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Review

Effects of the tyrosine kinase inhibitor Imatinib mesylate (STI571) on bone marrow features in patients with chronic myelogenous leukemia

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Summary. Preliminary data are available about bone marrow (BM) changes in patients with chronic myeloid leukemia (CML) who received the molecularly targeted and highly effective tyrosine kinase inhibitor Imatinib mesylate (STI571). This review is focused on a systematic assessment of BM features detectable at different stages of CML (stable, accelerated, blastic) following long-term (more than 10 months) treatment. By applying enzyme- and immunohistochemistry including monoclonal antibodies visualizing proliferating cell nuclear antigen (PCNA) and apoptosis (anti-apostatin), a more elaborate insight into alterations affecting hematopoiesis and the stroma compartment was gained. In patients with stable-phase CML therapy resulted in a significant reduction in cellularity, neutrophil granulopoiesis and number of megakaryocytes, accompanied by a retrieval of erythroid precursors. In patients with Imatinib as the only treatment morphometric analysis of $CD61^+$ megakaryopoiesis was in keeping with a significant decrease in maturation defects implying a lesser amount of atypical micromegakaryocytes almost consistent with normalization. Moreover, a reduction of the initially enhanced ($CD34^+$) microvessel density was detectable associated with a decrease in luminal distension. Regression of marked to moderate myelofibrosis was recognizable in about 70% of patients especially in the accelerated and blastic phases. The amount of myeloblasts, $CD34^+$ progenitor cells and lysozyme-expressing immature myelomonocytic cells declined with treatment, but recurred in about 19% of patients that developed a leukemic relapse after 21±6 months of therapy. Data on proliferative activity and apoptosis in general supported in vitro findings concerning the inhibitory effect of this agent on growth associated with a tendency for stimulated apoptosis, at least in responding patients.

Key words: Imatinib (STI571), neutrophil granulopoiesis, erythropoiesis, megakaryocytes, myelofibrosis, proliferation, apoptosis, bone marrow, CML

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Introduction

Chronic myelogenous leukemia (CML) is a clonal disease of hematopoietic stem cells characterized by excessive proliferation of the myeloid lineage and the reciprocal translocation between chromosome 9 and 22 (Rowley, 1973). This pathomechanism creates the Philadelphia chromosome (Ph^{1+}) and as a molecular consequence the replacement of the first exon of c-abl with sequences of the bcr gene (Bartram et al., 1983; Heisterkamp et al., 1983; Shtivelman et al., 1985). Following this translocation a bcr/abl fusion site is generated whose protein product displays increased tyrosine kinase activity (Konopka et al., 1984; Kurzrock et al., 1988; Faderl et al., 1999; Sawyers, 1999). In vitro experiments have shown that bcr/abl alone is sufficient to cause CML and that enhanced tyrosine kinase activity is required for its oncogenic activity (Daley et al., 1990; Heisterkamp et al., 1990; Kelliher et al., 1990; Lugo et al., 1990). For this reason, it has been postulated that an inhibitor of the specific bcr/abl tyrosine kinase should present a most effective and selective therapeutic strategy. The recent development of the tyrosine kinase inhibitor Imatinib (Imatinib mesylate, STI571, Gleevec®; Novartis, Basel, Switzerland) as a novel molecularly targeted, anticancer compound has heralded a major breakthrough in CML therapy (Buchdunger et al., 1996; Druker et al., 1996; Deininger et al., 1997;

Druker and Lydon, 2000). Until now a number of clinical trials have revealed encouraging results even when using Imatinib in patients resistant to other agents (Druker et al., 2001b; Goldman and Druker, 2001; Mauro and Druker, 2001; Braziel et al., 2002; Talpaz et al., 2002). However, contrasting the often dramatic and nearly complete hematological response (up to 95%) in chronic and accelerated phases of CML (Druker et al., 2001a; Kantarjian et al., 2002; Cervantes et al., 2003; O'Brien et al., 2003) major cytogenetic responses are less often encountered (range 60% to 87%).

