Translational Studies in Elderly Patients with Acute Myeloid Leukemia

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acute myeloïde leukemie

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CONTENTS

CHAPTER 1

Introduction

1.1 Acute myeloid leukemia

The production of blood cells (hematopoiesis) takes place in the bone marrow. Acute myeloid leukemia (AML) is a clonal disease, which is characterized by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation. This frequently results in a severe suppression of normal hematopoiesis (granulocytopenia, anemia and/or thrombocytopenia).[1,2]

AML is a heterogeneous disease, characterized by a diversity of morphologic, cytogenetic and immunophenotypic features. Until recently, the morphologic classification was according to the French-American-British group,[3-5] which distinguishes AML into nine distinct subtypes (FAB M0-M7, M4eo) that differ with respect to the particular myeloid lineage involved and the degree of leukemic-cell differentiation. This distinction is based on the morphologic appearance of the blasts and their reactivity with histochemical stains. In addition, immunologic methods have been incorporated into the diagnostic criteria for some FAB-groups, e.g. M0 and M7.[6,7] Cytogenetic abnormalities of the chromosomes in the leukemic blasts have also been shown to be associated with specific FAB subtypes, e.g. t(15;17) with acute promyelocytic leukemia (APL; AML M3).[8] Recently, the World Health Organization (WHO) has proposed a new classification for myeloid neoplasms.[9] In this classification, genetic features (cytogenetic and molecular genetic) and clinical features have been integrated with morphology and immunophenotype to define distinct disease entities. Within the category of AML, four main groups have been recognized: 1. AML with recurrent cytogenetic translocations; 2. AML with myelodysplasia-related features; 3. therapy-related AML and MDS; and 4. AML not otherwise specified. A further subdivision of these main groups is shown in Table 1.

1.2 AML in patients of older age

The incidence of AML increases with age, and is most frequently observed in older adults. According to the National Cancer Institute (NCI) Surveillance Epidemiology and End Results (SEER), from 2000-2003 the median age at diagnosis for AML was 67 years. Approximately 6% of patients were diagnosed under 20 years of age; 6% between 20 and 34; 7% between 35 and 44; 11% between 45 and 54; 15% between 55 and 64; 22% between 65 and 74; and more than 30% in individuals aged 75 years or older.[10]

Table 1. WHO classification of AML

AML with recurrent cytogenetic translocations
AML with t(8;21)(q22;q22), AML1(CBF-α)/ETO
APL (AML with t(15;17)(q22;q11-12) and variants, PML/RAR-α)
AML with abnormal bone marrow eosinophils (inv(16)(p13q22) or t(16;16)(p13;q11), CBFβ/MYH11X)
AML with 11q23 (mixed-lineage leukemia, MLL) abnormalities
AML with multilineage dysplasia
With prior MDS
Without prior MDS
AML and MDS, therapy-related
Alkylating agent-related
Epipodophyllotoxin-related
Other types
AML not otherwise categorized
AML minimally differentiated
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monocytic leukemia
Acute erythroid leukemia
Acute megakaryocytic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis

When untreated, AML progresses rapidly and in most cases is fatal in weeks to months.[11] The purpose of treatment of patients with AML is to induce a complete remission and thereafter to prevent relapse with post-remission therapy. For decades conventional induction chemotherapy consisted of cytarabine and an anthracycline, like daunorubicin or idarubicin.[1] While in patients of 18 to 60 years, complete response (CR) rates of 70-80% may be achieved,[12-14] the outcome of patients aged over 60 years is rather dismal, with CR rates of about 50% and 5-year overall survival (OS) of about 10%.[15-20]

1.3 Multidrug resistance

One of the major reasons for treatment failure in AML is clinical resistance to chemotherapy. Clinical resistance is apparent as failure of attaining a complete hematological remission on induction chemotherapy or recurrence of leukemia following an initial remission of limited duration. Resistance to chemotherapy is positively associated with the expression of multidrug resistance (MDR) proteins.[21] Among those, members of the family of adenosine triphosphate (ATP)-binding cassette (ABC) transporter proteins play a role as drug efflux pumps in the plasma membrane.[21,22]

Classical MDR results from expression of the *ABCB1* (*MDR1*) gene and its protein product, permeability-glycoprotein (P-gp). Increased P-gp expression and as a consequence enhanced drug efflux have been reported in leukemias in increasing incidence as a function of age.[23,24] In various retrospective studies, increased P-gp expression and function were associated with lower CR rates and decreased disease-free survival (DFS) and OS in AML.[25-28] Other genes associated with multidrug resistance are e.g. *ABCC1* (*MRP1*; multidrug resistance-related protein),[29] *LRP* (lung resistance related protein)[30] and *ABCG2* (*BCRP*; breast cancer resistance protein).[31]

P-gp-mediated efflux may be inhibited by noncytotoxic agents, such as the first-generation P-gp inhibitors Quinine, Verapamil or Cyclosporin A (CsA). Results of phase II or phase III trials as regards the therapeutic value of these first-generation agents were mostly nonconclusive.[32,33] Subsequently, initial results of phase I and phase II trials with the second-generation P-gp inhibitor PSC-833 (Valspodar, Amdray®; Novartis Pharma, Basel, Switzerland) were promising.[34,35]

1.4 Prognostic factors

Besides higher age and multidrug resistance features, various other prognostic factors have been correlated with poor clinical outcome in patients with AML. Among these are AML developing from an antecedent MDS, a clinical poor performance status of the patient, an increased white blood cell (WBC) count at diagnosis (e.g. WBC count $> 20 \times 10^9$/l), particular immunophenotypes, e.g. CD34 positivity, and particular cytogenetic abnormalities.[1] The prognostic value of cytogenetic abnormalities has been elaborately studied, but until recently these studies have been mainly conducted in younger AML patients.[36,37]

1.5 Aim of this thesis

The prognosis of patients with AML aged 60 years or older still remains unsatisfactory. With conventional chemotherapy, CR rates are between 45 and 60%, while the 5-year overall survival is about 10%. A particularly prominent problem in this elderly population is the clinical unresponsiveness to chemotherapy. Lack of clinical response is associated with the expression of MDR proteins. Since phase I and phase II trials with the second-generation P-gp inhibitor PSC-833 applied to circumvent P-gp mediated drug resistance had shown promising results, we set out to further investigate PSC-833 in patients with AML of 60+ years of age in a randomized phase III trial. In this thesis we present the results of the latter trial and a series of correlative investigations dealing with various aspects of the clinical problem of chemotherapy resistance in this context.

