have also been characterized as prognostic indicators for some type of tumours (18, 19). Kallikrein 6, which has been proposed as a novel biomarker of ovarian cancer (15, 20), has been shown *in vitro* to cleave substrates involved in carcinogenesis (21, 22). Kallikrein 6 is also among the members of the kallikrein family with high levels of expression in the brain and related fluids (23); however, knowledge of the expression of kallikreins by brain tumours is limited. A recent study has suggested an association of kallikrein 7 with aggressive brain cancer and shorter overall survival of brain cancer patients (24). The possible prognostic impact of the expression of kallikrein 6 in gliomas has not been examined thus far.

CD68 is a 110 kDa transmembrane glycoprotein, expressed by monocyte/macrophage lineages and serves as a marker for microglia (25). There are a significant number of publications emphasizing macrophage and microglia infiltration within astrocytomas (26-30). In human glioma, intratumoral microglia density is higher than in peritumoral and normal brain, and microglia increase in number according to the grade of malignancy (29, 31). Klein and Roggendorf (32) reported that microglial cells in astrocytic brain tumours not only proliferated, but also exhibited different proliferative activities at different grades of malignancy, with the highest rates of proliferating microglia shown in pilocytic astrocytomas. It has been evidenced that this microglial accumulation in diffuse glial tumours does not merely represent a nonspecific reaction to tissue injury but reflects participation of these cells in supporting and promoting the invasive phenotype of astrocytoma cells (33).

Notably, tumour cells can occasionally be reactive to some macrophage markers (27, 34). Leenstra *et al.* (34) investigated six specimens of cultured astrocytoma cells and reported that nine macrophage markers, including CD68, were clearly reactive in neoplastic astrocytes, whereas astrocytes in normal brain specimens were not reactive. This study suggested that the demonstration of macrophages within astrocytomas by using macrophage-specific antibodies alone must be cautiously considered. In accordance to quoted studies, we also found strong CD68 expression in tumour cells of U87 human glioblastoma cell suspension, in U87 spheroids (both prepared from the U87 human glioblastoma cell line, without microglia), as well as in induced rat tumours (35).

In this study we therefore considered the possibility that human malignant astrocytes may adopt a macrophage phenotype and aimed to evaluate the possible prognostic value of CD68 expression for the survival of brain tumour patients. The second objective of the study was to evaluate the prognostic impact of serine protease kallikrein 6. Furthermore, we also aimed to compare both potential new biological markers with known histopathological and proliferation markers, as well as the previously established biological marker Cat B.

Patients and Methods

Patients. Fifty-one patients (25 male and 26 female) with primary tumours of the central nervous system (CNS), operated on at the Department of Neurosurgery, University Clinical Centre Maribor between 1997 and 2004, were studied. The ages of the patients ranged from 20 to 75 years (median of 53 years). The histological slides of all cases were reviewed and classified according to the WHO classification of brain tumours (36). Our cases included 11 benign (A) and 40 malignant tumours (17 AA and 23 GBM). The median ages of patients with malignant and benign tumours were 56 and 42 years, respectively.

One neurosurgical resection of the tumour was performed in 43 of the patients, while 8 patients were operated on twice. In this latter subgroup of patients, one of the cases had an astrocytoma at first presentation which developed later into malignant tumour (AA). The rest of the patients had a malignant tumour at the initial operation. In all 51 cases, surgery was followed by presentation to the oncologist to decide on postoperative treatment. Follow-up of the patients was regularly performed in our Outpatients' Department.

Our survival analysis included only primary tumours (n=51), but in the descriptive analysis of immunohistochemical (IHC) staining we included the eight cases of secondary tumours from the re-operated patients (n=59).

Immunohistochemical analysis. Immunostaining was performed using a standard technique (37), according to protocols designed at the Department of Pathology at University Clinical Centre Maribor, Slovenia. In brief, 5-μm thick sections were mounted on aminoethoxysilane-coated glass slides. The slides were dried overnight at 37˚C and then at 57˚C for 8 hours. After deparaffinizing in xylene and washing in a graded series of ethanol, the sections were placed in a 10 mmol/l sodium citrate buffer (pH 6) and boiled for 12 minutes on 110˚C in a Microwave Vacuum Histoprocessor (Milestone RHS-1; Shelton, CT, USA) to facilitate antigen retrieval. The slides were subsequently incubated with monoclonal antibodies against Ki-67, a marker of proliferation (1:50 dilution; DAKO, Glostrup, Denmark); cathepsin B (Cat B), a cysteine protease (1:100 dilution; clone 3E1, gift from Dr. Janko Kos, University of Ljubljana, Slovenia); CD68, a macrophage and microglia marker (1:50 dilution; DAKO); as well as with a kallikrein 6 (KLK6) polyclonal antiserum (1:400 dilution; Mount Sinai Hospital, Toronto, ON, Canada). All incubations were carried out overnight at 4˚C. After a short washing in Tris-buffered saline, immunoperoxidase staining was performed by an EnVision antibody complex method using the ENVISION kit (DAKO) (38). After rinsing in tap water, the sections were counterstained with Mayer's haematoxylin and mounted.