Regarding bone marrow (BM) histopathology prominent changes may be expected in patients under treatment with Imatinib reflecting their response and outcome. Therefore, it seems to be interesting to study appropriate specimens, i.e. sequential biopsy samples in correlation with clinical findings. In this context it is noticeable that previous reports on BM changes induced by this agent therapy were focused on short-term treatment (up to 37 weeks) and did not clearly differentiate between phases of disease at presentation and associated lesions (Beham-Schmid et al., 2002; Hasserjian et al., 2002) or included only a small series of patients (Frater et al., 2003). The scant knowledge, in particular about the long-term effect of this innovative drug that has been applied in recently conducted major clinical trials, certainly warrants a review on corresponding data concerning BM morphology in CML patients (Kantarjian et al., 2002; Cervantes et al., 2003; O'Brien et al., 2003). This aim was achieved by comparing previous findings with results derived from an evaluation of 75 patients treated for a median period of 23 months (range 10-31 months) with Imatinib (Thiele et al., 2004a). As has been previously noted a more elaborate evaluation of BM features, especially megakaryopoiesis, has to include enzyme- and histochemical staining techniques including monoclonal antibodies (Thiele et al., 1999). For this reason processing of specimens in our study involved Hematoxylin-Eosin (H&E) as well as Giemsa, PAS (periodic acid Schiff reagent), naphthol-AS-D-chloroacetate esterase, Perls' reaction for iron and a silver impregnation method (Gomori's technique). Immunohistochemistry with monoclonal antibodies (Cordell et al., 1984) was applied for a proper identification of $CD34^+$ progenitors (Soligo et al., 1991) and endothelial cells - vascularity (Rumpel et al., 2003; Kvasnicka et al., 2004b), $CD61^+$ megakaryocytes (Gatter et al., 1988), nucleated erythroid precursor cells (Thiele et al., 1999), mature (residential) $CD68^+$ macrophages (Falini et al., 1993) and lysozyme-expressing myelomonocytoid cells (Pinkus and Said, 1977). Determination of cellularity (degree of hypoplasia) has to explicitly regard the age-related changes in this population. Semiquantitative evaluation of the different cell lineages should be performed by an easy-to-reproduce grading system principally based on the corresponding incidence in the normal BM: 0 - no increase in comparison to the normal state; +1/-1 - mild increase/decrease; +2/-2 - marked increase/decrease; and +3/-3 - marked increase/decrease. Grading of BM fibrosis (reticulin and collagen) followed a generally acknowledged semiquantitative scoring system (Bauermeister, 1971) modified by corresponding morphometric data on the density of argyrophilic (reticulin-collagen) fibers (Thiele et al., 1996). Accordingly, for the purpose of practicability the following modified scoring was applied: 0 - no increase in fibers compared to the normal BM; 1 - minimal to mild increase in reticulin; 2 - marked increase in reticulin throughout the section plus some bundles of collagen; and 3 - coarse collagen fibrosis. To calculate proliferative capacity a monoclonal antibody against proliferative cell nuclear antigen (PCNA) was applied (Hall and Levison, 1990; McCormick and Hall, 1992) by following methods reported in detail in previous communications (Thiele et al., 1993). Apoptosis was visualized by using the specific monoclonal antibody (dilution 1:100) anti-ss DNA/Apostatin (Bender MedSystems, Vienna, Austria).

Hematopoiesis

Results on Imatinib-induced bone marrow (BM) changes after therapy for a median period of about three months are generally in keeping with a quantitative normalization of cellularity and erythropoiesis, a marked reduction of neutrophil granulopoiesis, a significant decrease in the number of megakaryocytes and a striking regression of the fiber content (Beham-Schmid et al., 2002; Hasserjian et al., 2002; Frater et al., 2003) as well as the Ph^{1+} hematopoiesis (Thiele et al., 2004b). Although these findings suggest that this drug affects the differentiation of CML cells in vivo to exhibit features resembling normal BM in genetic responders (Frater et al., 2003; Thiele et al., 2004b), it is apparent that a more systematic evaluation based on immunohistochemical staining results, in particular regarding the $CD34^+$ precursor cell population and blasts as well as proliferation and apoptosis is lacking. Moreover, because so far only patients with a history of previous treatment (mostly resistance to interferon by different therapeutic regimens) were included in these investigations, a control group without prior drug administration preceding Imatinib should be entered into the study design.

In comparison with the short-term therapy effect in the majority of cases our cohort of patients with stable-phase CML revealed a significantly reduced or normal cellularity mostly associated with a marked decrease in neutrophil granulopoiesis (Fig. 1a,b) and megakaryopoiesis (Fig. 1c, d) after at least 10 months of Imatinib therapy, while changes of the red cell lineage were less conspicuous (Fig. 2a,b). These alterations of hematopoiesis were in particular apparent when reducing the different grades of our semiquantitative scoring system to the fraction of patients who revealed any change (Table 1). Remarkable were changes of

