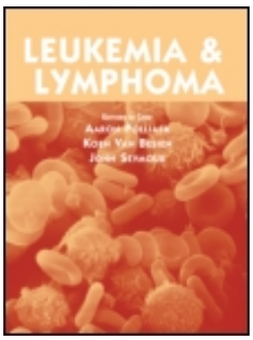

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Bone Marrow Features and Clinical Findings in Chronic Myeloid Leukemia – A Comparative, Multicenter, Immunohistological and Morphometric Study on 614 Patients

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(In final form July 10, 1999)

A multicenter, immunohistochemical and morphometric study was performed on diagnostic pretreatment bone marrow biopsies in 614 adult patients with Ph^{1+} chronic myeloid leukemia (CML) to compare histological features with clinical findings. For identification of megakaryopoiesis we used the monoclonal antibody CD61 and additionally the PAS reaction to determine the subfraction of atypical micromegakaryocytes and precursors. Labelling of erythroid precursors was carried out by a monoclonal antibody directed against glycophorin C. In order to selectively stain macrophages and their activated subset we applied CD68 and the GSA-I lectin. Density of argyrophilic fibers (reticulin plus collagen) was measured following Gomori's silver impregnation method. In accordance with laboratory data morphological variables revealed a comparable amount of congruence in the various groups of CML patients derived from different sources. In about 26% of patients early (reticulin) to advanced (collagen) fibrosis was detectable. Significant correlations were calculated between the extent of myelofibrosis with splenomegaly, anemia and increasing numbers of erythroblasts and myeloblasts in the peripheral blood count. These features were assumed to indicate more advanced stages of the disease process with ensuing transition into myeloid metaplasia and consequently were associated with an unfavorable prognosis. Significant relationships were revealed between the number of $CD61^{+}$ megakaryocytes and more important, also their precursor fraction with the degree of fibrosis. This result extends previous experimental findings regarding the impact of immature elements of this cell lineage for the generation of myelofibrosis. The significant association of erythroid precursors with the number of mature (resident) macrophages including their activated GSA-I subset may shed some light on their functional involvement in iron turnover and hemoglobin synthesis. A modified histological classification of predominant bone marrow features is introduced. This simplified synthesis staging system (Cologne Classification) is not only associated with certain sets of laboratory data, but also with different survival patterns.

Keywords: chronic myeloid leukemia, histological features, laboratory data, multicenter study, staging systems, clinicopathological correlations, survival, bone marrow biopsies

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INTRODUCTION

According to molecular biological features and clinical studies chronic myelogenous leukemia (CML) has become the best-characterized subtype of the chronic myeloproliferative disorders.[1] However, there is still a remarkable lack of some basic information, in particular concerning the striking heterogeneity of bone marrow morphology and corresponding hematological data.[2] Any attempt to determine the impact of histological parameters on prognosis has to be based on a proper identification of the various cell lineages involved in this myeloproliferation. This rationale must implicate an elaborate quantification procedure including immunohistochemistry and morphometry. Although anemia (hemoglobin level) has repeatedly been recognized as one of the more distinctive factors of predictive value [3–6], until now little attention has been paid to the assessment of erythroid precursors. In addition to granulopoiesis, dwarf-like atypical megakaryocytes constitute a cytological hallmark of the CML bone marrow.[7–15] In this context, megakaryocyte proliferation has frequently been associated with the development of myelofibrosis and consequently with an unfavorable indicator for survival.[16–21] However, routine staining methods (i.e. Giemsa) are not considered as a suitable mean to identify all the elements composing this cell lineage, in particular the immature and abnormal micromegakaryocytes.[10,12] There is mounting evidence regarding the influence of megakaryocyte precursors (pro- and megakaryoblasts) and so-called activated cells of the mononuclear macrophage system [22] on the cytokine- and growth factor-mediated generation of the myelofibrotic extracellular matrix.[23-27] Finally, conflict and discussion also arises regarding a peculiar sub-population of (activated) macrophages, the so-called pseudo-Gaucher cells.[28] Their frequency [29] and predictive value has been repeatedly debated.[20,21,30,31]

For practical purposes a semiquantitative histological classification has been proposed to distinguish certain subtypes of CML.[9,13] This staging system was assumed to define differences in the clinical course and eventual survival. However, the question now arises, whether this classification may easily be correlated with distinct clinical findings and whether it is reproducible following a more refined and comparative evaluation using immunohistochemistry and morphometry. Previous immunohistological studies which focused on a quantitative analysis of certain bone marrow features in CML are further biased by the relatively small number of patients investigated.[20,21] Moreover, the inherent failure to definitively exclude a selection bias, when examining samples taken from one institution, has also to be taken into account. Considering these inconsistencies in the interpretation of bone marrow findings and the associated clinical data in CML, the purpose of this study was to recruit a large number of pretreatment biopsies from different sources and, by applying immunohistochemistry, to determine histological features which are assumed to establish relevant correlations with laboratory data and patient's survival.

SUBJECTS AND METHODS

Patients

This retrospective study is based on 614 adult patients with non-blastic (stable phase) Philadelphia chromosome-positive (Ph^{1-}) CML derived from three different institutions (referral centers covering large parts of South- and Northwestern Germany) over a 14-year period (1984–1997). Diagnosis of CML was made by conventional criteria: relevant laboratory and cytogenetic findings (Ph^{1+}, bcr/abl translocation), characteristic peripheral blood (myeloblasts plus promyelocytes $\leq$ 15%) and bone marrow morphology as well as representative (pretreatment) bone marrow trephine biopsies with corresponding pathology and a close clinical follow-up (repeated bone marrow trephines in 218 patients).