In **chapter 2** the results are presented of an international, multicenter, randomized phase III trial, that was planned under auspices of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) and the United Kingdom Medical Research Council (UK MRC). The objective of this study was to compare standard induction chemotherapy with or without the addition of PSC-833 in newly diagnosed patients with AML of 60 years or older. The relationship of clinical outcome with P-gp status at diagnosis was also investigated. This P-gp status was assessed with P-gp function, or if not available, by measuring P-gp expression.

In the next two chapters we focus on mRNA expression. mRNA expression is yet another method to quantify MDR. **Chapter 3** deals with co-expression of *ABCB1* and *ABCG2*, while in **chapter 4** the clinical prognostic value of *ABCB1*, *ABCC1*, *LRP* and *ABCG2* mRNA expression is investigated.

In **chapter 5** the relationship of the single-nucleotide polymorphisms (SNPs) *C1236T*, *G2677T* and *C3435T* of the *ABCB1* gene with P-gp function and expression, *ABCB1* mRNA expression, as well as with clinical outcome is evaluated. SNPs are genetic variations in a gene which involve only one base pair, and several SNPs have been shown to be associated with altered drug metabolism.

In **chapter 6** the prognostic impact of the acquired cytogenetic abnormalities characteristic of the leukemia at diagnosis is evaluated. The impact of these results is considered in relationship to two other recently published reports on cytogenetic aberrations in elderly patients with AML.

In **chapter 7** the results are summarized and discussed, while some suggestions for future clinical research will be considered.

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CHAPTER 2

The value of the MDR1 reversal agent PSC-833 in addition to daunorubicin and cytarabine in the treatment of elderly patients with previously untreated acute myeloid leukemia (AML), in relation to MDR1 status at diagnosis

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Abstract

To determine whether MDR1 reversal by the addition of the P-glycoprotein (P-gp) inhibitor PSC-833 to standard induction chemotherapy would improve event-free survival (EFS), 419 untreated patients with acute myeloid leukemia (AML) aged 60 years and older were randomized to receive 2 induction cycles of daunorubicin and cytarabine with or without PSC-833. Patients in complete remission were then given 1 consolidation cycle without PSC-833. Neither complete response (CR) rate (54% versus 48%; *P* = .22), 5-year EFS (7% versus 8%; *P* = .53), disease-free survival (DFS; 13% versus 17%; *P* = .06) nor overall survival (OS; 10% in both arms; *P* = .52) were significantly improved in the PSC-833 arm. An integrated P-gp score (IPS) was determined based on P-gp function and P-gp expression in AML cells obtained prior to treatment. A higher IPS was associated with a significantly lower CR rate and worse EFS and OS. There was no significant interaction between IPS and treatment arm with respect to CR rate and survival, indicating also a lack of benefit of PSC-833 in P-gp-positive patients. The role of strategies aimed at inhibitory P-gp and other drug-resistance mechanisms continues to be defined in the treatment of patients with AML.

Introduction

The overall outcome of treatment of patients of older age with acute myeloid leukemia (AML) has remained highly unsatisfactory. In patients older than 60 years, complete response (CR) rates are 45 to 60% only, while median disease-free survival (DFS) values have been estimated at less than 12 months and the 4- to 5-year overall survival (OS) rates are approximately 10%.[1-5]

A potentially important biologic factor that may account for chemotherapy resistance of AML in patients of higher age is the high incidence of the intrinsic multidrug resistance (MDR) phenotype of leukemic blast cells.[6] The MDR phenotype results from expression of the *MDR1* gene[7,8] and its 170-kDa protein product, P-glycoprotein (P-gp),[9] also designated as adenosine triphosphate (ATP)-binding cassette (ABC) transporter B1 (ABCB1).[10] P-gp is a transmembrane protein that acts as an energy-dependent drug efflux pump for chemotherapeutic drugs such as the anthracyclines and epipodophyllotoxins, commonly used in AML therapy.

Increased P-gp expression and enhanced drug efflux have been reported with increasing age: from 17% in patients under the age of 35, 27% at 35 to 50 years, and 39% in patients over 50 years[11] to 71% in a group with median age 68 years (range, 56 to 88 years).[6] In retrospective studies MDR1/P-gp expression was associated with lower CR rates and decreased OS and DFS in AML.[12-16] Also P-gp positivity of AML is associated with other adverse prognostic factors such as CD34 expression, secondary leukemia, and unfavorable cytogenetics.[6,13,14,17]

Based on these studies a rationale was developed for MDR1 modulation as a therapeutic approach.[18] A variety of noncytotoxic agents, such as verapamil, quinine and cyclosporin A (CsA), inhibit the P-gp transporter through competition with other substrates for the binding sites of P-gp. These agents block P-gp-mediated efflux of drugs from the intracellular compartment and increase the intracellular accumulation of MDR-related drugs in MDR-positive cells.[19,20] Many of these P-gp reversal agents also block the elimination of cytostatic drugs from the molecular pores in the hepatobiliary canaliculi. By doing so they reduce hepatic elimination of antileukemic drugs like anthracyclines, which results in a prolonged half-life, an increase of the plasma area under the curve (AUC), and potentially increased toxicity from these agents.[21]

Randomized phase 2 and phase 3 studies with first-generation P-gp inhibitors in AML were mostly nonconclusive because of poor therapeutic benefit or unexpected interactions with the pharmacokinetics of the cytostatic agent.[22,23] Quinine and verapamil[24] have a cardiotoxic risk profile that prohibits adequate dosing.