Ki-67 immunodetection was considered as an indicator of positive nuclear staining. The Ki-67 staining index (SI) was defined as the percentage of positive nuclei of a total of 2,000 tumour cells counted using an eyepiece grid (39). Immunoreactivity for the other markers listed above was evaluated in the tumour cells on the basis of their morphological characteristics. The frequency of CD68, cathepsin B and kallikrein 6 immunoreactivity in tissue sections was considered negative when no positive tumour cells were observed within the boundaries of the tumour, weak when fewer than 30% of the tumour cells were positive, moderate when 30-60% of the tumour cells were positive, and strong when more than 60% of tumour cells were stained as positive. The frequency of staining was thus evaluated as 0, 1+, 2+, and 3+ for no immunoreactivity, weak, moderate and strong immunoreactivity, respectively. The intensity of staining was similarly evaluated as 0, 1+, 2+, and 3+ for no staining, weak, medium and strong staining, respectively. Twenty representative fields were counted for each slide. The IHC staining score was determined as the sum of the frequency and intensity scores. The results of staining were further subdivided into three groups according to negative staining (0 total score), weak staining (total score between 1+ and 3+) or more intense staining (total score between 4+ and 6+). Slides stained with omission of the primary antibody served as negative controls for the Ki-67 staining. Liver tissue sections were additionally used as positive controls for Cat B, brain sections for kallikrein 6 and spleen sections for the macrophage surface marker CD68.

Statistical analysis. Statistical analysis was performed using the program Statistica for Windows 6 (StatSoft, Inc., Tulsa, OK, USA). Variables used in the analysis included Ki-67 staining index and CD68, Cat B and kallikrein 6 IHC final scores. Descriptive statistical methods (*e.g.* mean value, median value, frequency tables) and *t*-test were used. Correlations between the studied markers were also determined by using by Statistica (StatSoft, Inc.). Overall survival probabilities were calculated by the Kaplan-Meier method (40); log-rank test was used to evaluate the association between survival and each of the selected markers. The survival time was determined as the interval between initial operation and the patient's death or final outcome at a determined endpoint (August 2008) for those surviving to this date.

RNA isolation and cDNA production. Biopsies from 9 patients with primary brain tumours, namely 6 GBM, 2 AA and 1 A patient (according to the WHO classification), were subjected to total RNA extraction. The astrocytoma sample (benign) was taken as a reference. Tumour samples were placed into Trizol (Invitrogen, Carlsbad, CA, USA) at the time of operation and kept in liquid nitrogen until processing. Samples were subsequently homogenised and total RNA was isolated according to the protocol recommended by the manufacturer of Trizol (Invitrogen). cDNA was synthesized using 1 μg of total RNA with cDNA High Capacity Archive Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions.

Real-time quantitative polymerase chain reaction. CD68 and kallikrein 6 were PCR amplified using pre-made Taqman Gene Expression Assays (Applied Biosystems): Taqman Gene Expression Assay Hs00154355-m1 was used for amplification of CD68 and Hs00160519-m1 for the amplification of kallikrein 6. Quantification of gene expression was performed using PCR (ABI 7900 HT Sequence Detection System; Applied Biosystems). Amplification of GAPDH probe was also performed as an internal control. The conditions for PCR were 50˚C for 2 min, 95˚C for 10 min, and 45 cycles of 95˚C for 15 s and 60˚C for 1 min. The data obtained from Taqman Gene Expression Assays were analyzed using ΔΔCt algorithm and normalized according to GAPDH expression in each sample.

Results

Immunohistochemical analysis. Immunostaining was evaluated in 59 slides comprising 51 primary tumours and 8 cases from re-operations (relapses). These slides included 11 cases of benign astrocytomas, 40 malignant tumours from initial operation (comprising 17 AA and 23 GBM) and 8 malignant relapses (3 AA and 5 GBM). The immunostaining pattern of the selected biological markers is shown in Figure 1 and their distribution among benign and malignant tumours is presented in Table I.

In this study, the majority (about 85%) of the 59 primary CNS tumours (48 malignant, 11 benign) expressed CD68 to various extents (Figure 1A and B). Staining for CD68 in malignant tumours was significantly more pronounced than in the benign tumours (mean score of 4.2 *versus* 2.8, p=0.036) (Figure 2A). High CD68 IHC scores (4+ to 6+) in slides from the malignant group were observed in 80% of the cases, while in benign tumours, a high IHC score for CD68 was found in 55%. However, in both groups a low IHC score (1+ to 3+) was found in a comparable percentage of patients (10% in malignant and 9% in benign group). No staining for CD68 was detected in 36% of benign tumours, compared with 10% of malignant tumours (Table I).

Cat B immunostaining was found in almost all (91%) of the evaluated samples (Figure 1C and D). Immunolabeling of Cat B in tumour cells of malignant tumours was stronger

