# Expression of angiopoietins and their receptor Tie2 in the bone marrow of patients with acute myeloid leukemia

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Background and Objectives. Angiopoietin-1 (Ang-1) and its natural antagonist angiopoietin-2 (Ang-2), both ligands for the receptor tyrosine kinase Tie2, are known to play an essential role in normal and pathological angiogenesis.

Design and Methods. We investigated the expression of Ang-1, Ang-2 and Tie2 by immunohistochemical analyses in bone marrow biopsies of 64 adult patients with newly diagnosed acute myeloid leukemia (AML) and correlated angiogenic factor expression with clinicopathological variables and long-term survival.

Results. Expression of Ang-2 was significantly higher in the bone marrow of AML patients than in 16 control patients. In contrast, the levels of Ang-1 expression in AML patients did not differ from those found in controls. Thus, we observed a reversal of the Ang-1 and Ang-2 expression balance in the neoplastic bone marrow. Furthermore, Tie2 was significantly overexpressed in leukemic blasts. Patients expressing high levels of Ang-2 had significantly longer overall survival than those with low Ang-2 levels (52.7 vs. 14.7 months). Multivariate analysis revealed that karyotype and Ang-2 expression were independent prognostic factors for overall survival (hazard ratio [CI]: 3.06 [1.39-6.70] and 0.31 [0.14-0.69], respectively).

Interpretation and Conclusions. These data provide evidence that the alteration of angiopoietin balance in favor of Ang-2 may play a critical role in the pathophysiology of AML. Furthermore, high pre-therapeutic levels of Ang-2 in the bone marrow indicate a favorable prognosis in AML patients treated with polychemotherapy, although the mechanism is not yet known.

Key words: angiopoietin, Tie2, AML, leukemia, angiogenesis.

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Angiogenesis, the formation of new blood vessels from pre-existing ones, is required for the growth of solid tumors and hematologic neoplasias such as acute myeloid leukemia (AML). We and other groups have demonstrated increased neovascularization in the bone marrow of AML patients.[1-3] The mutual interplay of bone marrow endothelial cells and growth factors derived from leukemic blasts contributes to the pathogenesis of hematologic malignancies.[4,5] Moreover, it has become increasingly clear that angiogenic factors produced by leukemic blasts may also act in an autocrine or intracrine fashion, thereby stimulating cell proliferation and survival through a mechanism independent from angiogenesis.[6,7] Several angiogenic inducers have been identified to exert the angiogenic phenotype observed in AML bone marrow or to establish autocrine signaling pathways in leukemic blasts. Vascular endothelial growth factor (VEGF) expression is significantly upregulated in the bone marrow of AML patients[4,5,8] and represents an adverse prognostic factor in AML.[3] Blocking both paracrine and autocrine VEGF/VEGFR-2 pathways induced long-term remission in a leukemia mouse model.[6] In patients with refractory AML VEGF receptor antagonists showed biological activity.[9-11] Our group also found significant overexpression of basic fibroblast growth factor (bFGF) in the bone marrow of patients with newly diagnosed AML.[12]

The angiopoietins (Ang) constitute a novel family of angiogenic mediators, which have been shown to be important regulators of neovascularization, vascular stability and maturation.[13,14] Ang-1 and its naturally occuring antagonist Ang-2 act via the Tie2 receptor tyrosine kinase, which is broadly expressed in the endothelium of adult vasculature[15] and in a subset of hematopoietic stem cells.[16,17] Although very similar in protein structure, Ang-1 and Ang-2 elicit opposing responses when binding to endothelial Tie2.[14,18] Binding of Ang-1 causes autophosphorylation of Tie2 and ensures the integrity of blood vessels by strengthening interactions between endothelial cells and periendothelial support cells. In contrast, Ang-2 specifically disrupts Ang-1-mediated receptor activation resulting in vessel

destabilization, thereby facilitating the angiogenic response to mitogenic factors such as VEGF or leading to vessel regression in their absence.[19] With its role in both angiogenesis and vascular maintenance, Tie2 seems to have a dual function defined by the quantitative balance between Ang-1 and Ang-2 activity.[20] While Ang-1 is constitutively expressed throughout adult tissues providing a stabilizing signal, normal postnatal Ang-2 expression is only observed at sites of active vascular remodeling.[14]

Most of the studies on the role of angiopoietins in tumor-related angiogenesis have focused on solid tumors. With regard to AML, Watarai *et al*. detected Ang-1, Ang-2 and Tie2 mRNA in AML cell lines and in, respectively, 27, 12 and 15 of 36 AML patient samples.[21] In another study, peripheral blood cells from patients with acute or chronic myeloid leukemia showed elevated RNA expression of Ang-1 and Tie2 in 11 of 17 cases, while Ang-2 expression was not examined.[22] In the first study investigating the prognostic relevance of the Tie2/angiopoietin system in hematologic neoplasias, Loges *et al*. recently demonstrated that high Ang-2 mRNA expression in peripheral blasts was an independent favorable prognostic factor for overall survival in AML.[23] In summary, the role of angiopoietins in AML remains unclear. Existing studies are usually based on mRNA expression in peripheral leukemic blasts or cell lines. To our knowledge, there is no study investigating the *in situ* protein expression pattern of angiopoietins and Tie2 in AML bone marrow, i.e. the site where the process of angiogenesis takes place. Therefore, we determined the expression of Ang-1, Ang-2 and Tie2 in the bone marrow of adult patients with newly diagnosed AML by immunohistochemical analyses. Furthermore, we analyzed the correlation between Ang-1, Ang-2 and Tie2 expression and clinicopathological features, bone marrow microvessel density (MVD) and long-term survival.