The second-generation P-gp inhibitor PSC-833 (Valspodar, Amdray; Novartis Pharma, Basel, Switzerland) is a cyclosporin analog that is 7- to 20-fold more potent than CsA in increasing daunorubicin (DNR) retention in MDR cells, while lacking the immunosuppression and nephrotoxicity. The dose-limiting toxicity is cerebellar ataxia, which, however, is transient and fully reversible.[25-27] Phase 1 and phase 2 studies suggest that substantial inhibition of P-gp can be achieved in vivo at clinically tolerable doses of both PSC-833 and DNR.[28,29]
Here we report the results of an international, multicenter, open-label, randomized phase 3 trial of PSC-833 plus standard chemotherapy in 419 previously untreated elderly patients with AML, performed under the auspices of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) and the United Kingdom Medical Research Council (UK MRC). Two remission induction regimens of DNR/cytarabine (Ara-C) and DNR/Ara-C/PSC-833 were compared for their effect on CR rate, event-free survival (EFS), DFS and OS. The effect of PSC-833 plus chemotherapy on these outcome parameters in relation to the P-gp status at diagnosis was also investigated.

Patients, materials and methods

Patients

Patients 60 years or older with previously untreated primary or secondary AML (M0 to M2 and M4 to M7, French-American-British [FAB] classification adapted from Cheson *et al*[30]) and World Health Organization (WHO) performance status 2 or below were eligible for this study. Patients with secondary AML progressing from antecedent myelodysplastic syndrome (MDS) were eligible if they had not been given chemotherapy previously. Antecedent MDS was defined by a duration of at least 4 months. Patients with promyelocytic leukemia (M3), blast crisis of chronic myeloid leukemia, previous polycythemia rubra vera, or primary myelofibrosis were not eligible. Other exclusion criteria were cytopathologically confirmed central nervous system (CNS) infiltration, neurosensory toxicity grade 2 or above, neurocerebellar toxicity grade 1 or above (National Cancer Institute of Canada [NCIC] Expanded Common Toxicity Criteria [CTC]), known positivity for HIV, impaired hepatic or renal function (alanine aminotransferase [ALT] and/or aspartate aminotransferase [AST] 2.5 or more times the institutional upper limit of normal (IULN), alkaline phosphatase [AP] 2.5 or more times the IULN, serum total bilirubin 1.5 or more times the IULN, and serum creatinine 1.5 or more times the IULN after adequate hydration), those receiving treatment interacting with CsA, previous surgery within 2 weeks or investigational therapy or

radiotherapy within 4 weeks of study entry, other primary malignancy except basal cell carcinoma of the skin or stage 1 cervical carcinoma within the last 5 years, concurrent severe and/or uncontrolled medical condition, psychological, intellectual, or sensory dysfunction that was likely to impede ability to understand and comply with study requirements, or severe cardiac or pulmonary disease.

The study was approved by the ethics committees of the participating institutions and was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent.

Registration and randomization procedures

Patients were randomly assigned to 1 of 2 induction chemotherapy regimens without or with PSC-833, using a validated, voice-activated telephone system, and stratified according to age (60 to 65, 66 to 70, and 71 or above) and secondary AML (no or yes).

Treatment

Induction chemotherapy consisted of 2 cycles of 45 mg/m^2 DNR, 15 minutes of infusion on days 1 through 3, and 200 mg/m^2 Ara-C every 24 hours, continuous infusion on days 1 through 7 (arm A); or a similar regimen but with a lower dose of DNR (35 mg/m^2) and with PSC-833, 2 mg/kg in 2-hour loading dose followed by 10 mg/kg continuous intravenous infusion every 24 hours for 72 hours days 1 through 3 (arm B). The 22% dose reduction of DNR was based on the results of the pilot study,[28] which had established 35 mg/m^2 DNR as the maximum tolerated dose when administered concurrently with 10 mg/kg PSC-833 per day. Cycle 2 was given to all patients who achieved a normocellular marrow with less than 5% blasts, no Auer rods, and no evidence of extramedullary involvement with full peripheral blood (PB) recovery (absolute neutrophil count [ANC] above 1.0×10^9/L and platelets above 80×10^9/L) within 60 days of start of induction cycle 1. In patients with evidence of persistent AML, the second cycle was administered independent of PB recovery.

Patients in both arms who attained a CR were to receive 1 consolidation cycle without PSC-833 consisting of Ara-C 1 g/m^2 6-hour infusion on days 1 to 4, mitoxantrone 6 mg/m^2 by slow intravenous bolus on days 1 to 4, and etoposide 80 mg/m^2 1-hour infusion on days 1 to 4.

Definition of endpoints

In this analysis, CR was defined as a normocellular bone marrow (BM) with less than 5% blasts, no Auer rods, and no evidence of extramedullary involvement. Data on PB recovery within 60 days after start of a cycle were not always available and were not considered as a criterion for CR. When the BM blast cell count remained between 5 and 25% but was reduced by at least 50% in comparison with the initial value, a patient was considered in partial remission (PR). Patients who relapsed or died within 28 days after CR were considered as not having achieved a CR. Patients who did not enter CR following induction therapy were classified as induction death if the patient died within 30 days after start of induction cycles 1 or 2 or as having refractory disease otherwise.
Early death was defined as death during the first 7 days of the first induction cycle.
EFS was determined from the date of randomization until no CR on induction therapy, relapse after CR, or death in CR, whichever came first. Patients who did not attain a CR were considered a failure at 1 day after randomization. DFS was determined for all patients who achieved CR and was calculated from the date of CR until relapse or death, whichever came first. OS was measured from randomization until death from any cause. Patients still alive at the date of last contact were censored.