Bone Marrow Biopsies

Fixation of bone marrow biopsies was performed in an aldehyde solution for 12–48 hours (2 ml 25% glut-

araldehyde, 3 ml 37% formaldehyde, 1.58 g anhydrous calcium acetate, and distilled water per 100 ml). Further processing included decalcification for 3–4 days in 10% buffered ethylen-diamine tetra-acetic acid (EDTA), pH 7.4, paraffin embedding, and employment of several routine staining techniques, involving Giemsa, PAS (periodic acid Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perl's reaction for iron and the silver impregnation method, following Gomori's technique.[32] For a specific staining of marrow cells three appropriate monoclonal antibodies were selected: CD61 (anti-platelet glycoprotein IIIa) for the identification of megakaryocytes including abnormal microforms and precursor cells (promegakaryoblasts as well as megakaryoblasts), Ret40f (anti-glycophorin C) to selectively mark erythropoiesis [33] and PG-M1 (CD68) for the staining of all mature, resident macrophages-phagocytic reticular cells.[34] So-called activated subpopulations of macrophages [22] were identified by their specific ability to express α-D-galactosyl residues [35,36], which caused an increased binding to the lectin from Griffonia (Bandeiraea) simplicifolia isotype I-B4 (GSA-I). Two other fractions of storage histiocytes were further distinguished: the iron-laden subpopulation and the pseudo-Gaucher cells [28]. Because both subpopulations were present only in a certain proportion of patients, incidence was determined by calculating percentages. Detection of pseudo-Gaucher cells was not only based on their characteristic fibrillar birefringence following polarization of Giemsa-stained slides, but also confirmed by their onion skin-like finely striated cytoplasmic pattern (PAS-reaction) and their distinctive GSA-I staining. Monoclonal antibodies and other reagents were purchased from Dako-Diagnostica GmbH (Hamburg, Germany). Details of staining procedures (APAAP-method) and proper identification of corresponding cell populations were reported in previous communications. [10,12,20,21,37]

Morphometry

Following immunostaining, morphometric analysis was performed by two manual optic planimeters (MOP-A-MO1-Kontron and VIDAS-Zeiss-Kontron) with a standard program set (Kontron software) on large trephine biopsies with an artefact-free mean marrow area of 9.6 ± 4.0 mm^2. Incidences of CD61-positive ($CD61^+$) megakaryocytes including atypical microforms and immature elements (pro- and megakaryoblasts), erythroid precursors (Ref. 40f) and macrophages ($CD68^+$, $GSA\text{-}I^+$) per square millimeter were determined at 500 × magnification by calculation of the evaluable hematopoietic area of the trephine biopsy (with a cellularity-hematopoiesis ranging between 93 to 100 % of the total marrow area)and the number of the corresponding cells. After employment of the periodic acid-Schiff reaction (PAS) the total number of mature megakaryocytes and of $CD61^+$ megakaryocyte precursors [12] was also assessed by the same method. Argyrophilic (reticulin and collagen) fiber density was measured following silver impregnation (Gomori's stain) and expressed as numbers of intersections (i) per square millimeter fat cell-free hematopoietic tissue (so-called point-intersection method). Morphometric measurements were made by eight individuals and regularly checked by two others for accuracy.

Statistics

All basic clinical and laboratory data as well as histomorphometrical findings were entered into a special designed computer-database via interactive programs providing intense consistency checks. Statistical analysis included calculation of Pearson's correlation coefficients as well as one way analysis of variance and multiple range tests (Scheffe's test) between the various clinical and histological variables. Calculation of survival was carried out by using Kaplan-Meier's product limit method for censored survival data.[38]

RESULTS

The clinical characteristics of the 614 patients entered into this study are listed in Table I. Except for the

lower (median) age of the cohort from Essen including patients who were partially referred with the option of bone marrow transplantation, no significant differences could be calculated regarding the other parameters. This failure to detect certain peculiarities in the various groups is probably due to the wide ranges of values. Regarding bone marrow morphology overview of erythropoiesis revealed strikingly variable features which ranged from disseminated tiny islets to normal-sized clusters of Ret40f-positive cells (Fig. 1a). Similarly $CD61^+$ megakaryocytes were characterized by significantly different frequencies including single dispersed cells of this lineage or clusters and sheets of atypical micromegakaryocytes as well (Fig. 1b). These abnormal dwarf-like megakaryocytes were hardly recognizable on routinely stained slides (Fig. 1c). Moreover, precursors (promegakaryoblasts) could only be identified and differentiated from the other immature myeloid cells (myeloblasts) by applying immunohistochemistry (Fig. 1d). In keeping with the cellular components of the bone marrow, density of argyrophilic fibers ranged from no increase to advanced myelofibrosis with bundles of collagen (Fig. 1e) and initial osteosclerosis. Macrophages constituted a very heterogeneous population (Fig. 2a-d), because the mature resident $CD68^+$ histiocytic – phagocytic reticular cells (Fig. 2a) could be divided into several fractions. These included pseudo-Gaucher cells with fibrillar birefringence following Giemsa staining and polarization (Fig. 2b) and a distinctive PAS-reactivity (Fig. 2c). Pseudo-Gaucher cells were included in the large, so-called activated and therefore GSA-I-positive subpopulation (Fig. 2d). Finally, about one third of patients showed iron-laden macrophages to a strikingly variable extent (Table II). A semiquantitative evaluation revealed a normal amount in only 3 %, a moderate decrease in 8.5 % and a significant reduction in iron-staining capacity in 24.5 % of patients.

