# Clinical significance of chemokine receptor (CCR1, CCR2 and CXCR4) expression in human myeloma cells: the association with disease activity and survival

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Background and Objectives. The capacity of multiple myeloma (MM) cells to home to and reside in the bone marrow implies that they must be equipped with appropriate adhesion molecules and chemokine receptors to allow transendothelial migration. We and others have previously shown that human MM cells express at least three different chemokine receptors that are functionally involved in MM cell migration, i.e. CCR1, CCR2 and CXCR4. In this study, we analyzed the surface expression of these chemokine receptors on primary MM cells from bone marrow samples.

Design and Methods. Chemokine receptor expression was analyzed on bone marrow samples from a large population of patients (n=80) by flow cytometric analysis. The chemokine receptor expression profile was compared with clinical characteristics. Statistical significance was evaluated by Fisher's exact test. Survival curves were constructed using the Kaplan-Meier method. Cox regression analysis was used to determine the effect of chemokine receptor expression on survival.

Results. A heterogeneous expression pattern was observed for the three receptors tested. The chemokine receptor status (CRS) (i.e. no expression versus expression of at least one chemokine receptor), as well as expression of individual chemokine receptors was analyzed in relation to clinical and laboratory features and evaluated for prognostic significance. Chemokine receptor expression was significantly inversely correlated with disease activity: patients with active disease showed a significantly lower expression of CCR1, CCR2, as well as CXCR4 as compared to patients with non-active disease. Furthermore, the chemokine receptor expression profile correlated with serum b2-microglobulin, C-reactive protein and hemoglobin. CRS, and the individual expressions of CCR1, CCR2 and CXCR4 in diagnostic bone marrow samples (n=70) correlated with survival. Multivariate analysis, using the Cox proportional hazard regression model, identified CRS, along with serum b2 microglobulin, as an independent prognostic factor.

Interpretation and Conclusions. This study indicates that the chemokine receptor expression profile of MM cells correlates with disease status and survival of MM patients. This observation might reflect impaired chemoattraction and retention of MM cells within the bone marrow microenvironment, resulting in disease progression.

Key words: multiple myeloma, homing, chemokine receptor expression, survival.

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Multiple myeloma (MM) is a monoclonal B cell malignancy, arising from a late stage differentiated B cell, sharing many characteristics with immunoglobulin (Ig)-secreting plasma cells. A striking feature of this disease is the accumulation of plasma cells, with a low proliferative index and an extended life span, in the bone marrow, in close contact with stromal cells.[1] The bone marrow microenvironment plays a crucial role in the pathogenesis of MM by influencing tumor growth, survival, and drug resistance.[2-4] Although MM cells are mainly localized in the bone marrow during the early stages of the disease, extramedullary growth can be observed in more advanced stages. Several reports also describe the presence of small amounts of MM cells in the peripheral blood of many patients, suggesting that these circulating MM cells are responsible for tumor spreading.[5,6]

The capacity of MM cells to home to and reside in the bone marrow implies that these cells must be equipped with appropriate adhesion molecules and chemokine receptors that allow their migration across the vascular endothelium and the subsequent interaction with different stromal elements of the bone marrow. Trafficking and homing of leukocytes occurs by a multistep cascade, involving the sequential and coordinated activation of numerous adhesion and signal molecules.[7,8] The expression of chemokines and the presence of specific chemokine receptors on different leukocyte subsets control selective recruitment of immune effector cells from the peripheral circulation and homing of lymphocytes to the secondary lymphoid organs.[9,10] The homing process is initiated by a tethering and rolling phase, during which lymphocytes in

the blood transiently and reversibly interact with vascular adhesion receptors (including selectins and integrins) and sample the endothelium for activating factors (often chemokines). On activation, a combination of additional adhesion molecules, chemokines, and other signals will lead to an arrest of the lymphocyte, followed by transmigration across the endothelium and further migration directed by tissue-associated chemokine gradients.[11] Chemokines are a subgroup of cytokines with selective chemoattractant properties and are classified into four subfamilies, depending on the position of their $NH_2$-terminal cysteine residues (CXC, CC, CX, $CX_3C$). They bind to G-protein-coupled receptors, whose two major subfamilies are designated CCR and CXCR. Although many studies have been performed to elucidate the pathophysiology of MM, the homing mechanisms of MM cells are not fully understood. In accordance with the mechanisms used by normal lymphocytes for trafficking and homing between the blood and the lymphoid tissues, one can hypothesize that MM cells also use a chemokine-mediated mechanism for homing to the bone marrow and for remaining within the marrow stroma.