Analysis of P-gp expression and function in AML samples by flow cytometry

A BM aspirate of 3 to 10 mL was collected in a tube containing 0.5 mL Hanks balanced salt solution (HBSS; Gibco, Paisley, United Kingdom) with 625 U/mL sodium heparin. These samples were transported at 4°C or cryopreserved at –160°C and then transported to the central laboratory in Rotterdam.
Mononuclear BM cells were collected from patient BM aspirates by centrifugation over Lymphoprep (Nycomed, Oslo, Norway). They were frozen in 10% dimethyl sulfoxide (DMSO) and 20% fetal calf serum (FCS) and stored in liquid nitrogen. At the day of the analysis BM cells were thawed, washed, and resuspended in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 10% FCS, gentamicin at a concentration of 4×10^6 cells per milliliter.
Measurement of the expression of P-gp. For measurement of the expression of P-gp, cells were incubated (at room temperature) with monoclonal anti-P-gp antibody MRK 16[31] (Kamiya Biomedical, Tukwila, WA) at a concentration of 10 µg/mL or with UIC2 monoclonal antibody[32] (mAb) (Immunotech, Marseille, France) at a concentration of 12.5 µg/mL or an isotype-matched control antibody monoclonal immunoglobulin G2A (mIgG2a)

(Sigma Chemical; St Louis, MO) at a concentration of 10 μg/mL. The concentrations of antibodies were based on our quality control studies[33] and were also used in the pilot study.[28] Cellbound antibodies were detected by fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse immunoglobulin antibodies (DAKO, Glostrup, Denmark).

To measure expression of P-gp in $CD34^+$ cells, cells were labeled with phycoerythrin (PE)-labeled CD34 antibody or, as a control, PE-labeled mIgG1 antibody (Becton Dickinson, San Jose, CA). Cells were incubated with 0.1 μM TO-PRO-3 (Molecular Probes, Eugene, OR) to exclude nonviable cells. Fluorescence was measured using a FACScalibur (Becton Dickinson).

Results are given as the ratio of the mean of cell-associated fluorescence of cells incubated with the anti-P-gp antibody divided by the mean of cell-associated fluorescence of cells incubated with the control mIgG2a antibody.

Measurement of the function of P-gp. For measurement of the function of P-gp,[34,35] the fluorescent compound rhodamine 123 (Rho123; Sigma) was used as a P-gp substrate. Cells were incubated for 1 hour at 37°C at 5% CO_2 in the absence and presence of 2 μM PSC-833. After this incubation, 200 ng/mL Rho123 was added to the cells. A sample was taken at t = 0 minutes to correct for background fluorescence and at t = 75 minutes to measure intracellular rhodamine accumulation.

To measure function of P-gp in $CD34^+$ cells, cells were labeled with PE-cyanine 5 (Cy5)-labeled CD34 antibody or, as a control, PE-Cy5-labeled mIgG1 antibody (Immunotech).

Cells were incubated with 0.1 μM TO-PRO-3 to exclude nonviable cells. Fluorescence was measured using a FACScalibur.

Results are given as the ratio of the mean intracellular Rho123 fluorescence of cells exposed to PSC-833 divided by the mean intracellular Rho123 fluorescence of cells not exposed to PSC-833.

Interpretation. As controls in each analysis, the drug-sensitive human myeloma cell line 8226 S and the drug-resistant variant 8226 D6 cells[36] were used. Taken all experiments together, the mean efflux ratio (Rho123 + PSC-833 : Rho123) of the negative control cell line 8226 S was 0.92 ± 0.06 (mean ± SD; n = 88) and of the positive control cell line 8226 D6 was 6.12 ± 4.11.

Patient BM cells were considered positive for P-gp function if the efflux ratio was more than 1.05. This ratio of P-gp efflux is given for the whole population of blasts and also for the $CD34^+$ cells. Only patients with more than 10% P-gp-positive cells in all experiments were considered positive.

The mean of the MRK 16 expression ratio of the negative cell line 8226 S and of the positive cell line 8226 D6 were 1.28 ± 0.26 and 27.17 ± 6.37, respectively. The mean UIC2 expression

ratios were 1.16 ± 0.19 and 25.97 ± 7.05, respectively. Patient BM cells were considered positive for the expression of P-gp if the expression ratio was more than 1.65 for either MRK 16 or for UIC2. This ratio of the expression was given for the whole population of blasts and also for the $CD34^+$ cells together with the percentage of $CD34^+$ cells, which could be the leukemic tumor cell population. Only patients with a subpopulation of more than 10% of positive cells were considered positive. Some patients had P-gp expression but no function. These patients were considered negative because it is possible that these patients express a nonfunctional P-gp. Some patients showed P-gp function but no expression. These patients were considered positive because of the possible clinical relevance of this phenomenon.

P-gp assessment. For patients with P-gp data available, an integrated P-gp score (IPS) was based on the P-gp function or, if not available, on the expression ratios.[33] Patient BM samples were categorized as negative (efflux ratio between 0 and 1.05), low-positive (more than 1.05 to 1.40), intermediate-positive (more than 1.40 to 2.50), or high-positive (more than 2.50). Similarly, cut points for expression ratios were 1.65, 2.50 and 5.00. These cutoff values had been defined a priori and were chosen based on the efflux ratios observed with the doxorubicin-sensitive, P-gp-negative myeloma cell line RPMI 8226S and its doxorubicin-resistant cell lines 8226DOX1, 8226DOX6, and 8226DOX40, which exhibit increasing levels of P-gp function, P-gp expression, and cellular resistance (kindly provided by Dr W. S. Dalton, H. Lee Moffitt Center and Research Institute, Tampa, FL).