TABLE I Clinical characteristics (mean ± SD) of patients at diagnosis

Source	*Freiburg*	*Cologne*	*Essen*	*total*
n	264	260	90	614
Gender (male/female)	149/115	145/115	51/39	345/269
Age (median, years)	55	52	38	51
Leukocytes ($\times 10^9$/l)	107 ± 99	121 ± 109	151 ± 142	117 ± 111
polymorphonuclear granulocytes	47.8 ± 17.3	44.5 ± 15.0	39.8 ± 16.8	45.0 ± 16.4
promyelocytes (%)	3.7 ± 4.2	4.8 ± 5.7	4.5 ± 6.2	4.0 ± 5.4
myelocytes (%)	10.9 ± 8.7	11.6 ± 8.9	11.8 ± 7.8	11.4 ± 8.6
myeloblasts (%)	1.9 ± 5.4	2.6 ± 3.5	3.3 ± 4.6	2.4 ± 4.5
normo-erythroblasts (%)	1.3 ± 3.2	1.1 ± 2.4	1.0 ± 1.4	1.2 ± 2.7
Eosinophils (%)	2.2 ± 2.3	2.9 ± 3.1	3.2 ± 3.1	2.7 ± 2.8
Basophils (%)	3.6 ± 3.8	3.8 ± 4.1	5.5 ± 7.8	4.0 ± 4.7
Lymphocytes (%)	9.3 ± 8.1	8.4 ± 7.4	7.6 ± 6.0	8.6 ± 7.5
Erythrocytes ($\times 10^{12}$/l)	4.0 ± 0.7	4.1 ± 0.7	4.0 ± 0.8	4.1 ± 0.7
Hemoglobin (g/dl)	12.2 ± 2. 1	12.1 ± 2.3	12.7 ± 1.8	12.2 ± 2.1
Platelets ($\times 10^9$/l)	441 ± 362	469 ± 367	536 ± 340	469 ± 362
LDH (U/l)	657 ± 363	648 ± 349	708 ± 717	662 ± 440
ALP (score)[a]	18 ± 37	12 ± 23	20 ± 32	15 ± 29
Spleen size[b]	2.3 ± 2.5	4.4 ± 5.0	3.2 ± 4.4	3.4 ± 4.2
Liver size[b]	0.7 ± 1.2	1.7 ± 2.8	0.8 ± 1.3	1.1 ± 2.2

a. ALP-alkaline leukocyte phosphatase – normal index 20–80
b. Spleen- Liver size – cm below costal margin

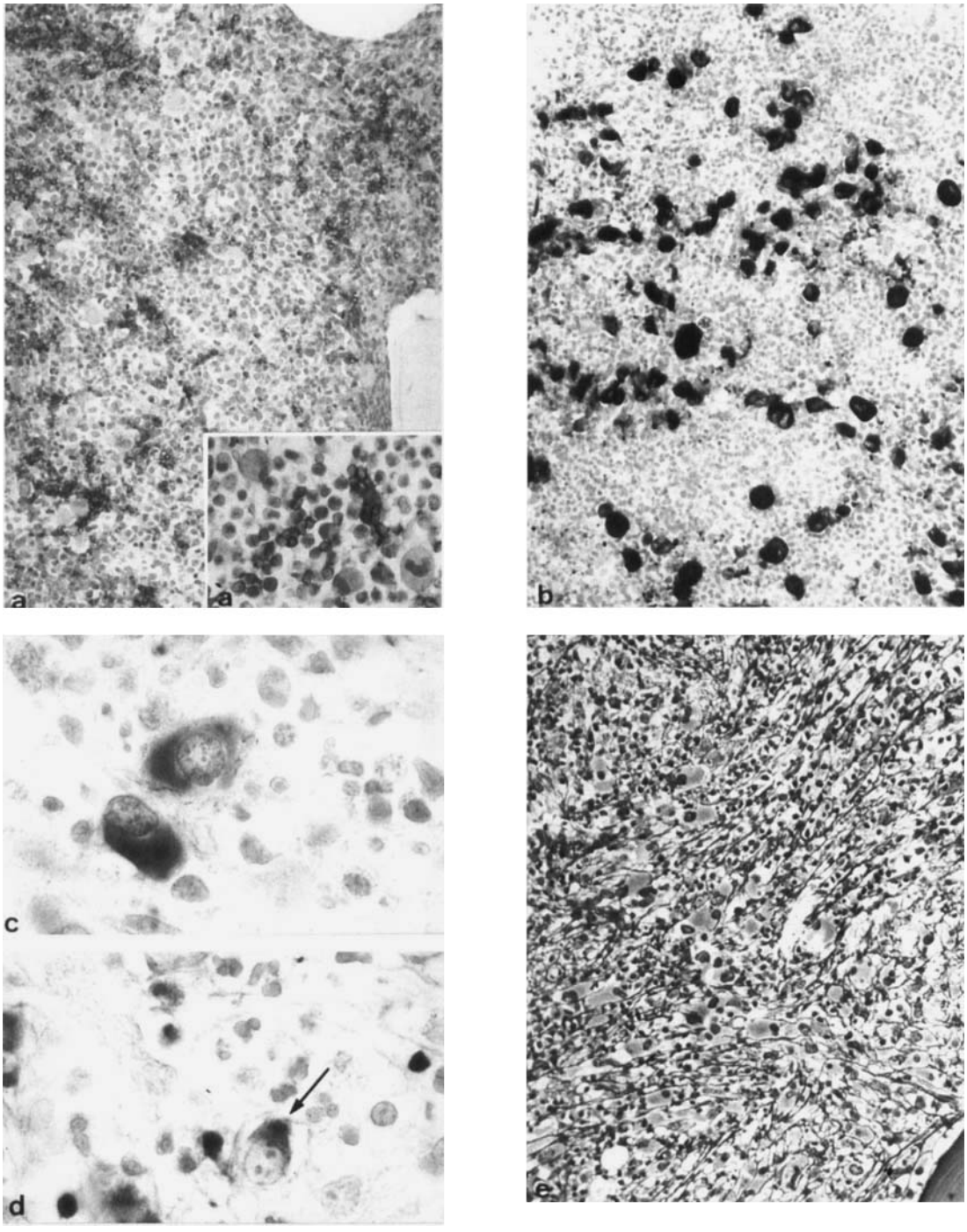

FIGURE 1 Bone marrow features in CML. Erythropoiesis shows a few and relative small islets (a) with tiny groupings of erythroid precursors (à). Some patients display a conspicuous increase and clustering of megakaryocytes, but predominantly microforms (b). $CD61^+$ atypical micromegakaryocytes without nuclear lobulation (c) or precursors (promegakaryoblast-arrow) are not recognizable by applying routine stains (d). Increased amounts of reticulin and collagen (argyrophilic) fibers associated with megakaryocyte growth may be observed in a number of patients (e). a, b, e × 170; inset a′ × 370; c, d × 870; a, a′ – Ret 40 f; b, c, d – CD61 immunostaining; e – Gomori's silver impregnation (See Color Plate VI at the back of this issue)