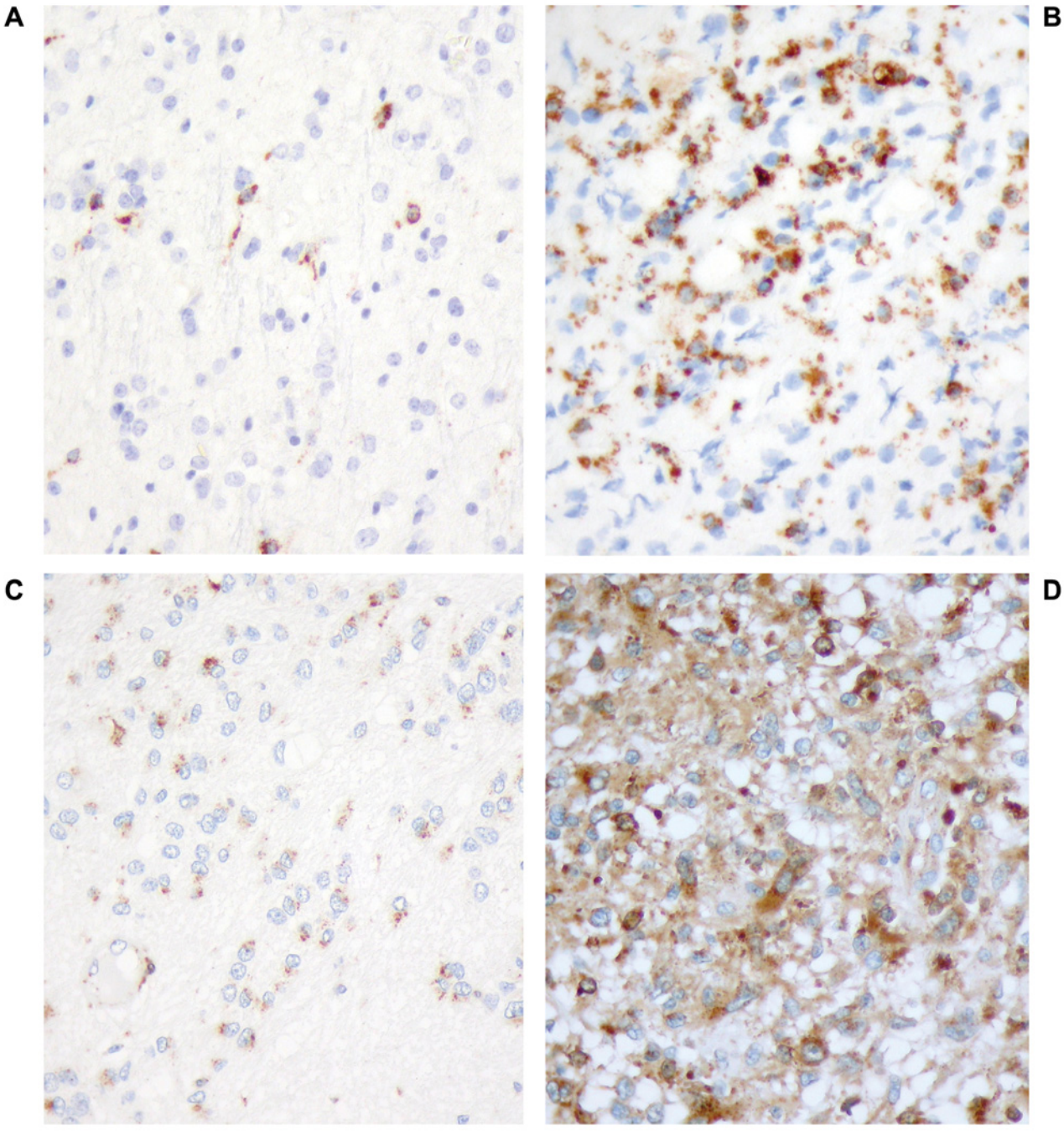

Figure 1. Continued

than that in benign tumours (mean score of 4 *versus* 2.6) with a significance of p=0.014 (Figure 2B). Within the malignant group, 68% of patients exhibited high Cat B IHC score (4+ to 6+), while the percentage was lower (36%) for the benign group (Table I).

Expression of kallikrein 6 was seen in 72.5% of all tumours (Figure 1E and F). Kallikrein 6 immunoreactivity was significantly (p=0.013) higher in benign than in malignant tumours (mean score of 3.6 *versus* 2.2) (Figure 2C). IHC scores ranging from 4+ to 6+ were expressed in 73% of benign tumours, but only in 30% of the malignant tumours. Furthermore, no kallikrein 6 staining was found in 30% of malignant tumours and 18% of benign tumours (Table I).