Cytogenetic analysis

Cytogenetic analysis of BM samples obtained at diagnosis was performed using standard cytogenetics techniques. All available cytogenetic reports were reviewed by 2 expert cytogeneticists. Chromosomal abnormalities were described according to the International System for Human Cytogenetics Nomenclature (ISCN 1995).[37] Favorable risk was defined as the presence of t(8;21), inv(16), or t(16;16). Unfavorable risk was defined by the presence of monosomies or deletions of chromosomes 5 or 7, abnormalities of the long arm of chromosome 3(q21;q26), t(6;9), abnormalities involving the long arm of chromosome 11 (11q23), or complex cytogenetic abnormalities (defined as at least 3 unrelated cytogenetic clones). Patients who did not meet the criteria for favorable or unfavorable risk were classified as being intermediate risk.

Statistical considerations

The primary objective of the study was to compare EFS between the 2 treatment arms on an intention-to-treat basis–that is, patients were analyzed according to assignment to treatment arm A (without PSC-833) or B (with PSC-833). To detect with a power of 80% an increase in 2-year EFS from 9.5% to 18% (2-sided significance level $\alpha = .05$) and assuming an accrual of 18 months and a follow-up time of 12 months, 400 patients were required and 331 events had to be observed.

Secondary end points were CR rate, DFS, and OS between the 2 treatment arms, safety and tolerability of the 2 treatment regimens, and the association between IPS and outcome and the interaction with additional PSC-833.

Patient characteristics between the 2 treatment arms were compared using the Fisher exact test[38] or the Pearson χ^2 test in case of discrete variables or the Wilcoxon rank sum test[39] in case of continuous variables.

The CR rate was compared between the 2 treatment arm using logistic regression.[40] The odds ratio (OR) was calculated with a 95% confidence interval (CI).

EFS, DFS, and OS were estimated by the Kaplan-Meier method,[41] and 95% CIs were constructed. Survival analysis was performed using Cox regression[42] to see whether there was a difference in survival between the 2 treatment arms. The hazard ratios (HRs) and corresponding 95% CIs were determined for all 3 survival end points. Kaplan-Meier curves were generated to illustrate differences between the 2 treatment arms and compared using the log-rank test.[43] Competing risk analysis was used to calculate cumulative competing risks of treatment failure among patients with a CR (either relapse after CR or death in first CR).

Safety was analyzed using descriptive statistics to summarize the incidence of adverse events (AEs) and laboratory findings. Toxicity of the 2 regimens was assessed by laboratory evaluation, physical examination, vital signs, and AE assessments. AEs were scored using the NCIC Expanded CTC.

All reported *P* values are 2 sided, and a significance level $\alpha = .05$ was used.

Table 1. Baseline characteristics of 419 elderly patients with AML by treatment arm

Characteristic	DNR/Ara-C No. (%)	DNR/Ara-C + PSC-833 No. (%)
Total no. of patients	211	208
Median age, y (range)	67 (58-85)	67 (60-83)
58-65	77 (36)	77 (37)
66-70	71 (34)	69 (33)
71-85	63 (30)	62 (30)
Secondary AML		
No	155 (73)	160 (77)
Yes	56 (27)	48 (23)
Sex		
Male	117 (55)	132 (63)
Female	94 (45)	76 (37)
FAB classification		
M0	14 (7)	17 (8)
M1	55 (26)	34 (16)
M2	70 (33)	74 (36)
M4	35 (17)	40 (19)
M5	14 (7)	19 (9)
M6	14 (7)	12 (6)
M7	4 (2)	1 (0)
Unclassified	5 (2)	11 (5)
WHO performance		
0	41 (20)	45 (22)
1	110 (53)	103 (51)
2	54 (26)	49 (24)
3	1 (0)	4 (2)
No data*	5	7
Median WBC count, $\times 10^9$/L (range)	7.0 (0.1-389)	11.3 (0.5-300)

Table 1. Baseline characteristics of 419 elderly patients with AML by treatment arm, continued

P-gp assessment: IPS		
Negative	37 (24)	45 (29)
Low-positive	50 (32)	40 (26)
Positive	43 (28)	42 (27)
High-positive	24 (16)	28 (18)
No data*	57	53
Cytogenetic study at diagnosis		
Not done	21 (12)	25 (14)
Done successfully†	146 (80)	147 (82)
Failure	15 (8)	8 (4)
No data*	29	28
Cytogenetic risk classification†		
Favorable	3 (2)	2 (1)
Intermediate	108 (74)	114 (78)
Unfavorable	35 (24)	31 (21)

WBC indicates white blood cell.

*Data not included when calculating percentages.

†Classification of cytogenetic abnormalities for 293 patients with successful cytogenetics. Favorable risk was defined as the presence of t(8;21), inv(16), or t(16;16). Unfavorable risk was defined by the presence of monosomies or deletions of chromosomes 5 or 7, abnormalities of the long arm of chromosome 3(q21;q26), t(6;9), abnormalities involving the long arm of chromosome 11 (11q23), or complex cytogenetic abnormalities (defined as at least 3 unrelated cytogenetic clones). Patients who did not meet the criteria for favorable or unfavorable risk were classified as being intermediate risk.