## Design and Methods

### Patients and bone marrow specimens

Expression of Ang-1, Ang-2 and Tie2 was determined in bone marrow specimens of 64 patients with newly diagnosed AML. This study population is an extension of a previously investigated cohort of 62 patients for whom the degree of bone marrow angiogenesis at presentation was reported.[2] Sixteen unselected controls were taken from the initial group of 22 patients who had also been analyzed for bone marrow MVD. These controls are adult patients with various diseases but normal bone marrow morphology as demonstrated by cytological and histological analyses. A bone marrow core biopsy (iliac crest) was obtained at presentation from all AML and control patients for histological diagnosis. After every core biopsy a bone marrow aspiration was obtained through a separate puncture for cytological analyses. A total of 57 patients received intensive chemotherapy according to the current protocol of the German AML cooperative group (AMLCG). The remaining seven patients not qualifying for intensive therapy were enrolled in different experimental protocols (thalidomide, SU5416) and were excluded from the analysis of survival. For remission induction, patients were treated according to a double induction protocol (either with TAD [thioguanine, standard dose cytarabine and daunorubicin] and HAM [high dose cytarabine and mitoxantrone] or with two successive courses of HAM), irrespective of response to the first course. The second cycle of induction chemotherapy was applied to patients older than 60 years only when a day 16 bone marrow puncture revealed significant residual blast infiltration (≥5% blasts in the hypoplastic bone marrow). All patients achieving complete remission according to standard criteria received consolidation therapy consisting of one cycle of TAD. Following consolidation, patients either received myelosuppressive maintenance therapy for 3 years or underwent autologous stem cell transplantation. In our study, 41 patients received TAD as the first cycle of induction therapy, 16 patients received HAM. Six patients underwent allogeneic stem cell transplantation and one patient had an autologous transplant. All patients treated within the AMLCG study gave their informed consent to the study after having been advised about its investigational nature. The study received approval from the local ethics board.

### Immunohistochemical staining

Bone marrow samples were fixed in paraformaldehyde, decalcified with EDTA and embedded in paraffin. Serial sections (4 μm thick) of each sample were processed immunohistochemically to demonstrate the expression of the angiopoietins and Tie2 with rabbit polyclonal Ang-1 (Alpha Diagnostic, San Antonio, TX, USA; working dilution 1:100), Ang-2 (Alpha Diagnostic; 1:500) and Tie2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA 1:200). As described by the manufacturers the angiopoietin antibodies are specific for Ang-1 and Ang-2 and do not show any cross-reactivity with each other or with other angiopoietins. The Tie2 antibody is also specific and does not cross-react with Tie1 or other receptor tyrosine kinases. Controls using non-immune mouse IgG or rabbit IgG (Santa Cruz Biotechnology) instead of the primary antibodies were consistently negative (*data not shown*). Immunohistochemical staining was performed using the alkaline phosphatase/antialkaline phosphatase double bridge technique (Dako APAAP-kit, Dako, Glostrup, Denmark) as described previously.[8,12] Briefly, tissue sections were deparaffinized in xylene and rehydrated in a graded ethanol series. Samples were pretreated to promote antigen retrieval in a microwave oven at 450 W twice for 7 min in 10 mM sodium citrate (pH 6.0; Dako). The primary antibodies were applied overnight at 4°C. Subsequent steps were performed according to the manufacturer's instructions. The fast red substrate (Dako) supplemented with 0.1% (w/v) levamisole was used for detec-

tion of phosphatase activity (30 min at room temperature). Sections were counterstained with 0.1% (w/v) erythrocin solution. The method for determining the degree of MVD by immunohistochemical identification of microvascular endothelial cells with anti-human thrombomodulin antibodies has been reported elsewhere.[2]