Chemokine receptor expression in MM cells has already been analyzed in some previous reports. MM cell lines show a heterogeneous expression of transcripts and surface protein for CCR1, CCR2, CCR3, CCR5, CCR6, CCR10, CXCR3, CXCR4 and CXCR6.[12-14] So far, surface expression on primary MM cells from patients' bone marrow samples has been confirmed for CCR1, CCR2, and CXCR4.[12, 13, 14] These last three receptors were also found to be functional since they mediate the *in vitro* migration of human MM cells to their specific ligands, ie. MCP-1 (for CCR2), MIP-1α (for CCR1) and SDF-1 (for CXCR4).[12,13]

In the present study, we studied the expression of the chemokine receptors CCR1, CCR2 and CXCR4 on primary MM cells of a large panel of MM patients and analyzed the clinical significance of this expression.

## Design and Methods

### Patients' samples and clinical details

Eighty patients with MM were included in this study. Patients were staged according to the criteria of Salmon and Durie.[15] These patients' characteristics are shown in Table 1. Bone marrow aspirates were obtained for routine diagnostic or evaluation purposes after informed consent. Parameters recorded at diagnosis were age, sex, Durie-Salmon stage, percentage of plasma cells in the bone marrow, immunoglobulin (Ig) class, blood hemoglobin, serum albumin, serum $\beta_2$-microglobulin, serum C-reactive protein and deletion of chromosome 13 (del 13). The patients were classified as having symptomatic (n=51) or non-symptomatic (n=29) disease at the time that the bone marrow samples were taken, based on the criteria recently defined by the International Myeloma Working Group.[16]

### Flow cytometric immunophenotype studies of chemokine receptor expression

Flow cytometry was used to analyze surface chemokine receptor expression on primary MM cells. The following monoclonal antibodies were used: mouse anti-human CCR2 (clone LS132.1D6) ($IgG_2a$), a kind gift from Dr. C. Clement (Millenium Pharmaceuticals, Cambridge, MA, USA); phycoerythrin-conjugated mouse anti-human CCR1 MoAb (clone 53504.111) ($IgG_2b$) and anti-human CXCR4 (clone 12G5) ($IgG_2a$), obtained from R&D systems (Abingdon, UK); Cy-5 conjugated mouse anti-human CD38 (clone HIT2) ($IgG_1$), from Pharmingen (Becton Dickinson, Erembodegem, Belgium). Chemokine receptor expression on the surface of primary MM cells was evaluated by a double-staining procedure as described previously.[12] Briefly, mononuclear cells isolated from bone marrow samples by Ficoll gradient centrifugation, were incubated for 30 min. at 4°C with the Cy-5 conjugated CD38 specific antibody in combination with mouse anti-human CCR2, CXCR4, CCR1, control mouse $IgG_2a$ or control mouse $IgG_2b$ monoclonal antibody (all at 10 μg/mL). In the second step, cells were incubated with phycoerythrin-conjugated goat anti-mouse $IgG_2a$/$IgG_2b$ antiserum (Southern Biotechnology ImTec, Antwerpen, Belgium) for 30 min at 4°C. After washing, cells were resuspended in phosphate-buffered saline and analyzed on an EPICS XL flow cytometer (Coulter Electronics, Analis, Namur, Belgium). The presence of monoclonal plasma cells among the $CD38^+$ gated cells was checked by intracytoplasmic κ/λ immunofluorescence using a three-color staining procedure (human Igκ-fluorescein isothiocyanate and Igλ-phycoerythrin $(Fab)_2$ fragments (both from Dako diagnostics, Heverlee, Belgium). Data were analyzed using WinMDI 2.8 FACS software. To compare the fluorescence-staining intensities of MM cells from different patients, we calculated the fluorescence intensity ratio (FIR). The FIR for a given antigen is defined as the fluorescence intensity of cells stained with the antigen-specific antibody, divided by the fluorescence intensity of cells stained with the isotype control antibody. The fluorescence intensity was calculated from the fluorescence histogram. As described in a previously published study, in which phenotypic features of MM cells were also examined in relation to different clinical parameters, we considered a FIR value of more than 1.4 as positive.[17]