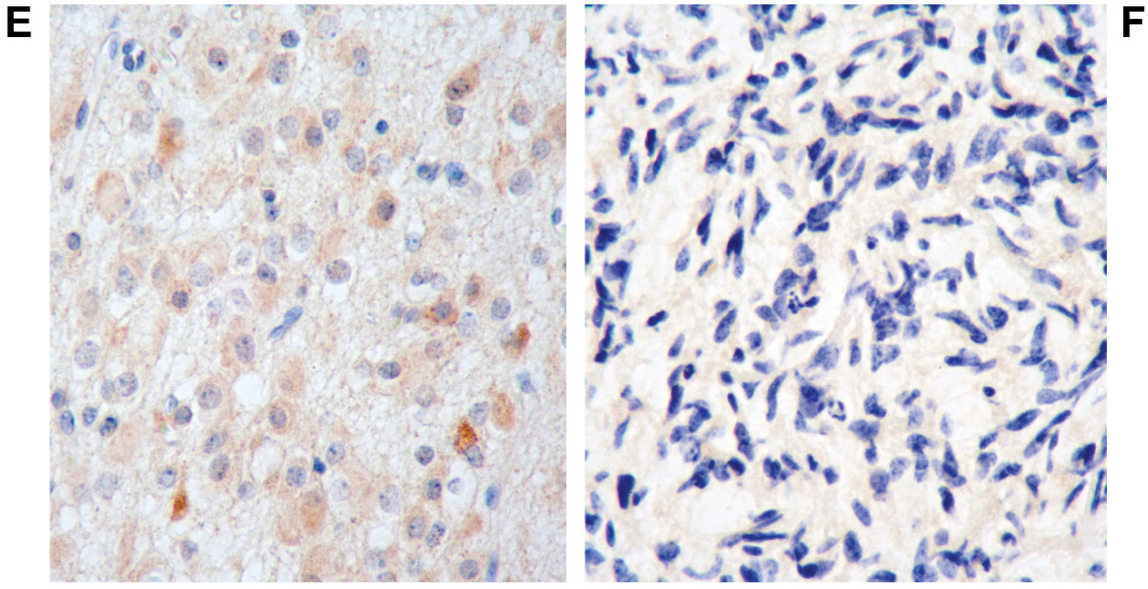

Figure 1. *Immunohistochemical (IHC) staining of biological markers in glioma samples. A, Astrocytoma (×40); treatment with CD68 antibody resulted in a few positively stained tumour cells. B, Glioblastoma multiforme (×40); treatment with CD68 antibody revealed strongly positive immunostaining in almost all tumour cells. C, Astrocytoma (×40); treatment with cathepsin B (Cat B) antibody resulted in a few positively stained tumour cells. D, Glioblastoma multiforme (×40); treatment with Cat B antibody resulted in strong positive staining in the majority of tumour cells. E, Astrocytoma (×40); staining with kallikrein 6 antibody revealed strong immunostaining in tumour cells. F, Glioblastoma multiforme (×40); staining with kallikrein 6 antibody resulted in some positively stained tumour cells.*

Staining for cell proliferation, by means of Ki-67, was significantly higher in malignant tumours than in benign tumours (mean score of 16.9 *versus* 1.5, p=0.035). In the benign group we observed only weak Ki-67 expression in approximately half of the tumours (45.5%) (percentage of Ki-67 positive nuclei was ≤20%), while the remainder of the cases were not stained for Ki-67 (Table I).

Real-time PCR analysis of CD68 and kallikrein 6 mRNA. Amplification of CD68 RNA was performed in all nine samples originating from primary brain tumour biopsies (6 GBM, 2 AA and 1 A) (Figure 3A). The expressions were normalised to the expression in the only benign astrocytoma sample (A). One of the glioblastoma samples expressed at least 500 times more CD68 than any of the other samples (data not shown). Expression analysis of kallikrein 6 in these 9 samples revealed a low gene copy number. Therefore, an additional step of preamplification was performed as recommended by the supplier of the Gene Expression Assay (Applied Biosystems 4384556). After this, kallikrein 6 expression was detected in all of the samples (Figure 3B); the highest level of expression was identified in one GBM sample (20-fold higher than the astrocytoma sample, data not shown), while one of the other GBM samples exhibited the lowest levels of expression (approximately 1,000 times less than in sample A1) (data not shown). Furthermore, kallikrein 6 mRNA expression copy numbers were comparable in both AA samples, reaching (numbers 2 and 6) approximately 20% of the levels measured in sample A1.

Correlations. In all tumour cases, a significant correlation was found only between the immunohistochemical score of CD68 and Cat B (correlation coefficient r=0.45, p<0.01) (Figure 4).

Survival analysis. The final outcome was considered on the determined endpoint (August 2008) and the follow-up time ranged from 0 to 125 months (median of 10 months). During this time about 92% of patients died (Table II). As expected, significantly (p<0.001) shorter survival (median of 8 months) was observed for patients with malignant tumours compared to those with benign tumours (median of 50 months). Adjuvant therapy had been used in 23 cases: among patients with malignant tumours, 17 (42.5%) were treated by irradiation and 4 (10%) by additional chemotherapy. Additional therapy was not considered for the remaining patients of the malignant group due to old age, the presence of other pathological conditions, or poor functional state according to the Karnofsky scale. In patients with benign tumours, 2 out of 11 patients (18%) had received irradiation.

IHC analysis for staining of CD68 in evaluated slides showed that patients with a score over 3 (ranging from 4+ to 6+) had a significantly poorer prognosis (p<0.01) than patients with lower IHC scores (Figure 5A). In the subgroup

Table I. *Immunohistochemical staining of biological markers, CD68, kallikrein 6 (KLK6), cathepsin B and Ki67 in glioma.*

Biological marker	Benign glioma	Malignant glioma
CD68		
Mean (median) IHC score*	2.8 (4.0)	4.2 (4.5)
IHC score group**		
0	36%	10%
1-3	9%	10%
4-6	55%	80%
Kallikrein 6		
Mean (median)	3.6 (4.0)	2.2 (2.0)
0	18%	30%
1-3	9%	40%
4-6	73%	30%
Cathepsin B		
Mean (median)	2.6 (2.0)	4.0 (4.0)
0	9%	7%
1-3	55%	25%
4-6	36%	68%
Ki-67***		
Mean (median)	1.5 (0)	16.9 (5)
0%	55%	21%
1-20%	46%	56%
21-40%	0	6%
41-100%	0	16%

*The mean and the median value of the IHC scores of each biological marker in the histologically benign and malignant tumours were calculated using descriptive statistics by Statistica (StatSoft, Inc.). **The relative abundance (percentage) of the antigens in samples with IHC scores: 0, 1-3 and 4-6 was determined for each biological marker within the group of benign and malignant tumours. ***Distribution of relative Ki-67 abundance (percentage of positive cells) was calculated as percentage of positive nuclei of a total of 2,000 tumour cells counted using an eyepiece grid.