### Evaluation of Ang-1, Ang-2 and Tie2 expression

Immunostaining was simultaneously assessed by two independent experienced investigators using light microscopy. Both investigators were blinded to the clinical characteristics and the bone marrow microvessel counts of the patients when performing the evaluation of protein expression. Expression of Ang-1, Ang-2 and Tie2 protein was semiquantitatively assessed by scoring the proportion and intensity of stained cells according to published methods.[8,12] At first, the entire bone marrow section was systematically scanned at ×100 and ×250 magnification and the percentage of positive cells stained with each specific antibody within cellular areas was estimated according to a three-grade scale (1: <10%, 2: 10-50%, 3: >50% positive cells). Subsequently, the intensity of positive staining was evaluated in three representative fields magnified ×500 selected from each section after the initial screening at ×100–×250 magnification. The degree of intensity of cell staining within these areas was classified in the following manner: 1: faint or negative, 2: <50% cells with moderate staining, 3: >50% cells with moderate staining, 4: <50% cells with intense staining, 5:>50% cells with intense staining. In order to achieve a reasonable assessment of the protein expression in the whole bone marrow section, the mean of the intensity of cellular staining from three representative fields magnified ×500 was subsequently multiplied by the number obtained when estimating the percentage of positive cells according to the three-grade scale in the entire bone marrow section. Results were expressed as arbitrary units (AU). In each biopsy sample, the expression of Ang-1, Ang-2 and Tie2 was evaluated in two or three sections processed in independent immunostains and the mean value was calculated. The median variability between the investigators for the immunostaining scores was 16.6% (lowest quartile-highest quartile [LQ-HQ]: 0.0-33.3%). Analysis of two or three marrow sections from a single biopsy specimen revealed a median intra-individual variability of less than 30% for each of the antibodies (median [LQ-HQ]: Ang-1: 25.0% [14.3-54.4%], Ang-2: 22.5% [8.1-45.4%], Tie2: 28.6% [10.9-56.3%]). To ensure the reliability of the quantification assay, marrow slides were randomly selected during the study and re-analyzed with excellent agreement. A bone marrow specimen was used as an internal control. Sections of this specimen were repeatedly immunostained for the different antibodies and quantified by the two investigators at different times.

**Table 1.** Patients' characteristics.

| *Features* | *AML patients* |
|---|---|
| No. | 64 |
| Median age (range), years | 63 (17–84) |
| Sex | |
| male | 36 |
| female | 28 |
| FAB classification | |
| M0 | 1 |
| M1 | 6 |
| M2 | 21 |
| M3 | 2 |
| M4 | 11 |
| M5 | 16 |
| M6 | 5 |
| M7 | 1 |
| non-classifiable | 1 |
| Percentage of bone marrow infiltration (range) | 78 (20-95) |
| Karyotype | |
| favorable: t(8;21), t(15;17), inv(16) | 6 |
| intermediate: normal, +8, +22, other | 36 |
| unfavorable: complex, -5, -7 | 13 |
| unknown | 9 |
| CR following induction therapy | 47 (82%) |
| Median follow-up (range), months | 20.2 (7.5-50.1) |

*FAB: French-American-British classification for AML; CR: complete remission.*

### Statistics

Data are presented as individual data plots or as medians, interquartile ranges (LQ–HQ), and ranges. Differences in the expression of Ang-1, Ang-2 and Tie2 between AML and control groups were analyzed using the Mann-Whitney rank sum test for independent groups. The statistical significance of overall differences between more than two groups was analyzed by the Kruskal Wallis one-way analysis of variance. Correlations between variables were assessed by the Spearman rank correlation coefficient ($r_s$). The distributions of the time-to-event variables were estimated by Kaplan-Meier life curves and compared using the log-rank test. Patients were categorized into high and low angiogenic factor expressing subgroups using the median value as the cut-off. Parameters independently associated with overall survival and relapse-free survival were identified in multivariate analyses by a Cox's proportional hazards model. Overall survival was calculated from the date of first diagnosis to death from any cause. Relapse-free survival was measured from the time of complete remission until relapse or death in complete remission. Patients who did not suffer an event within the follow-up period were censored at the date of last contact. Two-sided $p$ values <0.05 were considered statistically significant. All statistical analyses were performed with the SPSS package (SPSS Inc., Chicago, IL, USA).

## Results

Expression of angiopoietins and Tie2 was analyzed by immunohistochemical staining in bone marrow core sec-

tions of 64 AML patients at diagnosis. The patients' characteristics are presented in Table 1. The staining scores of these patients were compared with the degree of angiogenic factor expression in bone marrow biopsies obtained from 16 patients with different diseases but normal bone marrow morphology.

### Angiopoietin-1

Ang-1 protein was heterogenously distributed within the cellular areas of AML bone marrow with a similar staining pattern as that found in the controls (Figure 1A and 1B). In AML and control patients, staining intensity varied from weak to moderate with some scattered cells showing strongly positive signals. Immunoreactivity was primarily associated with the cytoplasm of leukemic blasts and normal hematopoietic cells. However, besides cellular staining, diffuse intercellular and matrix-associated immunoreactivity for Ang-1 antigen was observed in AML and control patients. The staining scores for Ang-1 protein expression in AML patients were low to moderate (median [LQ-HQ]: 2.5 [1.9–3.3] AU) and did not differ significantly from the Ang-1 expression found in bone marrow sections of control patients (3.6 [2.1–5.0] AU; $p$=0.105, Mann-Whitney test; Figure 2A). In specimens from both groups, megakaryocytes also stained positive and were easily recognized by their characteristic size and morphology.