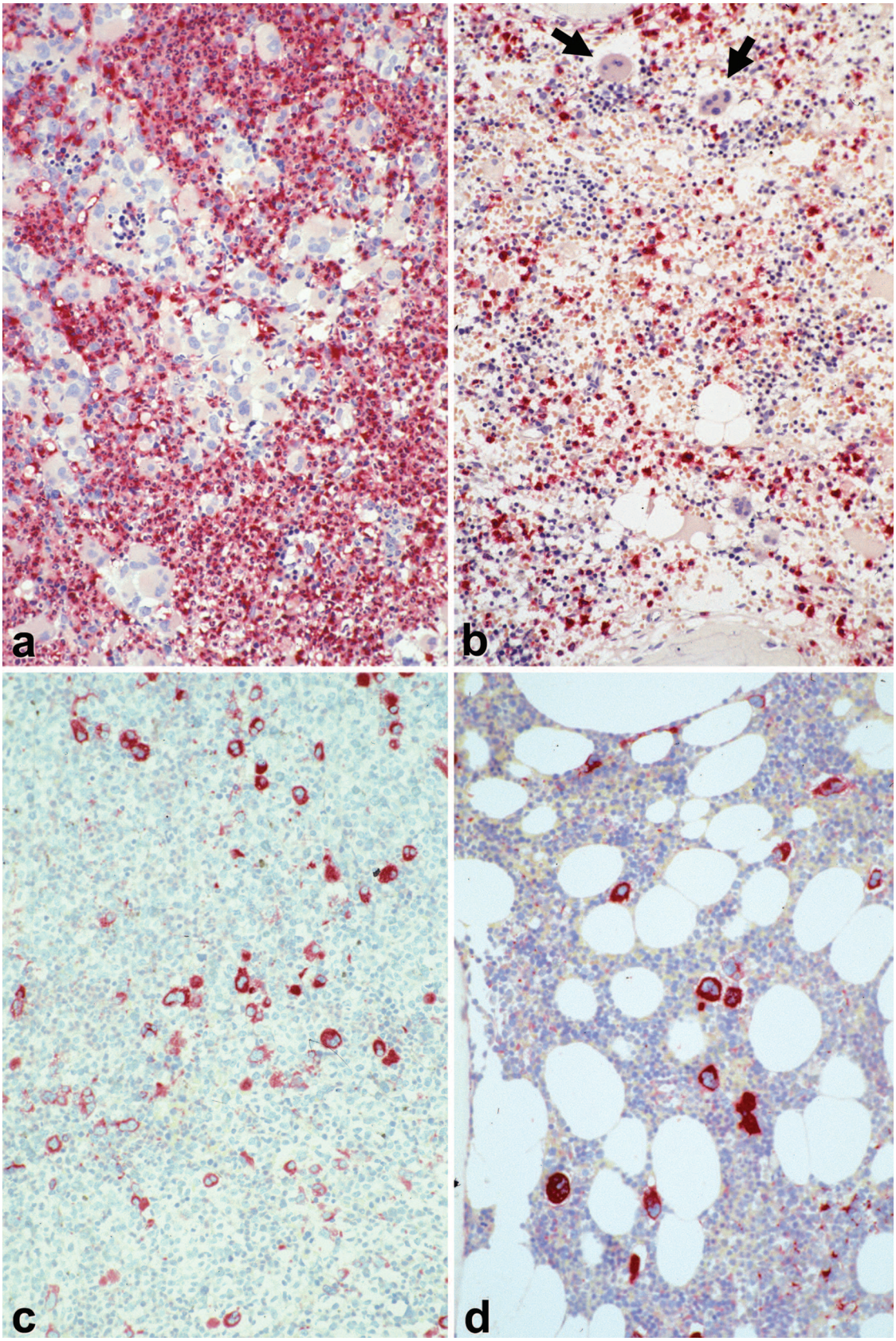

Fig. 1. Neutrophil granulopoiesis and megakaryopoiesis in the BM of CML patients before and after Imatinib therapy. **a.** Initial biopsy with so-called megakaryocyte-rich subtype of CML revealing a reduction in cellularity and only a few normal-looking megakaryocytes (arrows) after treatment **(b)**. **c.** CD61 immunostaining displays many micromegakaryocytes with rounded nuclei in pretreatment BM specimens and a retrieval of medium-sized to large megakaryocytes following therapy **(d)**. a and b: Chloroacetate esterase reaction; c and d: CD61 immunostaining.x 180