Table II. *Survival of glioma patients at the pre-determined endpoint.*

Patients	All (n=51)	Tumour type Benign (n=11)	Malignant (n=40)
Alive	4 (7.8%)	2 (18.2%)	2 (5%)
Dead	47 (92.2%)	9 (81.8%)	38 (95%)
Time from operation (in months)*	10 (0-125)	50 (2-125)	8 (0-87)

*Median time and total interval from the operation of primary tumour until the death or the pre-determined endpoint (August 2008).

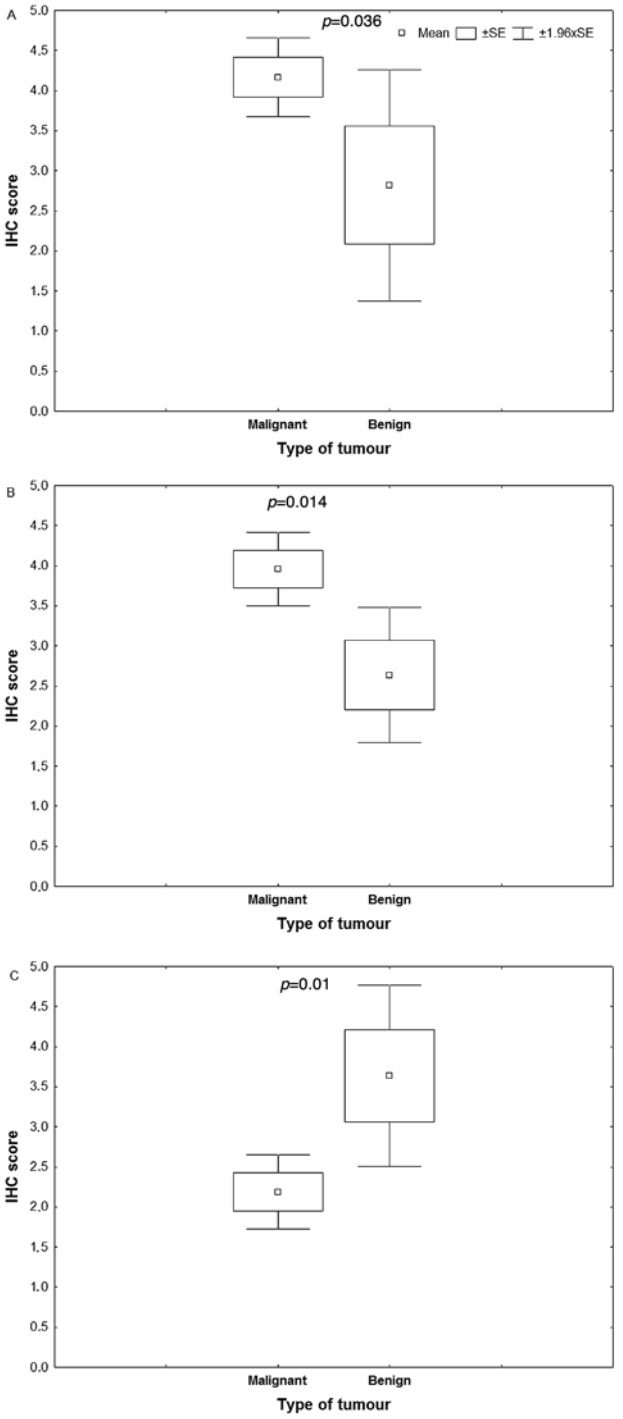

Figure 2. *Box and whiskers plot showing significantly different immunostaining of A, CD68; B, cathepsin B; C, kallikrein 6 in benign compared to malignant tumours. Statistical analysis was performed by Statistica (StatSoft, Inc.) using t-test.*

of patients with malignant tumours, a higher CD68 score (4+ to 6+) also indicated worse outcome, however the difference was of borderline significance (p=0.057) (Figure 5B). Survival analysis of the patients with AA, showed a potential prognostic value for CD68 IHC staining with low statistical significance (Kaplan-Meier curve not shown). Nevertheless, the exclusion of an outlier from the analysis (a case of AA, IHC score 4, survival time 83 months – outcome considered