### Angiopoietin-2

In contrast to Ang-1, Ang-2 expression was significantly greater in neoplastic bone marrow than in normal bone marrow. Ang-2 positive leukemic blasts were widely and uniformly observed in a homogeneous pattern throughout the cellular regions of AML bone marrow (Figure 1C). Signals were associated with the cytoplasm as well as with the nucleus of leukemic blasts. In contrast to its expression in AML, Ang-2 expression in control bone marrow samples was generally weak and limited to a few scattered positive cells, with most of the cells being completely negative (Figure 1D). All 16 control specimens displayed staining scores ≤2 AU. The staining scores for Ang-2 protein expression in bone marrow sections of AML patients were significantly higher than those for controls (median [LQ–HQ]: AML: 4.7 [3.3–5.7] AU; controls: 1.5 [1.5–1.8] AU; $p$<0.0001; Figure 2B). With only one exception (1.3 AU), all individual Ang-2 staining scores of the AML group were higher than the median Ang-2 expression in control patients (1.5 AU).

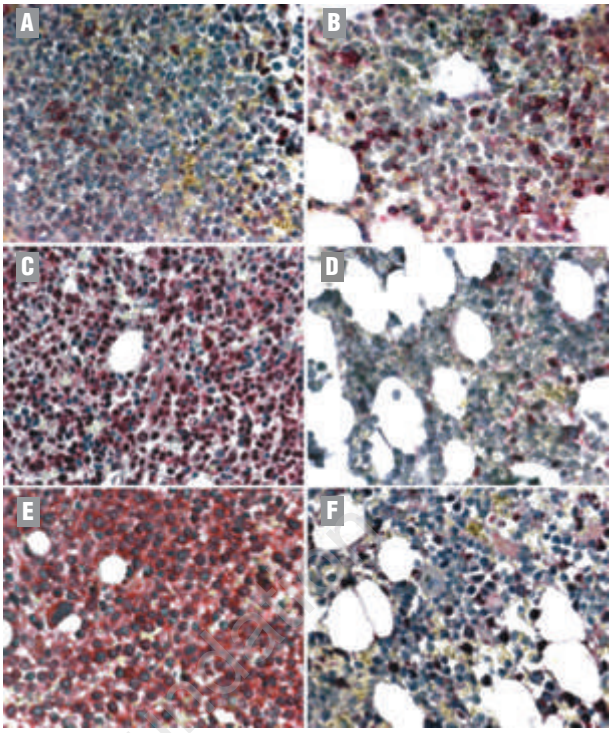


**Figure 1.** Immunohistochemical staining of bone marrow sections obtained at presentation from AML and control patients for Ang-1 (A,B), Ang-2 (C,D) and Tie2 (E,F). Immunohistochemical localization was performed using the respective specific antibodies and the alkaline phosphatase/anti-alkaline phosphatase technique (Dako-APAAP kit; Dako). Note the overexpression of Ang-2 and Tie2 and the homogeneous distribution of signals in the bone marrow sections of AML patients (C,E) compared to the controls (D,F). In contrast, similar staining patterns and intensity were observed for Ang-1 in the bone marrow of AML (A) and control patients (B). Original magnification ×500.

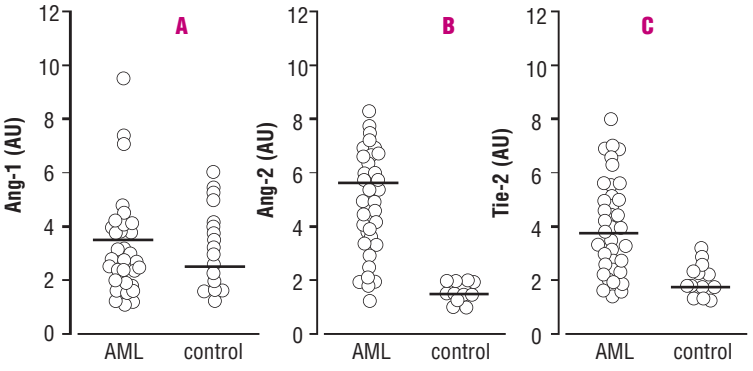


**Figure 2.** Expression of Ang-1, Ang-2 and Tie2 in the bone marrow of AML and control patients. Adjacent sections of bone marrow specimens from 64 AML patients at presentation and 16 control patients were stained in parallel for Ang-1 (A), Ang-2 (B) and Tie2 (C). Staining scores for each protein were calculated as described in *Design and Methods*. Data are presented as individual values (open circles) and medians (horizontal bars). Ang-2 and Tie2 levels were significantly higher in AML than in control patients ($p$<0.0001, Mann-Whitney test). No significant differences were detected between AML and control patients for Ang-1 ($p$=0.105).