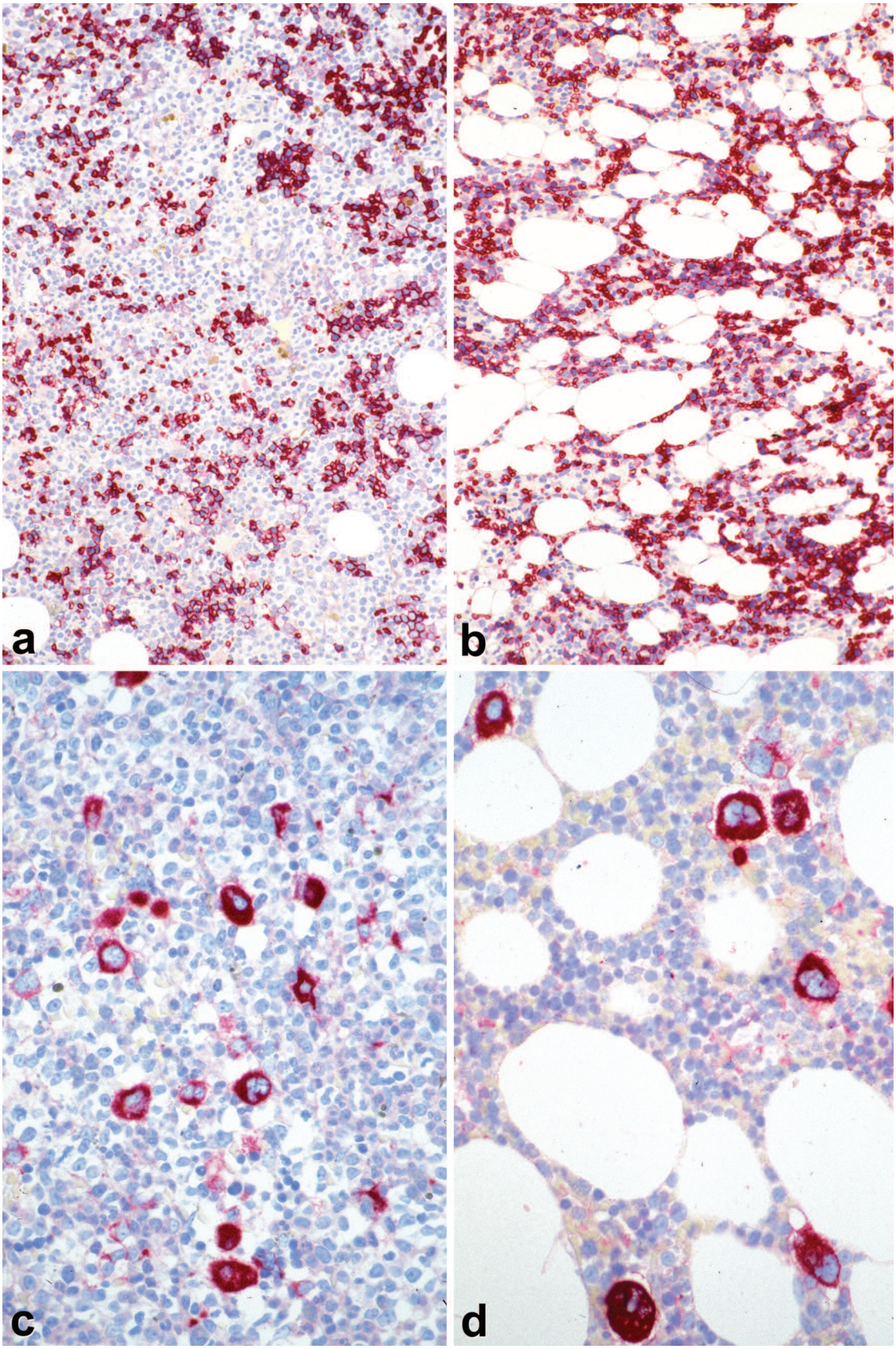

Fig. 2. Erythropoiesis and cytological alterations of megakaryocytes in the BM of CML patients before and after Imatinib therapy. **a.** In pretreatment biopsy specimens only small to tiny clusters of nucleated erythroid precursors are recognizable that become more confluent groups after therapy **(b)**. **c**. CML is characterized by dwarf-like megakaryocytes with compact nuclei. However, following treatment this peculiar appearance changes significantly to larger forms, exhibiting irregular shapes and lobulated nuclei, characterizing a normal morphology **(d)**. a and b: antiglycophorin C immunostaining; c and d: CD61 immunostaining. a, b, x 180; c, d, x 380

Table 1. Dynamics of bone marrow changes (semiquantitative scoring) concerning hematopoiesis in 41 patients with stable-phase CML and long-term Imatinib therapy (marked changes are indicated in bold).

PARAMETER	No. OF PATIENTS WITH CHANGES	SEMIQUANTITATIVE GRADING						
		-3	-2	-1	0	+1	+2	+3
Cellularity	40	**1**	**4**	**11**	**14**	3	16	
Granulopoiesis	35	**2**	**2**	**5**	**10**	4	12	0
Erythropoiesis	18	1	0	5	**7**	**4**	**1**	**-**
Megakaryopoiesis	25	**3**	**3**	**2**	**4**	9	3	1
maturation defects	25	0	0	0	**13**	10	12	0
bulbous nuclei	12	0	0	0	**8**	4	0	0
naked nuclei	8	0	0	0	4	3	1	0
clusters	12	0	0	0	**6**	6	0	0
Myeloblasts	3	0	0	0	0	1	**3**	0
CD34+ progenitors*	3	0	0	1	0	0	1	**2**

*: 3 patients developed a leukemic relapse.

Table 2. Morphometry of CD61+ megakaryopoiesis (median values) in 14 patients without any pretreatment following monotherapy with Imatinib.

	THERAPY	
	BEFORE	AFTER
Cell parameters		
size (µm²)	159	288
perimeter (µm)	48.2	67.1
maximal length (µm)	17.2	24.2
maximal width (µm)	113.4	148.2
roundness	0.71	0.64
aspect ratio	1.3	1.4
circularity	14.1	15.0
form factor	0.90	0.84
Nuclear parameters		
size (µm²)	40.0	87.8
perimeter (µm)	25.5	41.2
maximal length (µm)	9.3	14.6
maximal width (µm)	53.5	73.5
roundness	0.62	0.54
aspect ratio	1.4	1.5
circularity	14.7	17.5
form factor	0.84	0.66
Nuclear-cytoplasmic ratio	0.28	0.31
No. of evaluated megakaryocytes	3,323	1,468

Table 3. Dynamics of immature bone marrow cells (semiquantitative grading) in 34 patients with accelerated and blastic-phase of CML under long-term Imatinib treatment (marked changes are indicated in bold).

PARAMETER	No. OF PATIENTS WITH CHANGES	SEMIQUANTITATIVE GRADING			
		0	-1	-2	-3
Myeloblasts*	16	8	2	**5**	**1**
CD34+ progenitors	24	12	**8**	**4**	0
Lysozyme+ myelomonocytic cells	14	7	**4**	2	1

*: 11 patients revealed findings consistent with a leukemic relapse.

Table 4. Dynamics of bone marrow changes (semiquantitative scoring) in 34 patients with accelerated and blastic-phase of CML and long -term Imatinib therapy (marked changes are indicated in bold).