**Table 1.** Patients' characteristics.

| *Parameter* | *n=80* |
|---|---|
| Age, mean (range) | 64 (35-94) |
| Male/female ratio | 1.3/1 |
| Immunoglobulin type | |
| IgG | 46 |
| IgA | 26 |
| Bence Jones | 7 |
| Non-secretory | 1 |
| Bone marrow plasmacytosis (%), mean (range) | 19 (2-95) |
| Durie and Salmon stage | |
| Stage I | 20 |
| Stage II | 23 |
| Stage III | 31 |
| Plasma cell leukemia | 6 |

### Statistics

Statistical tests were performed using Medcalc® statisti-

cal analysis software (version 7.5.0.0) for Windows. The association between clinical and biochemical variables and the expression of the chemokine receptors CCR1, CCR2 and CXCR4 was studied by the use of contingency tables. Statistical significance was evaluated by the Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method. The statistical significance of differences in overall survival between groups of patients was estimated by the log-rank test. Overall survival was defined as the time from diagnosis until death from any cause, with those still alive censored at the time of the last follow-up. Univariate analyses were performed to screen for prognostic parameters, by using Cox proportional hazards regression. The Cox model was also used for multivariate analysis to assess the independent prognostic significance of the chemokine receptor expression profile. A $p$ value of <0.05 was considered significant in all statistical analyses.

## Results

### Chemokine receptor expression by primary MM cells

Primary MM cells from the bone marrow of 80 different patients were analyzed for the expression of the chemokine receptors CCR1, CCR2 and CXCR4 using flow cytometry. For all three receptors, the expression was found to be heterogeneous. CCR1 was present in 56% of MM patients (n=45); CCR2, and CXCR4 were present in 59% (n=47). The mean FIR for positive samples was 8.6 for CCR1, 6.6 for CCR2 and 7.1 for CXCR4. The expression level for the three chemokine receptors was unimodal in all patients tested.

### Association of chemokine receptor expression on primary MM cells with disease activity

The expression of the chemokine receptors CCR1, CCR2, and CXCR4 correlated with disease activity. The percentage of MM patients expressing at least one chemokine receptor was higher in the population with non-active disease than among patients with active disease ($p$=0.0001). The chemokine receptors CCR1, CCR2 and CXCR4 were expressed in 82%, 79% and 79% of patients with non-active disease, respectively, and in only 41%, 47% and 47% of patients with active disease ($p$=0.005; $p$=0.03 and $p$=0.005 for CCR1, CCR2 and CXCR4, respectively; Fisher's exact test).

### Association of chemokine receptor expression with clinical features

Several clinical features and biological parameters were compared in patients with and without expression of CCR1, CCR2 and CXCR4 (Table 2). Patients were also stratified into two groups according to their chemokine receptor expression profile (*chemokine receptor status* or CRS): one group had no chemokine receptor expression (CRS 0) whereas the other group expressed at least one chemokine receptor (CRS 1). For all three receptors separately, as well as the global CRS, a significant correlation was observed for the two groups between expression and markers of disease activity with prognostic significance, ie. serum β2 microglobulin ($p$=0.0002; $p$=0.0001, $p$=0.003 and $p$=0.000007 for CCR1, CCR2, CXCR4 and CRS, respectively) and C-reactive protein levels ($p$=0.01; $p$=0.01, $p$=0.0005 and $p$=0.01 for CCR1, CCR2, CXCR4 and CRS, respectively). Higher levels of β2 microglobulin and C-reactive protein were observed in patients without expression of CCR1, CCR2 or CXCR4. CCR1, CCR2, and CRS, but not CXCR4 expression correlated with hemoglobin level ($p$=0.04, $p$=0.04 and $p$=0.02 for CCR1, CCR2 and CRS, respectively). The chemokine receptor expression profile also correlated with the presence of del 13, although this was not statistically significant, due to a limited number of patients in which information about del 13 was available (n=27). The chemokine receptor expression profile did not correlate with age, gender, stage, percentage of bone marrow plasmacytosis or albumin. The majority (66%) of patients with primary plasma cell leukemia (n=6) showed no chemokine receptor expression.