### Ang-2:Ang-1 ratio

Ang-1 leads to activation of endothelial Tie2 while Ang-2 inhibits Ang-1-mediated receptor activation in endothelial cells. The degree of receptor phosphorylation is determined by the relative balance between agonistic and antagonistic ligands. We, therefore, calculated the Ang-2:Ang-1 expression ratio in normal and leukemic bone marrows as previously described.[24,25] The median Ang-2:Ang-1 expression ratio was 3-fold higher in AML patients than in controls (median [LQ–HQ]: AML: 1.73 [1.25–2.37]; controls: 0.51 [0.37–0.65]; $p<0.0001$), indicating a dominant influence of Ang-2 in the neoplastic bone marrow and of Ang-1 in the normal bone marrow microenvironment.

### Tie2

Areas of homogeneously positive staining for Tie2 were widely distributed in cellular regions of bone marrow from AML patients (Figure 1E). Signals were primarily seen in the cytoplasm of leukemic blasts with membrane accentuation. In contrast, normal bone marrow samples displayed weak and heterogeneously distributed immunoreactivity for Tie2. Here, Tie2 expression was limited to some positive cells, which were occasionally detected among many completely Tie2-negative bone marrow cells (Figure 1F). Tie2 protein staining scores of the 64 bone marrow specimens from AML patients ranged from 1.5 to 10.8 AU with a median value of 3.8 (2.8–4.9) AU, whereas control samples showed significantly weaker Tie2 protein staining (1.8 [1.6 – 2.3] AU; $p<0.0001$; Figure 2C). All individual staining scores from the control group were lower than the mean value of protein expression in AML patients. Beside cells of malignant and non-malignant hematopoiesis, occasionally observed capillary-shaped structures and arterioles were found to be Tie2-positive in AML and control patients. Again, megakaryocytes were stained with Tie2 antibodies in both groups.

### Ang-1, Ang-2 and Tie2 expression and clinicopathological features

No statistically significant association was found between the expression of angiopoietins and age at first diagnosis of AML. However, the scores for Tie2 expression were slightly higher in AML patients under 60 years old than in AML patients over 60 years old. Furthermore, there was no association between Ang-2 or Tie2 expression and patients' sex. In contrast, bone marrow Ang-1 expression was slightly higher in female AML patients than in male patients in our study cohort. The bone marrow of the AML patients studied was highly infiltrated by leukemic blasts. The median (LQ–HQ) percentage of blasts was 78 (50–86)%. Although Ang-1, Ang-2 and Tie2 were consistently detected in leukemic blasts, staining scores did not correlate with the percentage of leukemic blast infiltration determined by bone marrow aspiration (Ang-1: $r_s$=-0.098; Ang-2: $r_s$=0.131; Tie2: $r_s$=-0.123; $p>0.05$ for each variable).

**Table 2. Univariate analyses of the impact of bone marrow Ang-1, Ang-2 and Tie2 expression on relapse-free and overall survival in AML patients.**

| | *Relapse-free survival* | | *Overall survival* | |
|---|---|---|---|---|
| *Variables*[1] | *n* | *p* | *n* | *p* |
| Ang-1 | | | | |
| Low | 20 | 0.7374 | 25 | 0.7664 |
| High | 27 | | 32 | |
| Ang-2 | | | | |
| Low | 18 | 0.1048 | 24 | 0.0390 |
| High | 29 | | 33 | |
| Ang-2/Ang-1 ratio | | | | |
| Low | 21 | 0.5972 | 25 | 0.7460 |
| High | 26 | | 32 | |
| Tie2 | | | | |
| Low | 20 | 0.4265 | 24 | 0.8015 |
| High | 27 | | 33 | |

[1]*Median values of each variable were used as the cut-off levels to define low and high angiogenic factor expressing groups.*

We then analyzed the correlation between angiogenic factor expression and AML subtypes according to FAB classification, as defined by cytological analyses. Statistical analyses did not reveal any significant differences in Ang-1, Ang-2 or Tie2 expression between subtypes M0 to M7 (Kruskal-Wallis test, $p>0.05$ for each variable). From each bone marrow sample, adjacent sections to those processed in the present study were previously immunostained with specific markers for endothelial cells in order to assess the MVD.[2] The median MVD in the 64 AML patients of our cohort was 25.0 (21.5–28.9) microvessels/×500 field and 13.7 (12.7–15.0) microvessels/×500 field in the 16 control patients, respectively. Protein expression scores of Ang-2, Tie2 and the Ang-2/Ang-1 ratio, but not Ang-1 correlated with MVD in the entire study population (AML and control patients: Ang-2: $r_s$=0.521; Tie2: $r_s$=0.417; Ang-2/Ang1 ratio: $r_s$=0.514; $p<0.0001$ for each variable). However, when analyzing the AML and control group separately, no significant correlation was found ($p>0.3$ for each variable). Furthermore, we tested whether bone marrow Ang-1, Ang-2 or Tie2 expression at diagnosis could predict the response to induction chemotherapy assessed on day 16 or the achievement of a complete remission. No association was detected between the levels of expression of Ang-1, Ang-2 or Tie2 and the response to induction therapy ($p>0.3$ for each variable) or the achievement of a complete remission ($p>0.4$ for each variable).