PARAMETER	No. OF PATIENTS WITH CHANGES	SEMIQUANTITATIVE GRADING			
		0	+1	+2	+3
Fibers*	13	6	**4**	**2**	1
Macrophages	11	**4**	2	4	1
Pseudo-Gaucher cells	6	2	**3**	0	1
Lymphoid nodules	13	5	**6**	**2**	0

*: of the 10 patients with minimal to marked (reticulin) myelofibrosis at diagnosis 6 revealed a reduction, whereas density of fibers increased in 7 patients.

megakaryocyte cytology involving a pronounced decrease in maturation defects (deviation of nuclear cytoplasmic differentiation) and variations in the quantity of small forms displaying hyperchromatic and hypolobulated (compact, bulbous) nuclei (Fig. 2c,d). A more refined planimetric evaluation by morphometry showed that a clear-cut shift from micromegakaryocytes with rounded nuclei (so-called dwarf forms) to normally-sized cells containing irregularly-shaped (lobulated) and larger nuclei could be recognized (Table 2). These obvious changes in histological appearance (Fig. 2c,d) were accordingly reflected by parameters characterizing not only sizes, but also the shapes of the megakaryocytes and their nuclei such as circularity, aspect ratio or form factor. A corresponding alteration of the nuclear-cytoplasmic ratio suppported this transformation (Table 2) and most important, the loss of

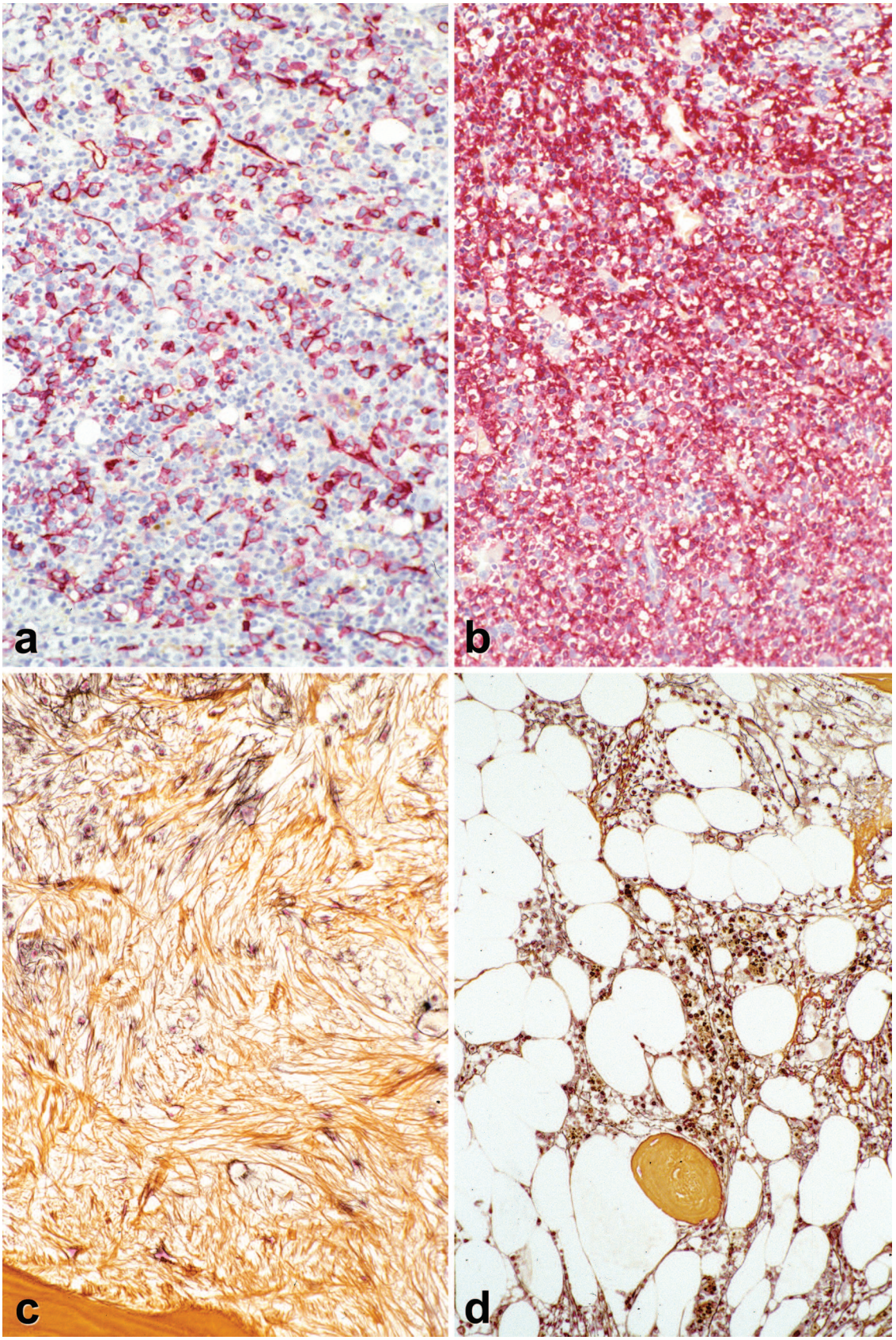

Fig. 3. Leukemic relapse and myelofibrosis in the BM of CML patients before and after Imatinib therapy. **a.** Increase in $CD34^{+}$ progenitor cells indicating an initial leukemic relapse following treatment. **b.** Manifest blastic crisis characterized by an overgrowth of immature myelomonocytic cells. **d.** Marked (grade +3) myelofibrosis before therapy is resolved to grade +1 after treatment and associated with hypoplasia and expansion of adipose tissue **(c)**. a: CD34 immunostaining; b: Lysozyme reaction; c, and d: Silver impregnation. x 180