TABLE II Morphometric and immunohistochemical analysis of bone marrow features in 614 patients with Ph^{1+}-CML (mean ± SD, range in brackets)

Source	*Freiburg*	*Cologne*	*Essen*	*total*
No. of patients	264	260	90	614
Erythroid precursors/mm^2 (Ref 40f)	501 ± 324 (29 – 1,567)	402 ± 334 (32 – 1,855)	337 ± 213 (21 – 933)	437 ± 321 (21 – 1,855)
Megakaryocytes /mm^2 total ($CD61^+$)	89 ± 90 (4 – 745)	82 ± 75 (3 – 568)	61 ± 38 (9 – 88)	82 ± 79 (3 – 745)
precursors	33 ± 36 (1 – 340)	25 ± 34 (1 – 265)	20 ± 15 (1 – 167)	28 ± 33 (1 – 340)
Macrophages /mm^2 total ($CD68^+$)	310 ± 92 (107 – 649)	300 ± 90 (76 – 650)	323 ± 72 (167 – 523)	308 ± 88 (76 – 650)
activated subpopulation ($GSA\text{-}I^+/mm^2$)	185 ± 60 (74 – 441)	166 ± 49 (80 – 377)	200 ± 46 (107 – 321)	180 ± 55 (74 – 441)
Pseudo-Gaucher cells present (%)	28.5	34.3	25.3	30.4
Iron-laden subpopulation present (%)[a]	41.7	35.7	27.0	36.0
Fibers ($i \times 10^2/mm^2$)	44 ± 24 (0.5 – 148)	37 ± 24 (0.5 – 151)	35 ± 22 (0.5 – 135)	39 ± 24 (0.5 – 151)

a. In general, macrophages contained only small amounts of stainable iron

In accordance with the laboratory data, morphological variables displayed a similar amount of congruence in the different groups of patients under study. Although the larger Freiburg group revealed a tendency for a slightly higher incidence of megakaryocytes including precursors and an increase in fiber density than the smaller cohort from Essen, these differences were not significant (Table II). On the other hand, a number of significant correlations between histological and clinical variables could be ascertained. Significant relationships were calculated between the number of erythroid precursors on the extent of myelofibrosis with anemia (reduction of hemoglobin level), splenomegaly, myeloblasts in the peripheral blood count and LDH value (Fig. 3). Moreover, there was an adverse correlation between density of fibers and numbers of erythroid precursors ($r = -0.20$, $p \leq 0.001$). All these features probably indicate a more advanced stage of disease or ensuing transition into myeloid metaplasia. Noteworthy are not only significant ($p \leq 0.001$) interactions between fiber content and $CD61^+$ megakaryocytes ($r = 0.30$), but also the striking correlation between megakaryocyte precursors and this variable ($r = 0.32$). Comparison of two groups of patients presenting either with a normal platelet count ($\leq 350 \times 10^9/l$) or thrombocytosis (platelet count exceeding $600 \times 10^9/l$) disclosed significant differences ($p \leq 0.001$) regarding the incidence of $CD61^+$ megakaryocytes, independently from fiber density. Finally, the relevant association between erythroid precursors with the number of macrophages including their activated ($GSA\text{-}I^+$) subpopulation ($r = 0.27$, $p \leq 0.001$) may reflect the functional involvement of this cell lineage in iron turnover and hemoglobin synthesis.

Discrimination into different histological subgroups was carried out by trying to select certain cut-off points for the parameters $CD61^+$ megakaryocytes and fiber density, because these variables are the two major constituents of the so-called Hannover Classification.[9,13] This procedure was performed by taking into account values derived from patients without hematological pathology and with respect to the corresponding appearance of the bone marrow as well. Following this rationale, a more than three-fold increase in normal (mean) reticulin fiber density ($50\ i \times 10^2/mm^2$) was recognized as early myelofibrosis, whereas a four-fold density compared to the normal could be clearly characterized as a manifest (reticulin) myelofibrosis. Advanced fibrosis that included single or broad bundles of collagen fibers usually started with a five-fold increase (cut-off point $80 \times 10/mm^2$) and ranged up to a nine-fold increase in density compared to the normal.[20] This grading of reticulin-stain measured fibrosis was generally comparable with corresponding semiquantitative scoring systems. Biopsies showing varying degrees of fibrosis were usually associated with a megakaryocyte growth exceeding the (mean) normal value of $25 \pm 5\ /mm^2$.[10] On the other hand, in a number of specimens without relevant myelofibrosis, a significant increase in this cell lineage was also detectable. For this reason, a granulocytic subtype was separated from the megakaryocyte-rich group which revealed at least a more than