### Ang-1, Ang-2 and Tie2 expression and overall and relapse-free survival

We subsequently investigated the relationship between pre-therapeutic Ang-1, Ang-2 and Tie2 expression and

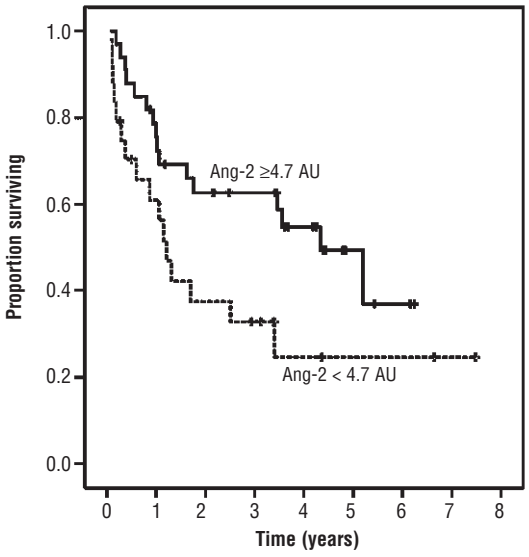


**Figure 3.** Kaplan-Meier curves of overall survival of AML patients stratified for bone marrow Ang-2 expression. The overall survival rates of patients expressing high levels of Ang-2 (≥4.7 AU) in the bone marrow were significantly higher than those in patients with low Ang-2 expression (<4.7 AU) ($p$=0.039).

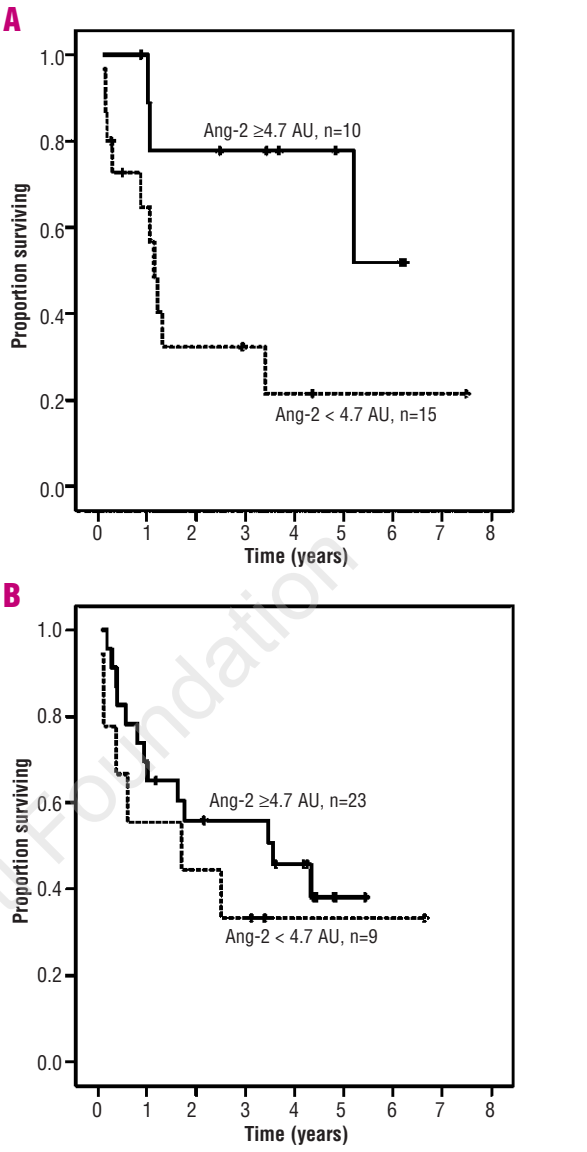


**Figure 4.** Kaplan-Meier curves of overall survival stratified for Ang-2 expression in the low Ang-1 expressing (A) and high Ang-1 expressing (B) subgroups. The impact of Ang-2 on overall survival was especially evident in the low Ang-1 expressing subgroup ($p$=0.0298) and was diminished in the high Ang-1 expressing subgroup ($p$=0.3310).

prognosis in AML (Table 2). Univariate analysis revealed that patients expressing high levels of Ang-2 in the bone marrow had significantly longer overall survival than those with low Ang-2 levels ($p$=0.039). The median survival time of high Ang-2 expressing AML patients was more than 3-fold longer than that of patients with low Ang-2 expression (52.7 vs. 14.7 months). Figure 3 demonstrates the Kaplan-Meier curve for overall survival stratified according to Ang-2 expression. To exclude confounding influences of different treatment regimens, additional subgroup analyses were performed. The type of induction therapy did not influence patients' overall survival in our study (TAD vs. HAM, $p$=0.2088). Analysis of a possible impact of the type of maintenance therapy (3 years of myelosuppressive maintenance vs. autologous transplantation) was not informative given the low number of patients in the transplantation subgroup. After censoring patients who received allogeneic stem cell transplantation at the date of cell infusion to exclude the influence of transplantation-related death, the prognostic impact of Ang-2 on overall survival was even more pronounced ($p$=0.009). No association was detectable between Ang-1 or Tie2 expression and overall survival ($p$>0.7 for each variable).