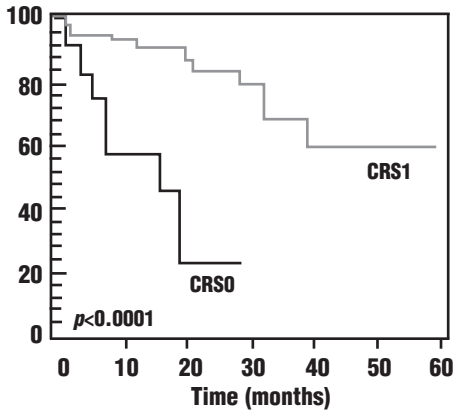


**Figure 1.** Survival probability of MM patients according to the chemokine receptor status. Kaplan-Meier survival curves for MM patients with CRS 1 (corresponding to the expression of at least one chemokine receptor) (bold line) compared with CRS 0 (corresponding to the expression of no chemokine receptor). The log-rank test revealed that MM patients with CRS 1 (bold line) had a significantly better prognosis than those with CRS 0 ($p$< 0,0001).

### The chemokine receptor expression profile at diagnosis correlates with survival

We next investigated whether the expression of CCR1, CCR2 and CXCR4 in 70 patients from whom bone marrow samples were taken at diagnosis was predictive of MM outcome using the Kaplan-Meier method. These patients represent a subset of the study population; their characteristics are shown in Table 3. Survival probabilities of subgroups with or without chemokine receptor expression were estimated, and then compared by the log-rank test. When patients were stratified into two groups according to their chemokine receptor expression profile (CRS 0 versus CRS 1), the survival difference between the two groups was highly significant ($p$<0.0001) (Figure 1). Expression of CCR1, CCR2 or CXCR4 individually also predicted a more favorable disease outcome ($p$=0.008, $p$=0.0006 and $p$=0.004, respectively) (*data not shown*). We then examined the prognostic value of serum β2 microglobulin and albumin levels at diagnosis. β2 microglobulin and albumin are factors recently used in the

**Table 3.** Characteristics of the patients whose chemokine receptor expression was assessed in bone marrow samples taken at diagnosis.

| *Parameter* | *n=70* |
|---|---|
| Age, 60 years or older | 46/70 |
| Male/female | 1/1.1 |
| Immunoglobulin type | |
| IgG | 43 |
| IgA | 21 |
| Bence Jones | 5 |
| Non-secretory | 1 |
| Bone marrow plasmacytosis (%), mean (range) | 24 (2-95) |
| $\beta_2$ microglobulin, 3 mg/L or higher | 34/70 |
| Albumin below 3 g/dL | 27/70 |

**Table 4.** Univariate analysis of the correlation of clinical factors and chemokine receptor expression with survival.

| *Variable* | *RR* | *95% CI* | *p** |
|---|---|---|---|
| Albumin | 3.22 | 1.28-8.08 | 0.01 |
| $\beta_2$ microglobulin | 5.91 | 1.93-18 | 0.002 |
| CCR1 | 2.95 | 1.12-7.76 | 0.03 |
| CCR2 | 4.32 | 1.69-7.41 | 0.003 |
| CRS | 7.10 | 2.61-19 | 0.0001 |
| CXCR4 | 3.59 | 1.41-9.15 | 0.008 |

*CI, confidence interval; RR, risk ratio; *by the chi-squared test.*

South West Oncology Group (SWOG)[18] staging model, which currently represents an accepted standard prognostication method in MM. Using univariate Cox regression survival analysis, serum $\beta_2$ microglobulin, albumin, CRS, as well as the expression of the chemokine receptors individually, were identified as variables associated with disease outcome in the MM patients whose diagnostic bone marrow samples were analyzed for chemokine receptor expression (Table 4). Subsequently, CRS and the expression of CCR1, CCR2 and CXCR4 were included in a multivariate Cox stepwise regression model with albumin and $\beta_2$ microglobulin as covariates. Multivariable analysis demonstrated that CRS and $\beta_2$ microglobulin were independent predictors of survival (Table 5).