the bcr/abl translocation gene (Thiele et al., 2004b). In three patients who experienced a leukemic relapse an increase in diffuse or loosely grouped $CD34^+$ progenitor cells and (immature myelomonocytic) blasts was found (Fig. 3a, b). In patients that initially presented with accelerated and blastic-phase CML a significant regression of the immature cell population was detectable (Table 3), although in about one third of this group a retrieval of blast crisis could be observed.

Stroma compartment

Regarding the stroma compartment (Table 1) and especially the fiber content, findings were generally in keeping with the maintenance of a stable state or a regression (Table 4), because of the 15 patients with moderate (+2) to marked (+3) fibrosis only 8 displayed a decrease (Fig. 3c,d). In this context it should be mentioned that myelofibrosis was more often encountered in patients presenting with an accelerated and blastic phase (Table 4). The result of an only partially expressed change does not totally conform with previously reported results after short-term therapy (Beham-Schmid et al., 2002). However, a more critical evaluation of the degree of myelofibrosis (grade 3-4 reticulin fibrosis consistent with grade 2-3 according to our scoring) showed that only 15 out of 21 (71%) of the investigated patients actually revealed a reduction after a median follow-up of 37 weeks (Hasserjian et al., 2002). This is in line with a fiber regression rate of 61% (2 grades) and 85% (1 grade) as recently described in 40 patients (Bueso-Ramos et al., 2004). These figures support our findings of a partial regression of myelofibrosis and the maintenance of a stable state in most patients (Table 4). Moreover, in a small number of patients, especially in those presenting an accelerated phase or blastic crisis, an increased quantity of lymphoid nodules (Fig. 4a), $CD68^+$ macrophages (Fig. 4b) and pseudo-Gaucher cells (Fig. 4c,d) were exhibited. Macrophages and their activated subtype, which are consistent with pseudo-Gaucher cells, may probably be regarded as scavanger cells, functionally associated with increased cell death (apoptosis - see below) and have been described as a prominent post-treatment feature (Frater et al., 2003). On the other hand, the meaning of lymphoid aggregates (Fig. 4a) is open to speculation. At least in patients with CML treated by interferon-α and chemotherapy there is a significant increase in the B- and T-lymphoid cell population observable including a higher frequency of lymphoid nodules (Thiele et al., 2000). Conspicuous changes of the microvascular density and architecture were described following Imatinib therapy (Rumpel et al., 2003; Kvasnicka et al., 2004b). In responding patients in chronic-phase CML a decrease of the initially enhanced vascularity was noticed (Fig. 5a,b). Concerning the vascular architecture there was an additional reduction of luminal distension similar to other therapeutic regimens (Kvasnicka et al., 2004a).

Proliferation and apoptosis

Experimental studies are in keeping with the assumption that Imatinib selectively inhibits the growth or sensitizes bcr/abl^+ cells to apoptosis and also induces a further differentiation of leukemic precursors (Deininger et al., 1997; Gambacorti-Passerini et al., 1997; Fang et al., 2000; Holtz et al., 2002). These pathomechanisms are assumed to be responsible for the hematological and cytogenetic effect of this agent. Moreover, in this context one should be aware that Imatinib therapy exerts an impact on different varieties of tyrosine kinases including the platelet-derived growth factor receptor-γ and c-kit (Druker et al., 1996; Buchdunger et al., 2000). Cell kinetics were characterized by an enhanced proliferative activity documented by increased PCNA expression (Fig. 5c) accompanied by a low incidence of apoptosis before Imatinib treatment (Table 5). These features were principally expressed in all groups of patients, but particularly in blastic crisis. However, after therapy a significant decrease in PCNA activity (Fig. 5d) and a tendency for a higher apoptosis rate (Fig. 5e) were noticed only in patients responding to therapy. On the other hand, in the groups with acceleration and blastic crisis including relapsing leukemia, in about one third of this series programmed cell death was not reduced (Table 5). Recently, several groups have reported data demonstrating ex vivo resistance of CML progenitor cells to Imatinib (Graham et al., 2002; Holtz et al., 2002; La Rosee et al., 2003). In this context, the existence of quiescent (bcr/abl^+) hematopoietic stem cells was

Table 5. Proliferative activity (PCNA expression) and apoptosis (median values and ranges) in patients with CML (stable versus accelerated and blastic phase) following Imatinib therapy.

Phase of CML	stable[a]		accelerated and blastic[b]	
No. of patients	41		34	
Treatment	before	after	before	after
PCNA/mm^2	505[c] (181 - 1,460)	315 (88 - 687)	702[c] (298 - 1,128)	267 (45 - 1,047)
Apoptosis/mm^2	5.3[d] (2.6 - 13.9)	9.4 (4 - 15)	5.1 (1.0 - 14.2)	5.0 (1.2 - 15.9)

[a]: 3 patients developed a leukemic relapse; [b]: 11 patients were non-responders or revealed a blastic transformation. Statistical analysis: [c]: p=0.003; [d]: p=0.06.