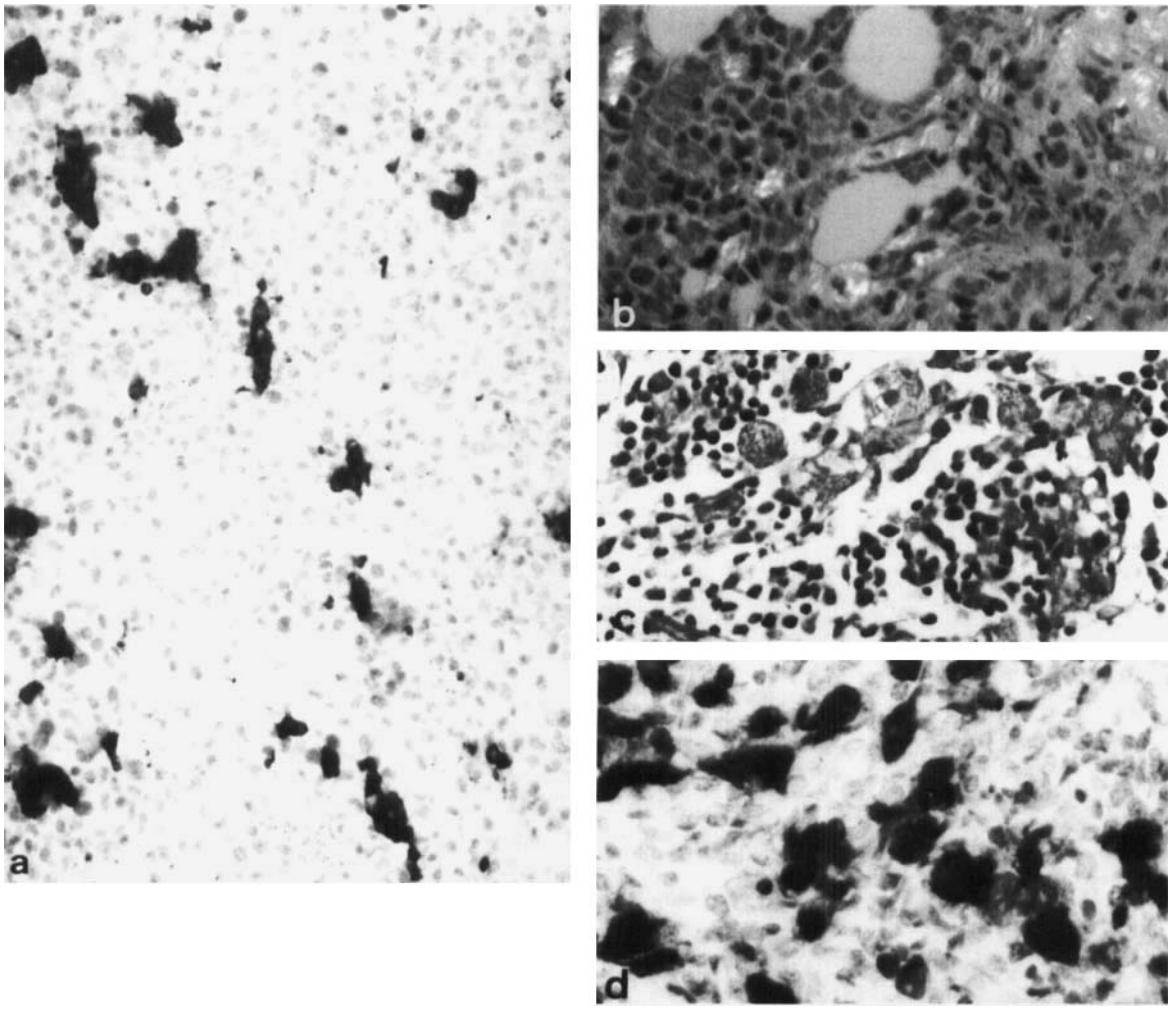

FIGURE 2 Macrophages in CML. The $CD68^+$ population reveals stellate cells of different size and shape (a). Pseudo-Gaucher cells are characterized by a fibrillar birefringence following polarization (b) and a positive PAS reaction (c). A certain subset of macrophages (activated subpopulation) including pseudo-Gaucher cells are expressing α-D-galactosyl-residues (d). a-d × 370; a – PG-MI and d – GSA-I immunostaining; b – polarization following Giemsa staining (See Color Plate VII at the back of this issue)

two-fold increase in $CD61^+$ megakaryocytes (> 50/mm^2), but with no fibrosis (Table III). It is noteworthy that the proposed criteria for histological classification regard certain sets of clinical findings with the platelet count as the starting point (root) of the decision tree-like construction (Fig. 4). This clinically determined staging system reflects our histological classification comprehensively (Table III) and is readily applicable on routinely stained slides as well and seems very suitable for practical purposes. Testing the clinical relevance of this staging system (Table III, Fig. 4), corresponding survival curves disclose that the different categories of our classification (Cologne Classification) exert significant different survival patterns (Fig. 5a,b). Independently of therapeutic regimens (busulfan/hydroxyurea) patients with granulocytic subtypes (I) show a more favorable prognosis compared to the megakaryocytic (II) and myelofibrotic (III) groups (Table III).

TABLE III Histological classification of predominant bone marrow features, erythropoiesis and macrophages and clinical findings (mean ± SD) in 614 patients presenting with stable phase Ph^{1+}-CML

Histological subtypes	*I. granulocytic*	*II megakaryocytic*	*III. myelofibrotic*	*Statistical analysis **
No. of patients	204	250	160	/
(%)	33.2	40.7	26. 1	/
Erythroid precursors (mm^2)	457 ± 350	479 ± 317	345 ± 265	p = 0.0113 (b,c)
Macrophages ($CD68^+/mm^2$)	297 ± 80	319 ± 93	304 ± 88	p = 0.0846 (a)
activated subpopulation ($GSA\text{-}I^+/mm^2$)	171 ± 46	189 ± 59	175 ± 57	p = 0.0018 (a,b)
Hemoglobin (g/dl)	12.4 ± 1.9	12.6 +2.1	11.3 ± 2.2	p = 0.0003 (b,c)
Leukocytes ($\times 10^9/l$)	104 ± 93	116 ± 117	144 ± 119	p = 0.0085 (a,b)
Thrombocytes ($\times 10^9/l$)	328 ± 227	572 ± 426	482 ±325	p = 0.00001 (a,b,c)
Peripheral blasts (%) myeloblasts	2.0 ± 3. 1	2.1 ± 3.9	3.6 ± 6.7	p = 0.08(b,c)
erythroblasts	1.0 ± 2. 1	1.0 ± 2.2	1.6 ± 2.9	n.s.
Splenomegaly (cm below costal margin)	2.7 ± 3.7	2.9 ± 4.0	4.6 ± 4.8	p = 0.0003 (b,c)