### *The chemokine receptor status in combination with $\beta_2$ microglobulin level predicts MM disease outcome*

We generated dichotomized combination variables based on $\beta_2$ microglobulin level and CRS. MM patients were divided into three groups and the Kaplan-Meier method was used to construct survival curves. One group consisted of MM patients with high $\beta_2$ microglobulin/CRS 0 (n=13), the second group consisted of MM patients with high $\beta_2$ microglobulin /CRS 1 (n=23) and a third group



**Table 2.** Relationships between chemokine receptor expression and clinical parameters.

| | *CCR1* | | | *CCR2* | | | *CXCR4* | | | *CRS* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *CCR1+* | *CCR1-* | *p* | *CCR2+* | *CCR2-* | *p* | *CXCR4+* | *CXCR4-* | *p* | *CRS 0* | *CRS 1* | *p* |
| Age | | | | | | | | | | | | |
| < 60 | 17 | 12 | 0.8 | 17 | 12 | 1 | 16 | 13 | 0.65 | 7 | 22 | 0.57 |
| ≥ 60 | 28 | 23 | | 30 | 21 | | 31 | 20 | | 9 | 42 | |
| Gender | | | | | | | | | | | | |
| Male | 24 | 18 | 1 | 25 | 17 | 1 | 22 | 20 | 0.26 | 6 | 35 | 0.27 |
| Female | 21 | 17 | | 22 | 16 | | 25 | 13 | | 10 | 29 | |
| Stage | | | | | | | | | | | | |
| I-II | 27 | 15 | 0.18 | 27 | 14 | 0.26 | 28 | 14 | 0.17 | 6 | 36 | 0.38 |
| III-plasma cell leukemia | 18 | 20 | | 20 | 19 | | 19 | 19 | | 9 | 28 | |
| Hb (g/dL) | | | | | | | | | | | | |
| ≥ 10 | 31 | 16 | **0.04** | 32 | 14 | **0.04** | 28 | 19 | 1 | 5 | 42 | **0.02** |
| < 10 | 14 | 19 | | 15 | 19 | | 19 | 14 | | 11 | 22 | |
| Bone marrow plasma cells (%) | | | | | | | | | | | | |
| < 25 | 30 | 21 | 0.64 | 30 | 19 | 0.65 | 31 | 20 | 0.65 | 7 | 40 | 0.26 |
| ≥ 25 | 15 | 14 | | 17 | 14 | | 16 | 13 | | 9 | 24 | |
| Albumin (g/dL) | | | | | | | | | | | | |
| ≥ 3.5 | 31 | 19 | 0.25 | 33 | 17 | 0.1 | 30 | 20 | 0.82 | 7 | 43 | 0.15 |
| < 3.5 | 14 | 16 | | 14 | 16 | | 17 | 13 | | 9 | 21 | |
| Deletion 13 | | | | | | | | | | | | |
| Normal | 11 | 8 | 0.47 | 13 | 6 | 0.26 | 11 | 6 | 0.72 | 5 | 7 | 0.18 |
| Deletion 13 | 5 | 7 | | 5 | 7 | | 8 | 4 | | 2 | 13 | |
| $\beta_2$ microglobulin (mg/L) | | | | | | | | | | | | |
| < 3 | 31 | 7 | **0.00002** | 31 | 7 | **0.0001** | 29 | 9 | **0.003** | 0 | 38 | **0.00007** |
| ≥ 3 | 14 | 28 | | 16 | 26 | | 18 | 24 | | 16 | 26 | |
| C-reactive protein (mg/dL) | | | | | | | | | | | | |
| < 6 | 33 | 15 | **0.01** | 34 | 14 | **0.01** | 36 | 12 | **0.0005** | 5 | 43 | **0,01** |
| ≥ 6 | 12 | 20 | | 13 | 19 | | 11 | 21 | | 11 | 21 | |

**By Fisher's exact test. Significant correlations (p<0.05) are in bold.*

included MM patients with low β2 microglobulin /CRS 1 (n=34). There were no MM patients with low β2 microglobulin and CRS 0. This approach allowed us to further explore the prognostic significance of CRS in MM. As shown in Figure 2, the dichotomized combination variables were highly predictive of MM outcome ($p<0.0001$; log-rank test). In addition, based on the dichotomized combination variables, we could separate the MM patients further into favorable and unfavorable groups when compared with the separation made by each variable alone (CRS and β2 microglobulin).