Results

Patient characteristics

Between May 1997 and February 1999, 428 patients from 99 centers in 15 countries were randomized for study treatment. Eight patients were not eligible because of previous treatment (n = 2), impaired hepatic or renal function (n = 2), or other (n = 4). One patient refused treatment after randomization and has been lost to follow-up since. One patient aged 58 years was accidentally randomized, but this was considered a minor protocol violation and

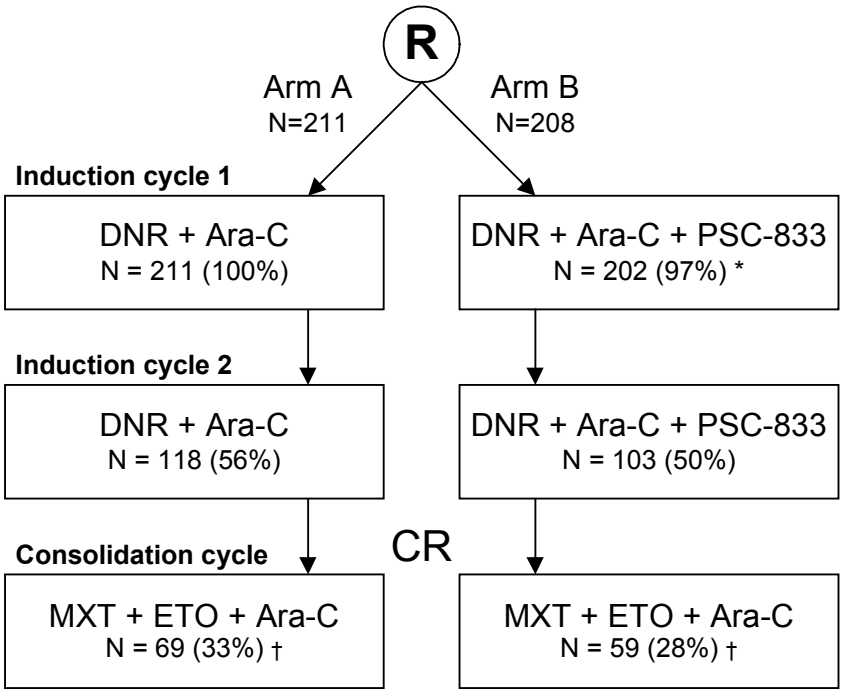

Figure 1. Flow diagram of 419 elderly patients with AML by treatment arm.

Per treatment arm, the number and percentage of patients who received a specific induction or consolidation cycle are shown. ® indicates randomization; MXT, mitoxantrone; and ETO, etoposide.

*Six patients in arm B did not receive any protocol treatment.

†Two patients, one in each treatment arm, received only induction cycle 1 and one consolidation cycle.

he has been included in the analysis. Of 419 remaining patients, 211 were randomized to arm A (control arm) and 208 were randomized to arm B (induction therapy with PSC-833). Median age was 67 years (range, 58 to 85 years). Patient baseline characteristics were comparable between the 2 arms (Table 1).

Cytogenetics

Successful cytogenetic data were available for 293 (70%) of the patients (Table 1). Five patients (2%) were classified as favorable risk and 66 patients (23%) as unfavorable risk, while the remaining 222 patients (76%) were classified as intermediate risk, equally distributed over the 2 treatment arms (P = .70). A total of 158 of 222 of intermediate-risk patients (71%) presented with a normal karyotype.

Table 2. CR in AML in elderly patients by treatment arm and by stratification factors age and disease status

Characteristic	DNR/Ara-C	DNR/Ara-C + PSC-833	Total
Total	101/211; 48% (41%-55%)	112/208; 54% (47%-61%)	213/419; 51% (46%-56%)
Age, y			
58-65	43/77; 56% (44%-67%)	53/77; 69% (57%-79%)	96/154; 62% (54%-70%)
66-70	36/71; 51% (39%-63%)	33/69; 48% (36%-60%)	69/140; 49% (41%-58%)
71-85	22/63; 35% (23%-48%)	26/62; 42% (30%-55%)	48/125; 38% (30%-48%)
Secondary AML			
No	79/155; 51% (43%-59%)	95/160; 59% (51%-67%)	174/315; 55% (50%-61%)
Yes	22/56; 39% (26%-53%)	17/48; 35% (22%-51%)	39/104; 38% (28%-48%)

Each cell contains the number of CR patients followed by the number of patients in the specific subgroup and the corresponding percentage, with its 95% CI.

Response to chemotherapy

Of 419 patients, 285 patients (68%) received induction cycles only and 128 patients (31%) received induction and consolidation treatment. Six of 419 patients (1%) did not receive any protocol treatment (Figure 1). Three of them received alternative induction therapy, resulting in a CR in 2 patients: one patient withdrew consent before cycle 1 and was treated with 6-mercaptopurine instead, and one patient had an AE before the start of treatment and was then treated with idarubicin instead of daunorubicin. Both patients were considered as CR patients according to the intention to treat.

The overall CR rate was 51%. CR was achieved in 101 of 211 patients (48%) in the control arm as compared with 112 of 208 patients (54%) in the PSC-833 arm (P = .22). A total of 80 patients in arm A and 87 patients in arm B achieved a CR after the first induction cycle. Table 2 shows the CR rates by stratification factor. Induction failures were classified as refractory disease or as induction death in 79 (37%) and 31 (15%) patients, respectively, in arm A, versus 63 (30%) and 33 (16%) patients, respectively, in arm B. Early death rates were similar in both arms: 4 (2%) and 5 (2%) patients, respectively.

Event-free survival, disease-free survival and overall survival

The survival end points are based on follow-up data available as of June 2004. The median follow-up of 56 patients still alive is 56 months; 35 of these patients were still in continuous first CR at last contact, including 19 in the control arm and 16 in the PSC-833 arm. Long-term

Table 3. Effect of PSC-833 on outcome at 5 years

	DNR/Ara-C N = 211		DNR/Ara-C + PSC-833 N = 208			
End point	No. of events	Probability of outcome at 5 y, % (95% CI)	No. of events	Probability of outcome at 5 y, % (95% CI)	*P*	RR* (95% CI)
CR rate	101	48 (41-55)	112	54 (47-61)	.22	1.27 (0.87-1.87)
Event-free survival	192	8 (5-12)	192	7 (4-11)	.53	1.07 (0.87-1.30)
Overall survival	181	10 (6-15)	182	10 (6-15)	.52	1.07 (0.87-1.32)
Disease-free survival after 1st CR	82	17 (11-26)	96	13 (8-20)	.06	1.33 (0.99-1.78)
Relapse after 1st CR†	72	72 ± 5	82	74 ± 4	.10	1.31 (0.95-1.79)
Death in 1st CR†	10	10 ± 3	14	13 ± 3	.36	1.47 (0.65-3.32)

RR indicates relative risk of event.