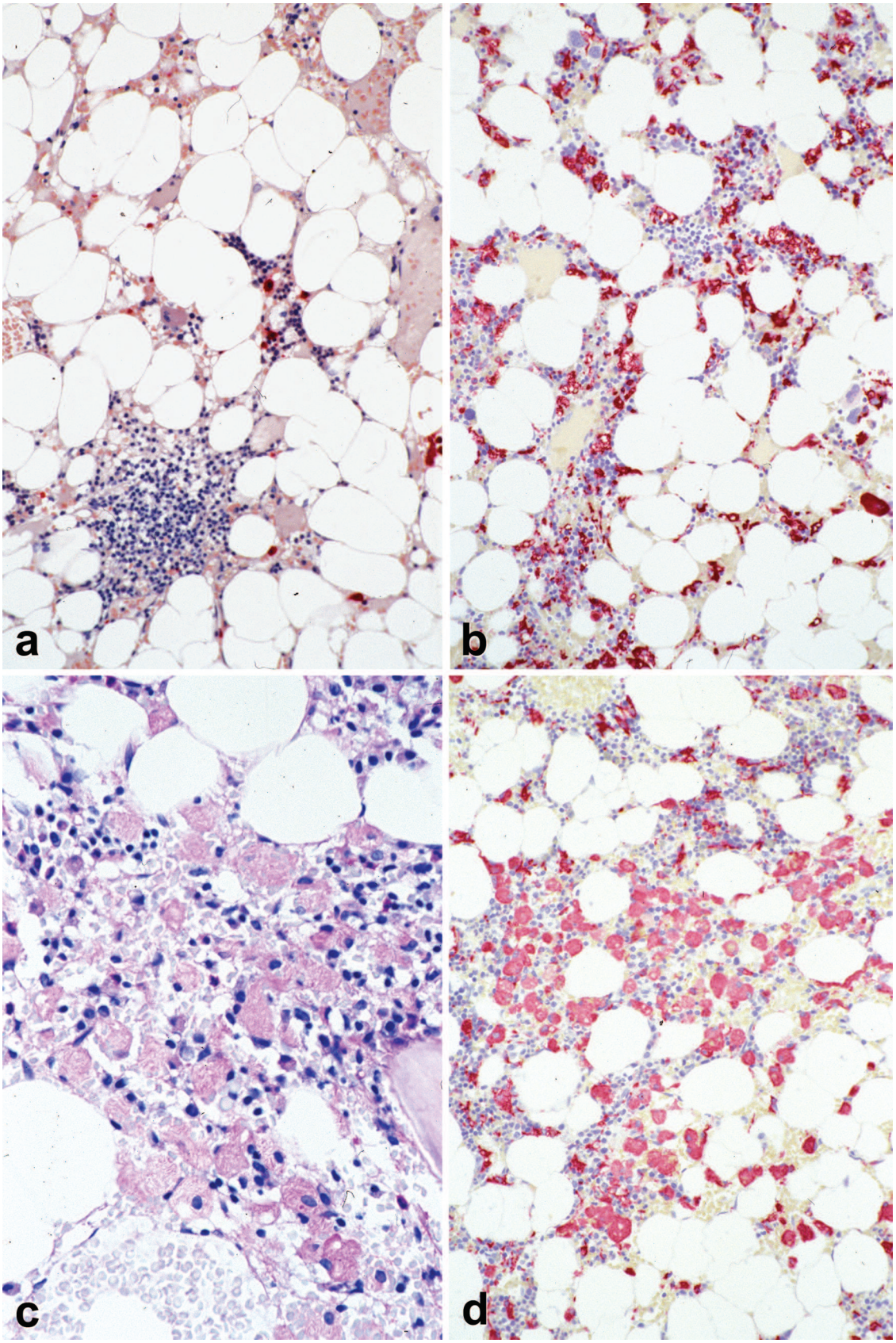

Fig. 4. Changes of the BM stroma after Imatinib therapy in BM biopsies of CML patients. **a.** Severe hypoplasia with reactive lymphoid aggregate. **b.** Abundant macrophages are frequently seen in post-treatment BM samples. **c.** Pseudo-Gaucher cells are characterized by the peculiar striated pattern of their cytoplasm and show extended loose groupings **(d).** a: Chloroacetate esterase reaction; b, d: CD68 immunostaining; c: PAS reaction. a, b, d, x 180; c, x 380