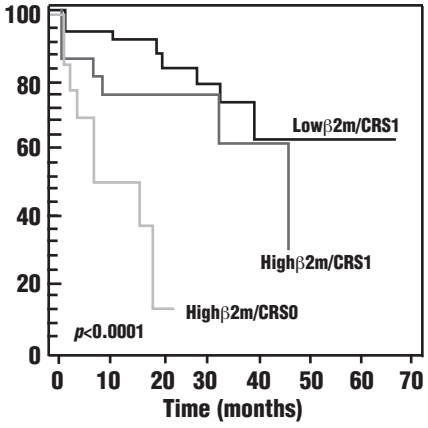


**Figure 2.** Dichotomized combination variables of CCR2 expression and β2-microglobulin (β2-m) level strongly predict outcome of MM. Dichotomized variables were generated based on CRS and β2m level. One group included MM patients with high β2m level (≥3 mg/dL) and CRS 0 (n=13), another group included MM patients with high β2 level and CRS 1 (n=23), whereas a third group consisted of MM patients with low β2m and CRS 1 (n=34). Survival probabilities of the different groups of MM patients defined by the dichotomized combination variables, β2m/CRS, were estimated by the method of Kaplan-Meier. The log-rank test was used to compare survival probabilities of the groups.

**Table 5.** Multivariate analysis: results of Cox stepwise regression analysis.

| *Variable* | *HR* | *95% CI* | *p** |
|---|---|---|---|
| β2 microglobulin | 3.86 | 1.11-13.45 | 0.03 |
| CRS | 3.73 | 1.29-10.83 | 0.02 |

*CI: confidence interval; HR: hazard ratio. *By chi-squared test.*

## Discussion

Chemokines and their corresponding receptors are key mediators of lymphocyte trafficking and homing. They are not only involved in cellular migration but mediate retention of migrated cells in extravascular tissue-compartments as well. Previous reports indicated that human MM cells express at least three different chemokine receptors, i.e. CCR1, CCR2 and CXCR4.[12-14] It has also been shown that the expression of these chemokine receptors is functional, since MM cells migrate *in vitro* to the following specific ligands: RANTES and MIP-1α (for CCR1), MCP-1, -2 and –3 (for CCR2) and SDF-1α (for CXCR4). Moreover, the chemokines MCP-1, MCP-2, MCP-3, as well as SDF-1 are abundantly produced by bone marrow stromal cells, suggesting that these chemokine receptors and their ligands are involved in the bone marrow homing and localization of MM cells.[12,13] All phenotypic and functional studies of chemokine receptor expression in human MM cells that have been reported so far were based on human myeloma cell lines or a limited number of patients' samples.[13,14]

In this study we determined the expression of these three chemokine receptors on MM cells in a large cohort of patients (n=80) and analyzed this expression in relation to disease activity and survival. Our study demonstrated that expression of the three receptors by primary MM cells is heterogeneous, although a majority of MM patients were found to be positive. In addition we found that chemokine receptor expression inversely correlates with disease activity. Patients with active disease less frequently expressed CCR1, CCR2 and CXCR4, suggesting that chemokine receptor expression can be altered when disease progresses. However, based on our findings it is not possible to conclude that chemokine receptor expression changes for each patient individually when disease becomes more active. Follow-up studies in different individual patients during disease progression are necessary to test this last possibility. Chemokine receptor expression also inversely correlated with laboratory parameters (β2 microglobulin, hemoglobin and C-reactive protein). MM patients with plasma cells negative for CCR1, CCR2 or CXCR4 displayed higher levels of serum β2 microglobulin and C-reactive protein. Survival analysis demonstrated that chemokine receptor expression was also significantly associated with better overall survival. Expression of at least one of the three chemokine receptors (CRS 1) predicted a more favorable clinical outcome among the MM patients whose chemokine receptor expression was assessed in bone marrow samples taken at diagnosis.

Because of its biological complexity, the clinical course of MM disease is quite variable. A subset of MM patients has a less favorable outcome. Various factors may help to identify these patients with poor prognosis including deletion of chromosome 13 and the serum parameters β2 microglobulin and albumin.[19] The univariate Cox regression survival analysis demonstrated that serum β2 microglobulin and albumin, CRS and the individual expressions of the CCR1, CCR2 and CXCR4 predicted disease outcome in our study group. These findings allow us to conclude that our study population is representative for evaluating the clinical significance of chemokine expression in MM. We also demonstrated that CRS and β2 microglobulin are statistically significant independent prognostic factors. CRS may therefore add independent information to standard prognostic factors.