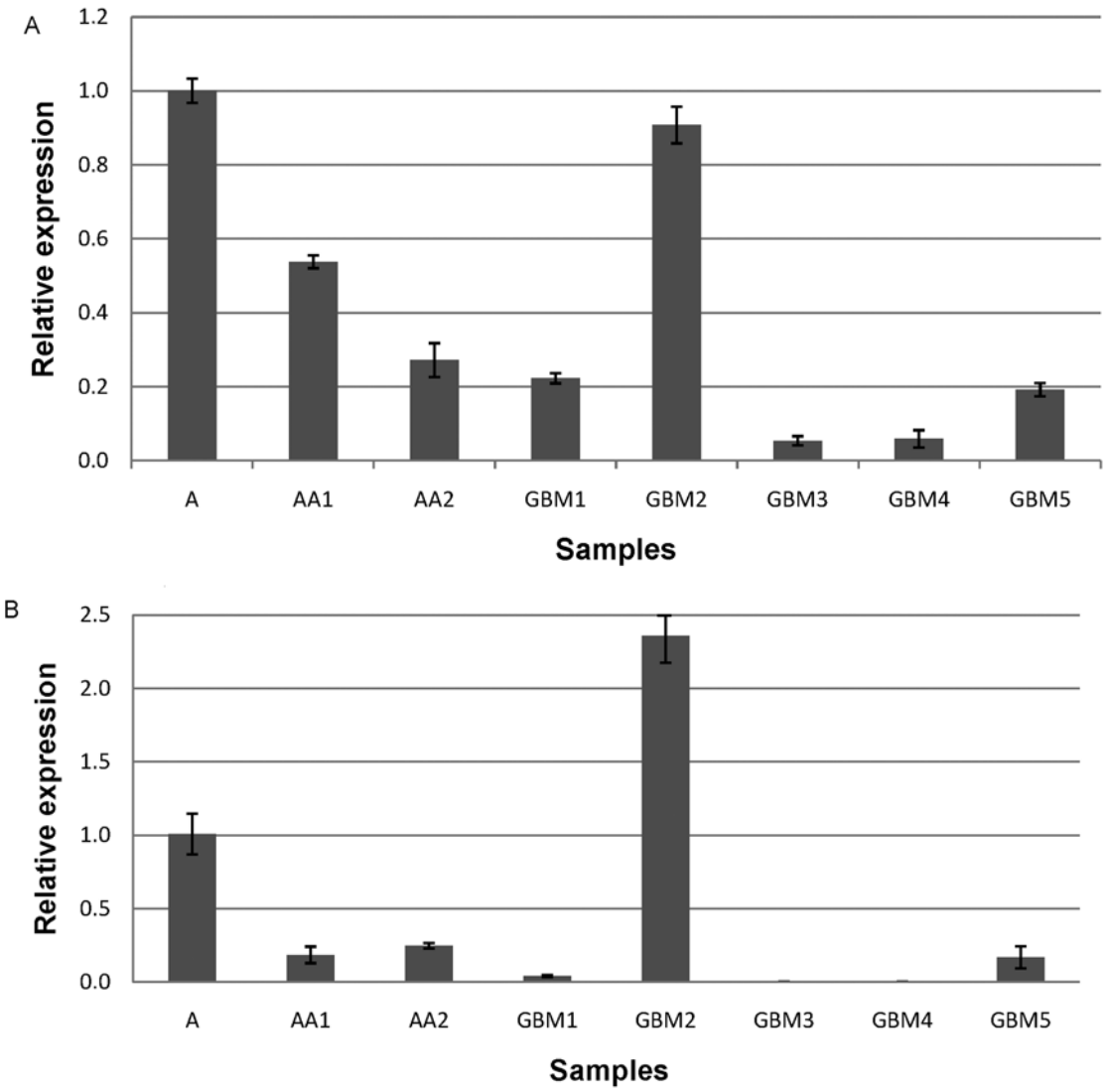

Figure 3. *RT-PCR analysis for CD68 (A) and kallikrein 6 (B) of brain glioma biopsy samples (n=9). Data were derived from at least two independently conducted experiments with two replicates. The expressions are shown relative to the expression of the astrocytoma sample (A) that was taken as 1. For the sake of clarity, one of the GBM samples was omitted from the graphs (see the Results). The expressions of CD68 and kallikrein 6 mRNA were generally lower in AA and GBM samples compared to those in A.*

as alive) revealed a significant prognostic impact of low CD68 IHC score for longer survival in patients with AA (p=0.021) (Figure 5C). The median survival time in the four AA patients exhibiting weak CD68 IHC was 45.5 months compared to 9.5 months in the twelve AA patients showing strong CD68 staining. We also observed that a Cat B IHC score over 3 was significantly associated with shorter overall survival (p=0.04) when all tumour cases were considered (Figure 5D). However, no prognostic impact of the tumour cell-associated Cat B staining was observed when the malignant group alone was included in the survival analysis (Figure 5E). In contrast to CD68 and Cat B, the IHC scores of kallikrein 6 and Ki-67 expression in brain tumours did not have any prognostic value (not shown).

Discussion

Cathepsins are among the proteolytic enzymes that have been associated with glioma progression. For instance, cathepsins B, L, H and S have been reported to be up-regulated in glioma, as observed at both the mRNA and the protein levels, more often marked at the invading front of the tumours, suggesting a role of these enzymes in glioma invasion. However, only Cat B has recently been demonstrated to have