A multivariate Cox regression analysis was then performed including the following parameters: age, FAB subtype, karyotype and Ang-2 expression. In this analysis, Ang-2 expression ($p$=0.004; hazard ratio 0.31, 95% CI 0.14–0.69) and karyotype ($p$=0.005; hazard ratio 3.06, 95% CI 1.39–6.70) emerged as independent prognostic factors for overall survival. In order to investigate whether the observed influence of Ang-2 expression on prognosis was angiogenesis-dependent, we correlated bone marrow MVD scores with overall survival. No differences were found between patients with low and high bone marrow microvessel counts ($p$>0.9). Regarding estimated relapse-free survival, there was a clear trend to a correlation between Ang-2 and this outcome, although this correlation failed to reach the level of statistical significance ($p$=0.1048). This is most likely explained by the lower number of patients available for analysis, given that 82% of the patients in this study achieved a complete remission. There was no association between Ang-1 or Tie2 expression and relapse-free survival ($p$>0.4 for each variable). Finally, we performed subgroup analyses to investigate potential interactions between Ang-2 and Ang-1 as

agonistic and antagonistic Tie2 ligands on patients' survival. Therefore, we divided our study cohort into two subgroups with Ang-1 expression above and below the median of 2.5 AU. Univariate analysis revealed that the impact of bone marrow Ang-2 expression on overall survival was most prominent in the subgroup of AML patients simultaneously expressing low levels of Ang-1 (n=25; $p$=0.0298; Figure 4A). However, in patients expressing both high levels of Ang-2 and Ang-1, Ang-2 lost its predictive value for survival (n = 32; $p$=0.3310; Figure 4B).

## Discussion

Our study clearly demonstrates an elevated expression of Ang-2 and its receptor Tie2 in the bone marrow of patients with newly diagnosed AML. Ang-1, Ang-2 and Tie2 have previously been detected in isolated blasts from AML patients as well as in AML cell lines. However, by using a semi-quantitative scoring system, our study gives the first unequivocal demonstration of significantly higher levels of *in situ* Ang-2 and Tie2 protein expression in the bone marrow of unselected, non-pretreated AML patients than in controls. Although the interpretation of these data is subject to the limitation of quantitative immunohistochemical methods, our blinded evaluation and several measures which were taken to ensure the reliability of the quantification assay protect our results from diagnostic bias. Our findings are in accordance with those of previous reports demonstrating Ang-2 mRNA expression in isolated blasts and AML cell lines. De Bont *et al*. detected Ang-2 mRNA in peripheral blasts from nine of 19 AML patients but not in normal bone marrow cells.[26] Another group found Ang-2 mRNA in 12 of 36 AML cells.[21] However, the frequency of AML bone marrow samples positive for Ang-2 in our study clearly exceeded the frequencies of Ang-2 mRNA expression in the two mentioned reports. The reasons for this discrepancy may lie in the method of Ang-2 detection or in the different material used for analyses. Indeed, there is evidence that blasts obtained from peripheral blood and bone marrow from the same patient can show differences in phenotype.[27] Furthermore, we cannot exclude that beside leukemic blasts other cellular components of the bone marrow stroma may have contributed to elevated Ang-2 expression. In fact, Ang-2 mRNA has been detected in stromal cells of normal bone marrow.[17] Paralleling our findings of Ang-2 in almost every investigated AML bone marrow specimen, Loges *et al*. recently detected Ang-2 mRNA in peripheral leukemic blasts in 98% of AML patients.[23]