*For CR rate, RR should be read as odds ratio (OR); OR and 95% CI are based on logistic regression. For all survival end points, RR should be read as hazard ratio (HR); HR and 95% CI are based on Cox regression analysis.

†Relapse after first CR and death in first CR are competing risks; plus-minus values are the actuarial means ± standard error.

EFS, DFS, and OS were similar for both treatment groups (Table 3; Figure 2). Five-year EFS was 7% (95% CI, 4%-11%) for the PSC-833 group as compared with 8% (95% CI, 5%-13%) for the control group (P = .53). Most patients who reached a CR relapsed afterwards. Ten versus 14 patients died in CR, resulting in 5-year DFS of 17% (95% CI, 11%-26%) in the PSC-833 arm and 13% (95% CI, 8%-20%) in the control arm; P = .06. The 5-year OS was 10% (95% CI, 6%-15%) in both treatment arms; P = .52.

P-gp assessment

Viable BM samples to assess P-gp status at diagnosis were available in 309 of 419 patients (74%). Most samples were transported at 4°C. Only samples from the United States, Canada, and Australia were cryopreserved before transportation. Ultimately, we had P-gp data of 35 patients with cryopreserved samples, which is 11% of all patients with P-gp data available. P-gp functional data were available in 282 patients, and P-gp expression data were available in 293 patients. These data were highly correlated. The Spearman rank correlation coefficient between the efflux ratio and MRK 16 expression ratio was 0.64 (P < .001), while between the

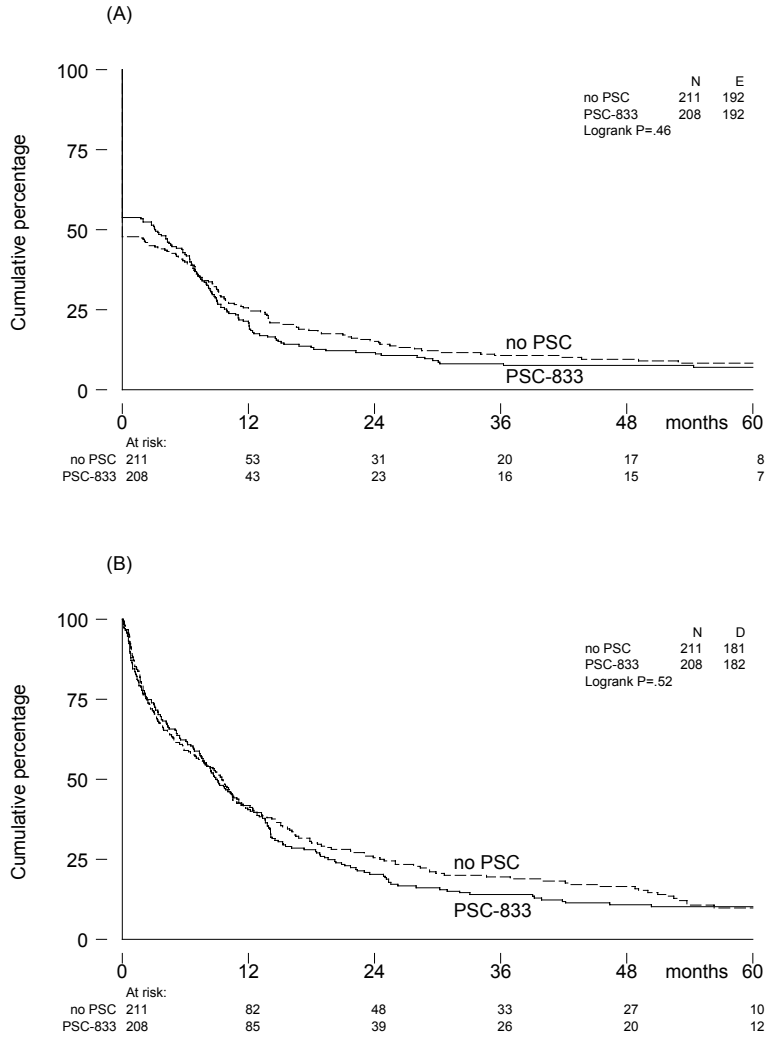

Figure 2. Kaplan-Meier survival curves of 419 elderly patients with AML by treatment arm.
(A) Event-free survival. (B) Overall survival. No PSC indicates patients in arm A.

MRK 16 and UIC2 expression ratio it was 0.81 ($P < .001$). Of the 265 patients with both P-gp functional and expression data available, 11 patients had positive function and negative expression, while in 30 function was negative and expression positive. The 309 patients were classified as IPS negative (27%), low-positive (29%), intermediate-positive (28%), or high-positive (17%), with no difference between the 2 treatment arms ($P = .91$; Table 1). An increased IPS was associated with a lower CR rate; the CR rate decreased from 61% (95% CI, 50%-72%) and 54% (95% CI, 44%-65%) in the IPS-negative and low-positive patients to 49% (95% CI, 38%-60%) and 40% (95% CI, 27%-55%) in the IPS-positive and high-positive patients, respectively ($P = .02$, test for trend). A higher IPS was also associated with significantly worse EFS and OS (Figure 3), and a trend for decreased DFS was observed.