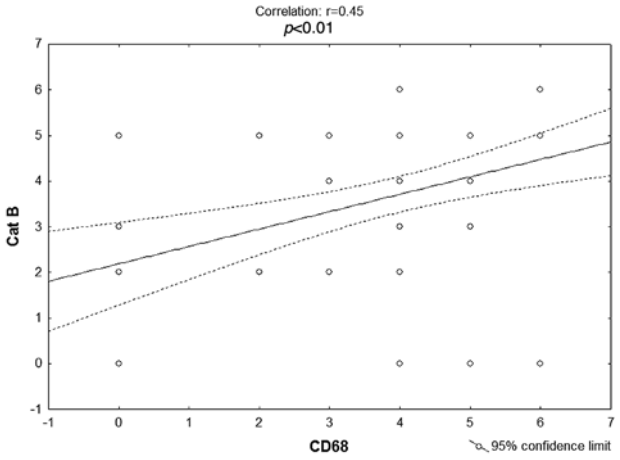

Figure 4. *Correlation between CD68 and cathepsin B (Cat B) IHC score in glioma samples. Statistical analysis was performed using correlation matrices in Statistica for Windows 6 (StatSoft, Inc.).*

a role in the invasion of glioma cells *in vitro* (41). We have also reported the high expression of Cat B in tumour vessels, which was a highly significant prognostic indicator of patient survival, supporting previously reported data (reviewed by Lah *et al*. (42)). Therefore, the present findings of high immunolabelling of Cat B and its prognostic signifficance confirmed our previous data and can be used as a standard to evaluate the relative importance of potential new prognosticators, such as kallikreins.

The expression of serine proteases of the kallikrein family by gliomas have not been studied thus far. Kallikreins are expressed by secretory epithelial cells of many organs and have been implicated in a range of normal physiological functions(14, 15, 43-45). They are also widely distributed in various areas of the human brain (23, 44, 46, 47), such as the cerebral cortex and grey matter, cerebellum, brain stem, thalamus, hypothalamus, anterior pituitary and choroid plexus. It has been suggested that the functional role of kallikreins is to assist in the normal turnover of brain proteins and the processing of peptide hormones, neurotransmitters and nerve growth factors that are essential for normal neuronal function and synaptic transmission (14, 15). Kallikrein genes/proteins are also aberrantly expressed in many cancer types and they may exert diverse and often contrasting effects on the tumour and its microenvironment. Therefore, high kallikrein expression has been associated with either poor or favourable patient prognosis. Some members of the kallikrein family are listed among the group of tumour-protecting proteases, as reviewed recently by Lopez-Otin and Matrisian (48), but even a single kallikrein may have a dual role in tumour progression. Kallikrein 6 is abundantly expressed by T-cells and macrophages, within multiple sclerosis lesions in the brain (49, 50). In these conditions, immune cells attracted to the site of injury may contribute to local increases in kallikrein 6 activity. It is intriguing to hypothesize a similar scenario in the case of brain tumourigenesis.

In the present study we demonstrated higher IHC expression of kallikrein 6 in the tumour cells of benign human gliomas comparing to malignant tumours. Real-time PCR of kallikrein 6 expression confirmed the immunohistochemical data, except in the case of one malignant tumour which exhibited higher kallikrein 6 expression than that of the astrocytomas. Our data support a potential role of kallikrein 6 in suppression of glioma progression, however, a prognostic value of kallikrein 6 was not revealed by this study. The possibility of a dual role (pro- and antitumour) of kallikrein 6 in tumour growth cannot be overlooked. Clinical studies with larger patient populations are needed to allow further evaluation of kallikrein 6 function in glioma progression.

Microglial cells function as resident immune cells and phagocytes in the CNS. Reactive microglia expresses a variety of cell surface molecules, including CD68 (51). In response to pathology, resident microglia follow a stereotyped pattern of first becoming activated and then phagocytic (52). On one hand, microglia, may represent components of the antitumour immune response in the CNS, which is inactivated by local secretion of immunonosuppressive factors by glioma cells. On the other hand, taking into account that microglia are capable of secreting a variety of immunomodulatory cytokines, they may be attracted by the gliomas to assist in tumour growth (53, 54). We evaluated the antigen expression of CD68 in glioma tissue by avoiding any region with necrosis and excluding foamy cells, possibly indicating the presense of macrophages. However, malignant astrocytoma cells were also highly CD68 positive, in accordance with the report of Leenstra *et al*. (34). These authors further emphasised that there may be biological properties shared by macrophages and astrocytoma cells, such as phagocytosis and production of the same growth and angiogenic factors. These common properties may be explained: (a) by genetic alterations during malignant transformation of astrocytes; (b) by fusion of astrocytes with macrophages; or (c) by another, as yet unknown, mechanism of gene transfer during glioma progression. These may lead to significantly higher CD68 immunostaining of the malignant glioma. Real-time PCR has also revealed differential CD68 expression in benign and malignant brain tumours, which is partially in agreement with the immunostaining data. We believe that these discrepancies were due to non-representative tissue selection for biopsy as opposed to the evaluation of the immunostaining. Survival analysis revealed that CD68 tumour staining has a prognostic value for glioma patients,