In contrast, the intensity and distribution of expression of Ang-1 protein in AML bone marrow did not differ significantly from that in normal bone marrow. In a previous study, Muller *et al*. found that Ang-1 mRNA was overexpressed in isolated leukemic blasts.[22] The apparent discrepancy between these findings may be explained by the different materials and methods used for detection or the smaller sample size in the mentioned study. Furthermore, the authors did not investigate the expression of Ang-2 as the second major Tie2 ligand, which seems to be mandatory since Tie2 function is unlikely to depend on a single angiopoietin. Previous studies have led to the proposal that Ang-1 mediates vascular stability, while Ang-2 induces vascular instability by overriding Ang-1-mediated Tie2 activation.[19] Thus, the balance between Ang-1 and Ang-2 determines the grade of endothelial Tie2 phosphorylation. In the present study the Ang-2:Ang-1 ratio changed from 0.5 in normal bone marrow to 1.7 in neoplastic bone marrow indicating that the normal angiopoietin balance is reversed and strongly altered in favor of Ang-2 in AML. Indeed, while Ang-1 appears to be the dominant Tie2 ligand in normal bone marrow, this balance strongly shifts towards Ang-2 during leukemic transformation. A high ratio of Ang-2:Ang-1 expression in association with high levels of mitogenic angiogenic factors stimulate angiogenesis in a wide range of malignant solid tumors.[28] Elevated levels of VEGF, bFGF and other factors are readily available in AML bone marrow[4,5,8,12] as the result of a bidirectional cross-talk between leukemic blasts and non-leukemic bone marrow stromal cells.[29] Collectively, our findings suggest that the reversal of the normal angiopoietin balance in favor of Ang-2 acting in concert with other angiogenic inducers may be essential for bone marrow angiogenesis in AML. The fact that a correlation between Ang-2 expression or the Ang-2:Ang-1 ratio and MVD was observed in the entire study population only and not in the AML group separately, may be a result of the absent mitogenic capacity of Ang-2. In fact, with its vessel destabilizing function, Ang-2 is considered a prerequisite for angiogenesis to occur.[19,20] The extent of neovascularization, as measured by the endothelial cell mass, is subsequently determined by VEGF or bFGF, which act as potent mitogens on vascular endothelial cells.

Cox regression analyses revealed that bone marrow Ang-2 expression represents a favorable prognostic factor for overall survival in AML. The prognostic impact of Ang-2 expression emerged as independent from the so far strongest prognostic variable, i.e. karyotype. Our finding of an inverse relationship between Ang-2 expression and survival duration in AML patients was somewhat unexpected. Indeed, Ang-2 expression has been associated with an adverse prognosis in solid tumors.[30-32] However, our results are in accordance with recently published observations that high expression of Ang-2 mRNA in peripheral blasts of AML patients is correlated with a better long-term survival.[23] In this sense, our results and those of the cited study can serve as mutually independent confirmation of the observed results employing different methodologies and underscore the relevance of Ang-2 as a favorable prognostic marker in AML. This is even more striking given that patients in the two studies were treated with different chemotherapy regimens.

The mechanisms responsible for the differences in prognosis between AML patients with high and low Ang-2 expression remain unclear. The authors of the above mentioned report[23] suggested a MVD-dependent mechanism but could not correlate Ang-2 mRNA expression with bone marrow angiogenic activity, because corresponding bone marrow specimens were not available. However, microvessel counts did not show any association with clinical outcome in our study. This fact might argue against the hypothesis that the observed differences in survival result from the modulation of bone marrow neovascularization. Instead, our observations suggest a potential alternative mechanism independent from angiogenesis, which might have greater impact on prognosis in AML patients intensively treated with chemotherapy. In fact, not only Ang-2 but also its receptor Tie2 was significantly overexpressed in leukemic blasts. As a result, Ang-2 may not just act in a paracrine fashion on vascular endothelium but may also be involved in an autocrine loop on leukemic blasts, as already demonstrated for other angiogenic ligand/receptor systems.[9-12] However, data on the function of an angiopoietin-based autocrine pathway in non-endothelial cells are limited. In normal hematopoesis, Ang-1 has been shown to regulate the adhesion of Tie2-expressing hematopoietic stem cells to the osteoblastic niche resulting in protection from apoptosis and enhanced cell survival.[33] Administration of Ang-1 protected hematopoietic stem cells from the myelosuppressive effects of 5-FU or radiation by a phosphatidylinositol 3-kinase (PI3-K)/Akt pathway-dependent mechanism.[33] Similarly, survival of AML cells requires PI3-K activation[34] and constitutive phosphorylation of Akt is associated with shorter overall survival in AML.[35] Furthermore, it has been recently demonstrated that Tie2 phosphorylation contributes to the activation of the PI3-K/Akt pathway in AML cells.[36] If Ang-2 conserves its antagonistic character in Tie2-expressing leukemic blasts, it is tempting to speculate that Ang-2, released for induction of angiogenesis, might reduce baseline activation of the Tie2/PI3-K/Akt axis in AML cells in an autocrine fashion. Reduction of PI3-K activity has been shown to increase the susceptibility of leukemic blasts to cytotoxic agents,[37] which might explain the favorable prognosis of AML patients with high expression of Ang-2. Our hypothesis might be further supported by the observation that the power of Ang-2 as a predictor of a favorable prognosis was especially evident in the subgroup of patients simultaneously expressing low levels of Ang-1. In turn, the outcome of AML patients with high Ang-1 expression was much less positively influenced by Ang-2 expression levels.

In summary, the present study provides evidence that AML is associated with increased expression of Ang-2 and Tie2, but not Ang-1 in the bone marrow. The switch in angiopoietin balance may play an important role in the angiogenic process occuring during leukemic transformation. Furthermore, our study identifies bone marrow Ang-2 expression as a favorable prognostic factor in chemotherapy-treated AML. Modulation of the autocrine angiopoietin/Tie2 axis may be a promising approach to improve the outcome of AML patients.

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