Statistical analysis:
Oneway analysis of variance and Multiple Range Test (Scheffe's Test). Letters denote pairs of groups significantly different at the 0.05 level. a: I vs. II; b: II vs. III; c: I vs. III

DISCUSSION

Clinical data

Although clinical findings together with peripheral blood films and bone marrow aspirates provide straightforward guidelines for the diagnosis of CML, they may also give the false impression that additional studies of cytogenetics or histopathology are superfluous. However, when evaluating non-disturbed (in situ) bone marrow tissue, a striking heterogeneity in addition to the granulocytic proliferation may be encountered.[7–9,13,14,20,21] In contrast to the flood of clinical trials on therapy and prognosis in CML (including laboratory data on large series of patients),[4,5,39–43] quantification of bone marrow features have been widely neglected until now. By pursuing a synoptical approach, in this study an attempt was made to correlate histopathology with hematological parameters and to classify CML into different subtypes or even stages according to their presenting features. There are only a limited number of studies on laboratory data in CML involving several hundreds of non-selected patients. [2,6] However, when comparing our data on 614 patients with those reported from various institutions [39], no significant differences were found. In particular, the younger age of the patients from the Essen-group is readily explained, since these were collected from a bone marrow transplantation center. Similar findings with a median age of only 34 years were recorded in a comparable series of patients.[44]

Myelofibrosis

The frequency of myelofibrosis at the time of diagnosis of CML ranges from 15 to 65 % in the pertinent literature [9,13,16–20] and may significantly be related to the different scoring systems used in semiquantitative evaluations. Assessment of fiber density by morphometry seems to be more accurate and discloses that in our large series, in comparison to corresponding normal bone marrow values, about 26 % of the patients present with early reticulin to advanced collagen fibrosis (Table III). According to data derived from the Hannover Classification [9] this proportion is slightly higher, probably because the initial stages of reticulin fiber increase are not easily recognizable by semiquantitative gradings. Studies during the last decade have substantially increased our knowledge of

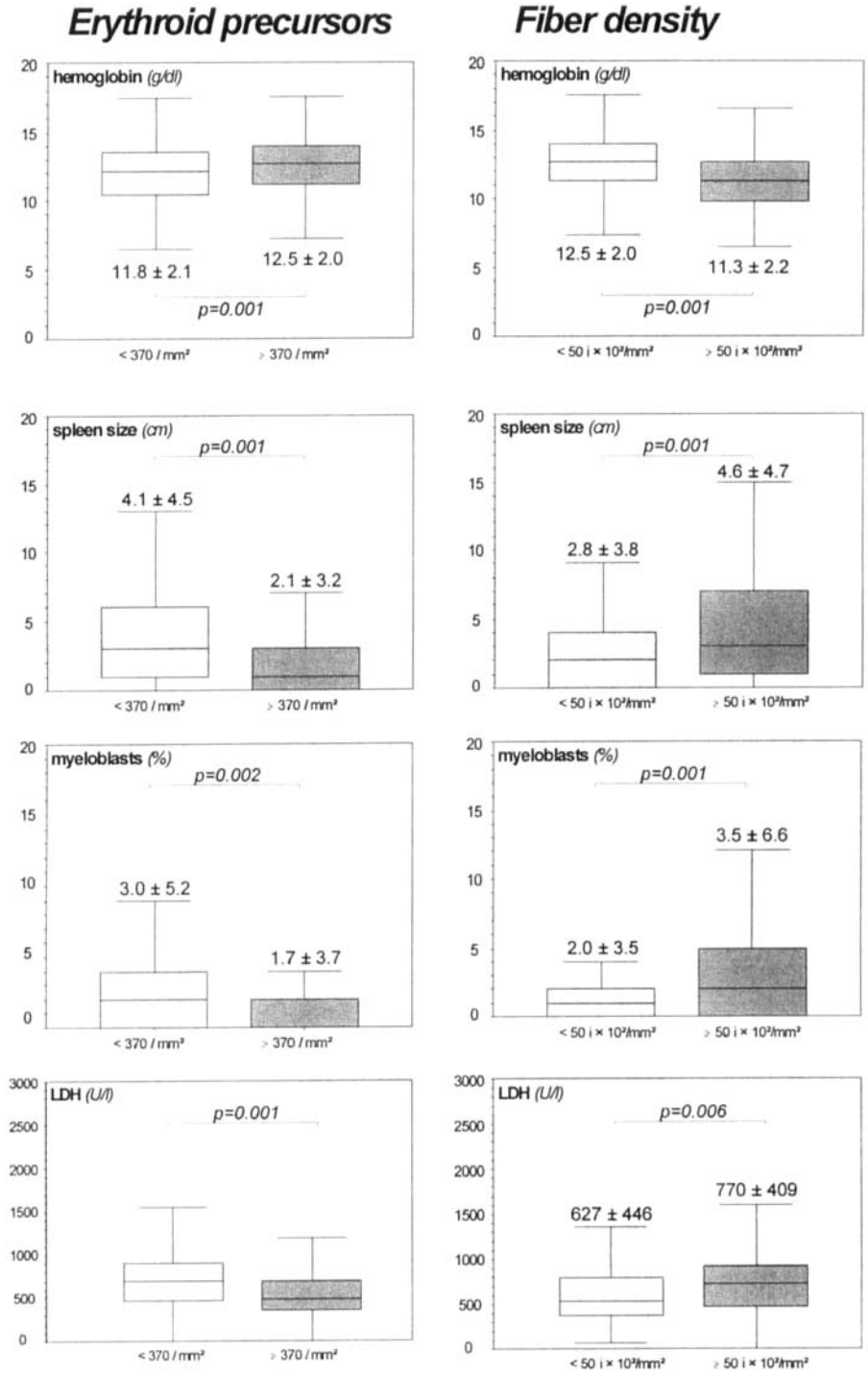

FIGURE 3 Relationships of relevant clinical parameters (hemoglobin, spleen size, myeloblasts, LDH) with erythroid precursors and fiber density in bone marrow specimens of CML. Box- and -Whisker plots were constructed for two groups of patients with cut-off points at median values (erythroid precursors: 370/mm^2, fibers: 50 i × 10^2/mm^2) determined by morphometry (compare with Table II)