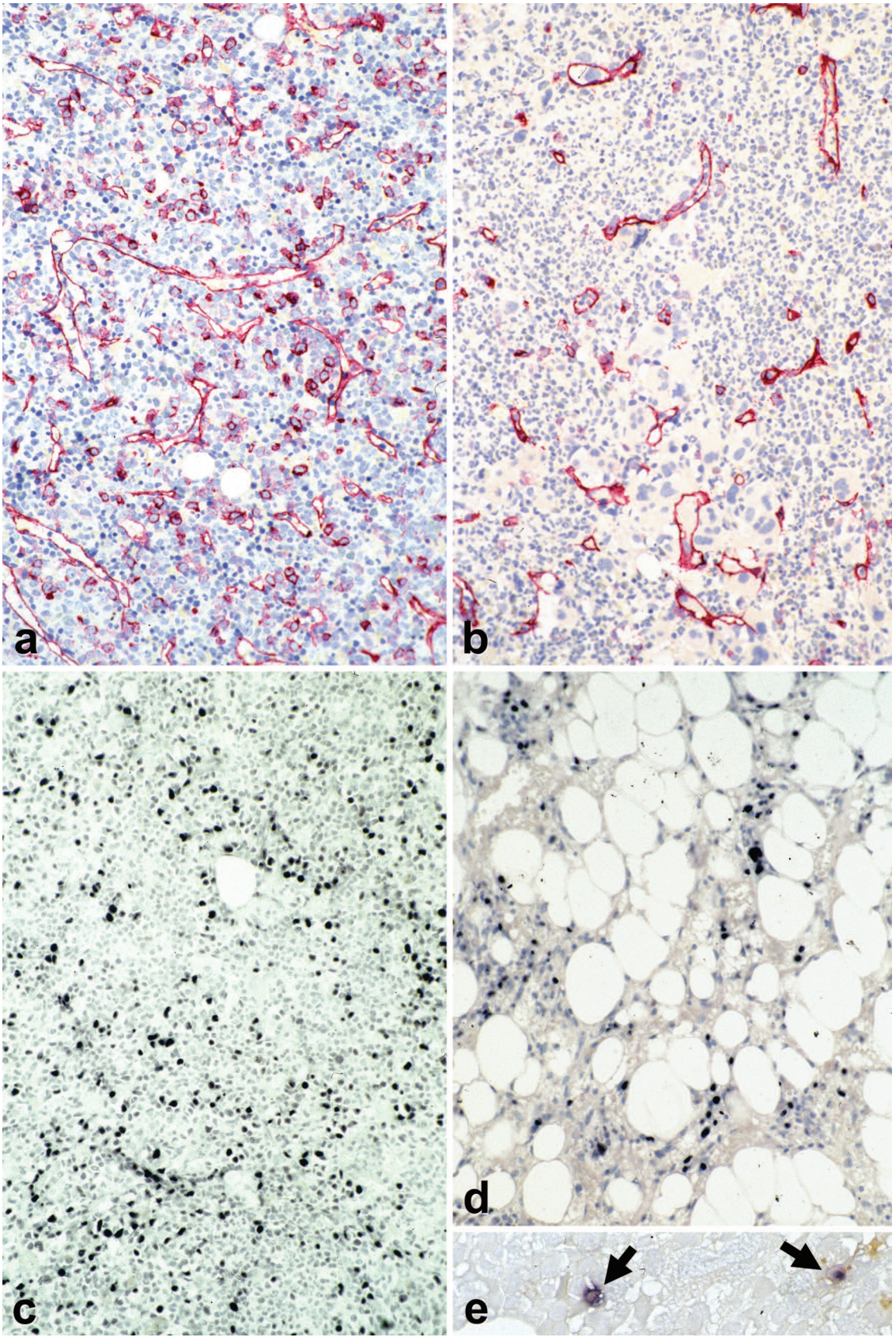

Fig. 5. Angiogenesis and proliferation-apoptosis after Imatinib therapy in BM biopsies of CML patients. **a.** In pretreatment BM samples a significant enhancement and branching of the microvasculature is noted that is significantly reduced following therapy **(b)**. **c.** Proliferative activity is markedly increased in CML. However, it declines significantly after treatment **(d).** On the other hand, there is an increase in apoptosis (arrows) in responding patients with stable-phase CML **(e)**. a and b: CD34 immunostaining; c, and d: PCNA reaction; e: anti-apostatin immunostaining. a-d, x 180; e, x 380

postulated that escape drug-induced apoptosis and thus may be the source of a later relapse (Graham et al., 2002). However, insensitivity to Imatinib has been shown to be the result of peculiar molecular properties of these progenitors (Gorre et al., 2001; Campbell et al., 2002; Roche-Lestienne et al., 2002; Shah et al., 2002) causing this drug resistance rather than a consequence of quiescence itself (La Rosee et al., 2003).

In conclusion, the success of Imatinib in CML has spurred clinical and translational research efforts that have led to the identification of the efficacy of this agent in other disorders. It is noteworthy that long-term Imatinib therapy generates prominent changes of BM histopathology in CML in the majority of responding patients. Amongst these, reductions in cellularity and neutrophil granulopoiesis are accompanied by an increase in nucleated erythroid precursors. The quantity of atypical micromegakaryocytes declines significantly and, as shown in patients without pretreatment, reveals features consistent with normalization including the loss of the Ph^{1+} chromosome (bcr/abl gene). Moreover, myelofibrosis is markedly reduced in only a fraction of patients while most show a stable state after prolonged treatment. Accordingly, microvessel density is changed to normal including the initially present distension of the vascular lumen. Finally, numbers of immature cells, including $CD34^{+}$ progenitors and myeloblasts declined rapidly in patients in accelerated and blastic phases and in those that did not experience a leukemic relapse during treatment.

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