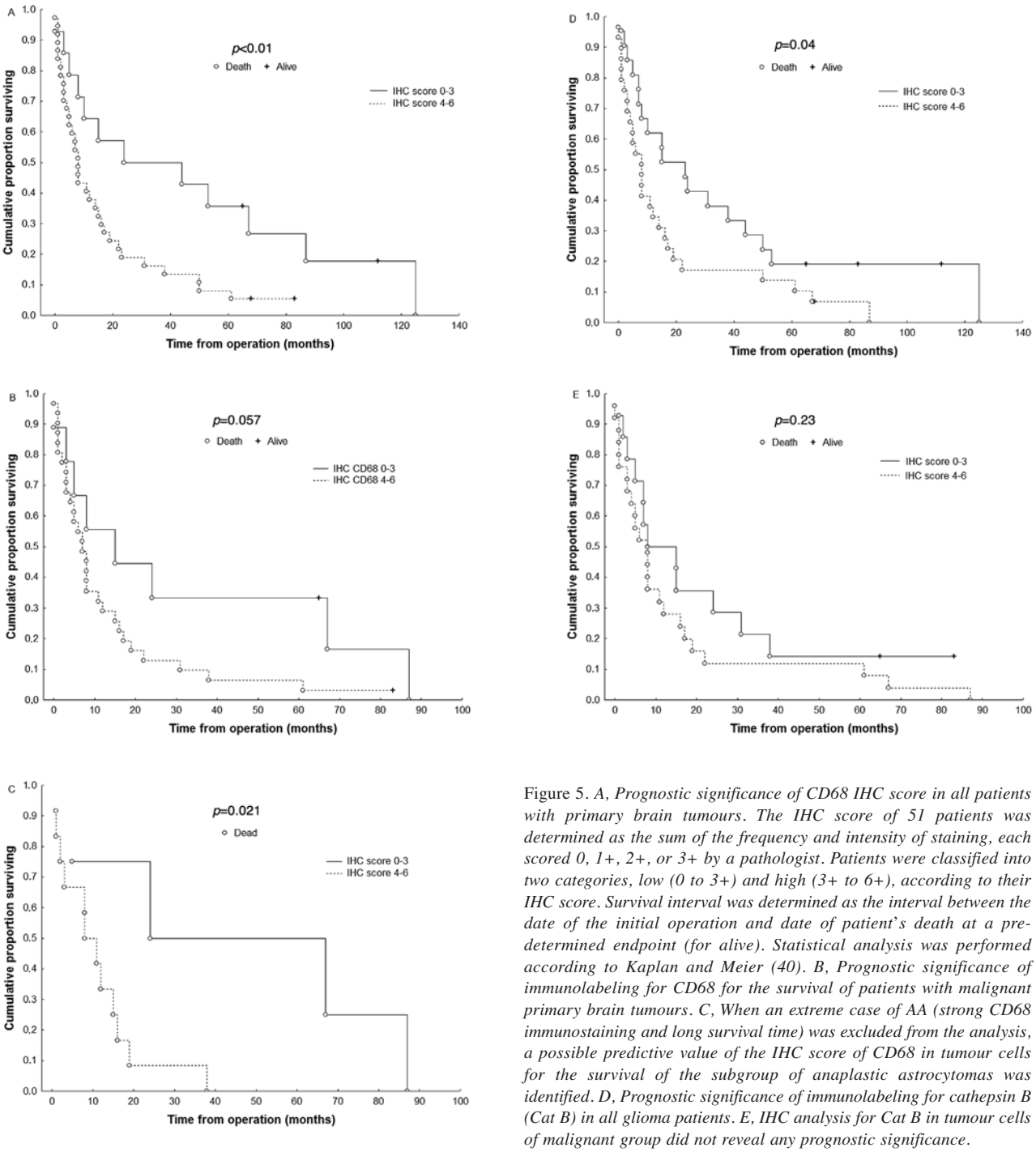

Figure 5. *A, Prognostic significance of CD68 IHC score in all patients with primary brain tumours. The IHC score of 51 patients was determined as the sum of the frequency and intensity of staining, each scored 0, 1+, 2+, or 3+ by a pathologist. Patients were classified into two categories, low (0 to 3+) and high (3+ to 6+), according to their IHC score. Survival interval was determined as the interval between the date of the initial operation and date of patient's death at a predetermined endpoint (for alive). Statistical analysis was performed according to Kaplan and Meier (40). B, Prognostic significance of immunolabeling for CD68 for the survival of patients with malignant primary brain tumours. C, When an extreme case of AA (strong CD68 immunostaining and long survival time) was excluded from the analysis, a possible predictive value of the IHC score of CD68 in tumour cells for the survival of the subgroup of anaplastic astrocytomas was identified. D, Prognostic significance of immunolabeling for cathepsin B (Cat B) in all glioma patients. E, IHC analysis for Cat B in tumour cells of malignant group did not reveal any prognostic significance.*

comparable to that of Cat B. Within the malignant group, intense CD68 staining was a marginally significant prognosticator for shorter survival, while this was not the case for Cat B staining of tumour cells. Notably, there was a significant prognostic value of CD68 tumour staining in the group of patients with AA. Further studies are necessary to investigate the possible mechanisms and consequence of macrophage phenotype expression of malignant astrocytomas, as well as possible role of microglia for tumour progression and patient prognosis.

In conclusion, the presented work shows that the protein levels of CD68 and Cat B are significantly higher in malignant compared with benign gliomas. We further conclude that specific immunostaining of CD68 and Cat B in tumour cells, along with nestin, a stem cell marker and previously reported strong prognosticator of brain cancer (55), can be used to predict the risk of overall death in patients with glioma. Noteworthy, we found prognostic value of CD68 immunostaining in tumour cells in AA, which may be important for the management of patients with longer survival than these with glioblastoma. We suggest that these novel prognostic markers should be evaluated and standardized in larger populations of patients to investigate their clinical value for the management of patients with glioma tumours and their application to the development of individualized therapeutic regiments.

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