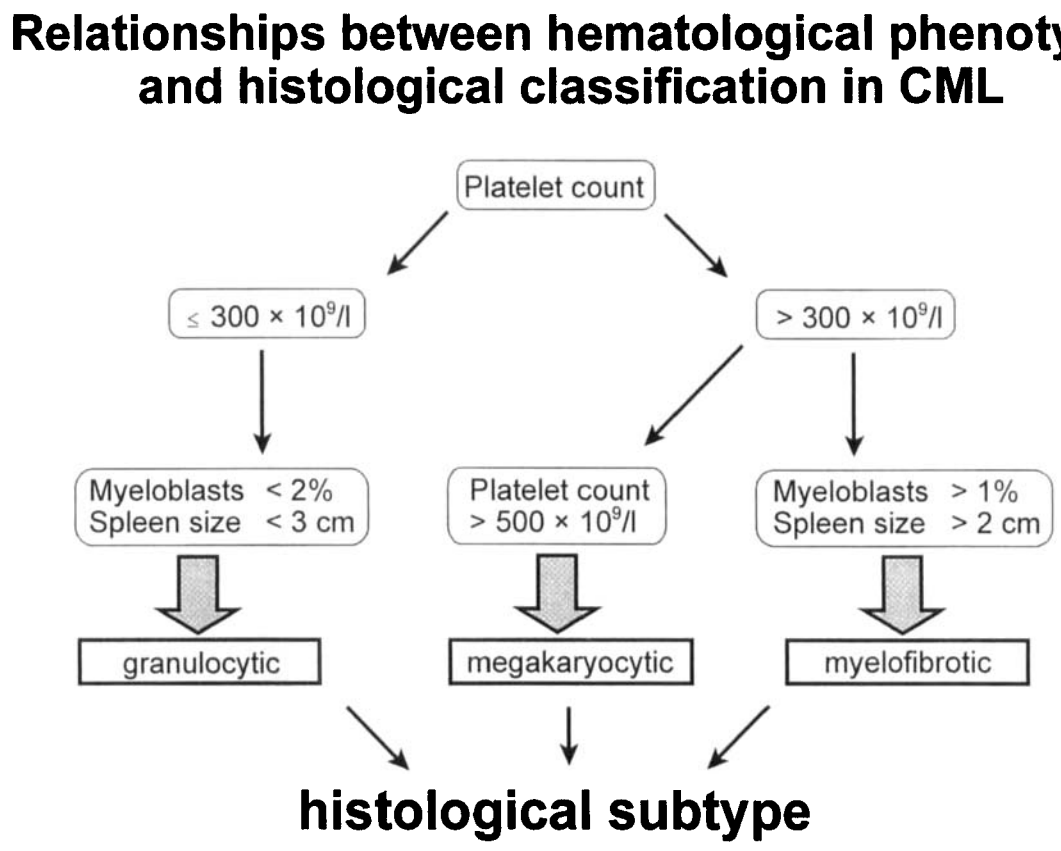

FIGURE 4 Relevant associations between laboratory data and our staging system of histological features in CML (Cologne Classification)

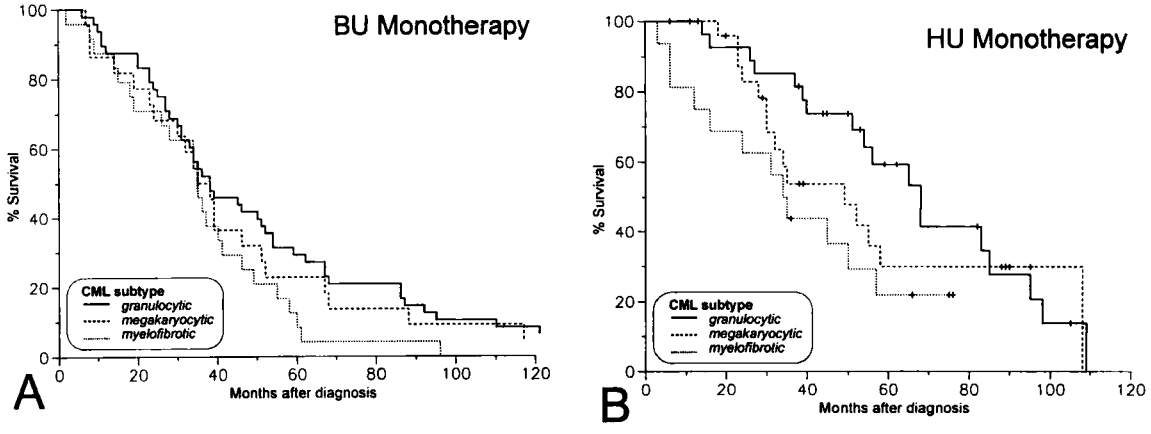

FIGURE 5A, B Survival curves according to our histological classification (Cologne Classification) for (a) historical cohort of patients having received busulfan (BU) and (b) another group with hydroxyurea (HU) monotherapy. In both groups the granulocytic subtype (I) reveals a significantly more favorable survival compared to the megakaryocytic (II) and myelofibrotic (III) sybtype