In the present study, we observed a clear correlation between low chemokine receptor expression and clinical parameters related to disease activity and survival, including high C-reactive protein and especially low albumin and high β2 microglobulin levels. Very recently, the international staging system for myeloma based on albumin

and $\beta_2$ microglobulin levels has been extensively confirmed and validated.[20] In addition, we found that the chemokine receptor status was an independent and additive prognostic factor that was able to identify, within the group of patients with high $\beta_2$ microglobulin, a subgroup with a different survival probability.

The reason why the absence of chemokine receptor expression is associated with a decreased probability of survival is unclear. It can be assumed that these three receptors not only mediate the migration of MM cells to bone marrow but that they also contribute to retaining the tumor cells in the bone marrow microenvironment. This means that the absence of chemokine receptor expression could favor dissemination of MM cells in the circulation, possibly contributing to accelerated disease progression. In MM, malignant plasma cells can be detected in the peripheral blood circulation and their numbers increase during disease progression.[21,22] The downregulation or absence of chemokine receptor expression could be involved in the (re)circulation/movement of MM cells from medullary to extramedullary sites, as a result of decreased retention within the bone marrow microenvironment. In this context it is important to mention that no chemokine receptor expression could be shown in the majority of bone marrow samples from MM patients with primary plasma cell leukemia. A recent study indicated that downregulation of CXCR4 on MM cells during mobilization of normal hematopoietic stem cells is associated with increased mobilization of tumor cells in the circulation.[23] The expression of chemokine receptors in malignant cells has been associated with prognosis and disease dissemination in different tumor types. Both positive as well as negative correlations have been reported. For example, CXCR4 expression in early-stage non-small cell lung cancer is associated with a better outcome,[24] while upregulation of CXCR4 in breast cancer cells is associated with tumor metastasis and more aggressive disease.[25] Also in some hematologic malignancies, a high expression of the chemokine receptor CXCR4 is associated with reduced overall survival as observed in acute myeloid leukemia[26] or with extramedullary organ infiltration as observed in childhood acute lymphoblastic leukemia.[27] In B-cell chronic lymphocytic leukemia, the expression of CXCR4 was found to be significantly increased in patients with lymphadenopathy and in patients with advanced disease.[28] Absence of chemokine receptor expression on neoplastic T-cells in mycosis fungoides is associated with metastasis into regional lymphatic tissue.[29] All these observations illustrate that the involvement of chemokine receptors in tumor pathogenesis is very complex and might result in opposite effects in different tumor types.

Which mechanisms are involved in the absence or downregulation of the chemokine receptors on MM cells is not known. Since the genetic background of MM is very complex and heterogeneous, some patients might lack or lose one or more chemokine receptors due to the intrinsic genetic profile of the tumor cells. Interestingly, it was demonstrated very recently that DNA methylation decreases CXCR4 expression in pancreatic cancer.[30] Aberrant gene promotor methylation is also a common phenomenon in MM, involving genes related to tumor suppression (p15, p16), apoptosis (death-associated protein kinase) or adhesion (E-cadherin).[31] It has also been suggested that methylation patterns might be useful prognostic indicators at diagnosis of MM.[32,0]Possibly, our observations reflect at least in part an epigenetic regulation of chemokine receptor expression in MM. Another explanation might be that the chemokine receptor profile on MM cells is influenced by stroma-associated factors. Changes in the composition and function of the tumor microenvironment during the course of MM might lead to chemokine receptor loss. Since chemoattraction to and retention in the bone marrow are essential for the paracrine growth regulation of MM cells in the initial phase of the disease, chemokine receptor loss at this stage would impair optimal stroma-mediated growth support. Subsequently, this would lead to a decrease in disease activity. Since we observed less chemokine receptor expression in MM patients with active disease, it could be concluded that this downregulation is associated with a decreased stroma-dependency of the tumor cells.

In conclusion, we demonstrated that chemokine receptor expression on malignant plasma cells is associated with disease activity and could be of prognostic value in individuals with multiple myeloma.

*IVB designed the study, interpreted all data, performed all the statistical analyses and wrote the manuscript; XL and TF provided a major part of the patient samples and contributed substantially to the analysis of the data by providing all clinical parameters related to disease status. Their contribution was essential for drawing the general conclusions of this study; RS contributed intellectually to the statistical analysis of the data and critically revised this manuscript; KV, BVC critically revised this manuscript and made some conceptual suggestions; IVR is the promotor of this researh work, contributed substantially to the conception of the manuscript and critically revised the final version.*

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