the complexity and diversity of the bone marrow microenvironment, particularly regarding the generation of myelofibrotic extracellular matrix. The current hypothesis involves an intramedullary release of growth factors from defective and abnormal megakaryocytes-platelets [24,26,27] and a stimulation of the cytokine cascade by complex interactions between the various constituents of the hematopoietic microenvironment.[25] In respect to the pathomechanism, immature precursors mostly found in acute micromegakaryocytic leukemia play a pivotal role.[23,25] In the initial stage of myelofibrosis in chronic phase CML, it has been assumed that atypical micromegakaryocytes may leak platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β).[26,27] It was suggested that these mediators stimulate non-clonal fibroblasts [45] to proliferate and deposit collagen as well as the non-collagenous glycoprotein fibronectin in the bone marrow stroma [26]. Moreover, in vitro studies point to an important functional role of the monocyte-macrophage system and certain adhesion molecules (CD44) in regard to the induction of fibrogenetic cytokines.[25] These speculations on the evolution of myelofibrosis in CML are generally supported by our findings of a significant correlation of CD61-immunostained megakaryocytes with the density of argyrophilic fibers. Moreover, in this context and even more noticeable, is the association of atypical dwarf megakaryocytes and precursors (pro- and megakaryoblasts) with the degree of myelofibrosis. The latter provides supportive evidence for the assumption that in particular the immature (atypical) elements of this lineage are responsible for growth factor release and ensuing myelofibrosis.[23,25]

Macrophages

Recently, interest has been reawakened in the macrophage population of the CML bone marrow.[46,47] The total $CD68^+$ mature resident compartment of phagocytic reticular cells may be divided into different subpopulations. These comprise not only storage macrophages, like the relatively small iron-laden fraction [48,49], but also the so-called activated population including the pseudo-Gaucher cells [28] which are characterized by the expression of α-D-galactosyl residues.[35–37,50,51] Experimental studies indicate that the normal bone marrow stroma selectively induces a lineage-specific commitment of stem cells.[52] The abnormal function of the CML bone marrow, in particular the disturbance of stem cell differentiation and proliferation has been linked to a defective adhesion of progenitors to stroma cells.[53,54]In accordance with their myelomonocytic progeny, stromal macrophages have been shown to harbour the Philadelphia chromosome [55] and are thus involved in the bcr/abl gene translocation.[46,56] In this context, clonally transformed (malignant) macrophages may significantly contribute to the microenvironmental dysfunction in CML and are assumed to take part in the selective expansion of the neoplastic cell clone accompanied by suppression of normal hematopoiesis.[46,47] In keeping with previously reported data[48,49] and in comparison with the normal bone marrow, there is a significant decrease in stainable iron in CML. However, iron stores and the number of sideroblasts were found to increase when patients entered into the accelerated or blastic phase of the disease, thus a careful determination of the marrow iron may be useful as a prognostic indicator of the disease state in CML.[57] A conflict of opinion should not be overlooked regarding the incidence of pseudo-Gaucher cells in the CML bone marrow which was reported to range between 20 and 70 %.[29] In the present investigation the frequency was 30 %. This finding is in agreement with corresponding values derived from smears of aspirates [30,31] as well as trephine biopsies.[20] The extreme values found by one group [29] may be related to the applied methodology, because mostly semithin, plastic embedded sections were evaluated without the option of a comparative identification by histochemistry, i.e. GSA-I reactivity (Fig. 2d).

Histological staging system

Considering a classification system of the histopathological features in the CML bone marrow, the question arises, whether and to what extent the proposed

categories represent stable subtypes or reliable entities in the course of disease. According to sequential bone marrow biopsies a megakaryocyte-rich subtype has to be considered as a prefibrotic stage of CML, since in follow-up examinations a predominant granulocytic growth fraction was very rarely found to develop myelofibrosis.[13]For this reason, it is debatable, whether the megakaryocytic and myelofibrotic subtypes are true entities which may be discriminated at any stage of the lengthy disease process or whether they should merely be regarded as morphological phenomena at the time of diagnosis, associated with certain sets of clinical data (Table III). Altogether, the different categories shown in Table III may be compared with a still frame in a motion picture depending on the dynamics of the disease progress. In keeping with major clinical findings [2,6,39,58] and their significant correlations with histological features in particularly spleen size, reduction of erythropoiesis[20,59] and the presence of erythroid precursors and myeloblasts in the peripheral blood films (Fig. 3), it seems that myelofibrosis reflects a later stage in the course of CML. Consequently, the adverse predictive value of this parameter for survival may be explained, [18,20,21] as well as its significant association with clinical acceleration and the development of blastic crisis.[16,17,19] Controversial issues have been repeatedly expressed regarding the clinical value of histological staging systems and their association with corresponding laboratory features and survival.[60,61] A comparative evaluation of these systems derived by using a significantly different approach – semiquantitative gradings of routinely stained slides versus histochemistry and morphometry in our study – are in keeping with the assumption that a clear-cut distinction of relevant laboratory data with subsequent assignment to histological categories, is only feasible by modifying the Hannover Classification.[9,13] It should be emphasized that until now, the latter staging system has not been validated by a proper correlation with clinical findings. On the other hand, when applying this staging system [9,13] on our bone marrow specimens and excluding the many so-called overlapping or intermediate subtypes, only the two last categories with manifest to advanced myelofibrosis revealed correlations grossly comparable with the data shown in Table III. We have tried to deal with these limitations with our simplified histological classification (Cologne Classification) which may also be easily applied on routinely processed specimens. In this context the granulocytic subtype (I) of CML reveals a significantly better prognosis than the megakaryocytic (II) and the myelofibrotic (III) group (Fig. 5a,b). Regarding the comparable survival of the latter subtypes, this finding may be explained not only by the strong correlation between the numbers of megakaryocytes and the density of argyrophilic fibers,[13,18,20] but also by the complex functional relationships between megakaryopoiesis and the evolution of myelofibrosis.[23–27]

In conclusion, in CML the histopathology of the bone marrow not only reveals significantly different features, but also an association with certain sets of laboratory findings thereby generating a constellation of findings which implicates a distinct improvement of their predictive value.

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