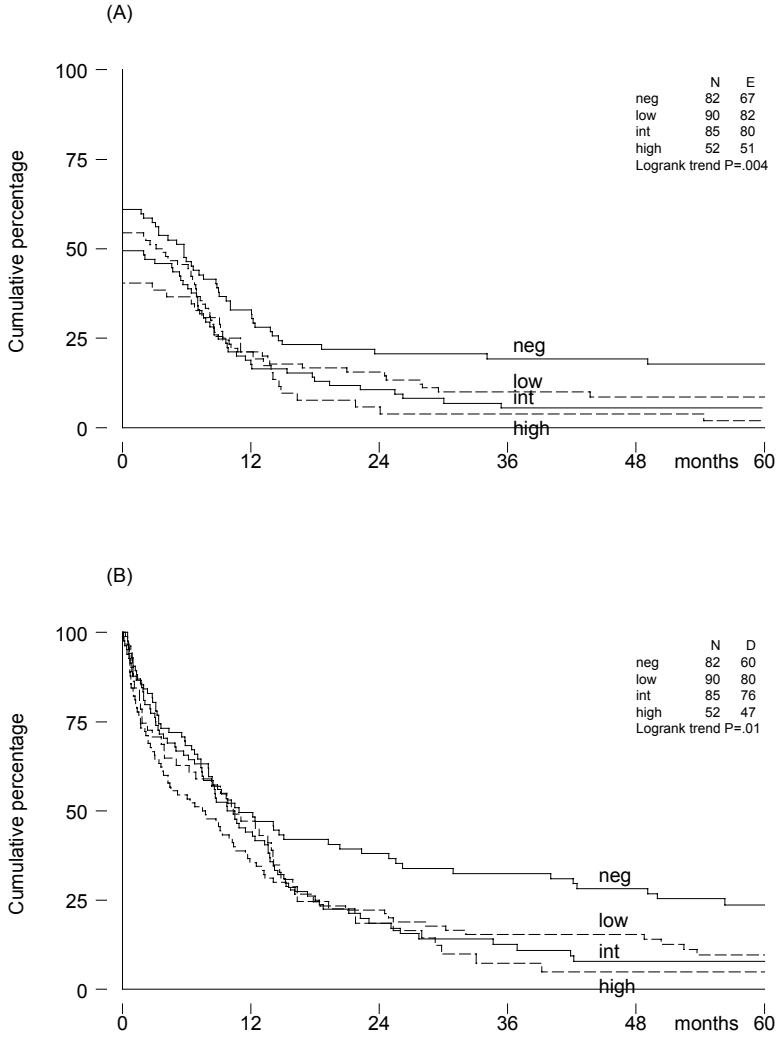

Figure 3: Kaplan-Meier survival curves of 309 elderly patients with AML by P-gp assessment (IPS).
(A) Event-free survival. (B) Overall survival.

We also evaluated the mutual effect of PSC-833 and IPS on CR rate and survival. A multivariate logistic regression analysis of CR rate was performed with treatment arm, IPS, and a treatment arm by IPS interaction term as covariates. EFS, DFS, and OS were also evaluated, using multivariate Cox regression analyses. Similar results as in the univariate analyses were obtained. No benefit of PSC-833 was seen, while a higher IPS suggested a lower CR rate (OR = 0.75; 95% CI, 0.54-1.03; *P* = .07), a significantly lower EFS (HR = 1.19; 95% CI, 1.01-1.40; *P* = .04), and a trend for worse DFS (HR = 1.22; 95% CI, 0.95-1.57; *P* = .12) and OS (HR = 1.12; 95% CI, 0.95-1.32; *P* = .17). Detailed results for CR rate, EFS,

Table 4. Multivariate analyses of CR rate, event-free survival, and overall survival in 309 elderly patients with AML using treatment arm, IPS, and treatment arm by IPS interaction as covariates

Variable	CR rate			Event-free survival			Overall survival		
	OR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
PSC-833 arm	1.49	0.71-3.15	.29	1.16	0.78-1.71	.46	0.96	0.65-1.44	.86
IPS*	0.75	0.54-1.03	.07	1.19	1.01-1.40	.04	1.12	0.95-1.32	.17
PSC-833 arm × IPS	1.05	0.68-1.63	.84	0.94	0.75-1.17	.57	1.06	0.85-1.33	.61

PSC-833 × IPS indicates the interaction term between these 2 variables.

*IPS was coded as 0, negative; 1, low-positive; 2, intermediate-positive; 3, high-positive.

and OS are shown in Table 4. However, none of the 4 analyses suggested an interaction between IPS and treatment arm. Especially no greater benefit of PSC-833 was apparent with increasing IPS. This was illustrated by a subgroup analysis of the 227 patients with low-, intermediate-, or high-positive IPS, who were expected to benefit most from PSC-833. Although the CR rate was somewhat higher in the PSC-833 arm (55% versus 44%; $P = .07$), survival was not better. HRs for PSC-833 were 1.02 (95% CI, 0.78-1.33; $P = .91$) for EFS, 1.34 (95% CI, 0.90-2.01; $P = .15$) for DFS, and 1.03 (95% CI, 0.78-1.35; $P = .84$) for OS.

Adverse events

AEs affecting the central and peripheral nervous system and liver and biliary disorders were more frequently reported in the patients treated with PSC-833 and DNR than in those receiving DNR alone. More AEs related to the nervous system were reported in patients treated with PSC-833 and DNR, such as paresthesia (16.3% versus 1.9%), ataxia (13.9% versus 1.4%), or dizziness (26.2% versus 11.3%), whereas excess liver AEs among the PSC-833 treatment group reflected more frequent reports of bilirubinemia (18.8% versus 7.1%). The patterns of other reported AEs were similar for the 2 treatment arms (Table 5).

Premature discontinuation of chemotherapy due to nonfatal AEs was more frequent in the group treated with PSC-833 and DNR than in the group treated with DNR alone (15.4% versus 9.5%). In both groups, the most frequently reported reasons for premature discontinuation were infections, while discontinuations due to ataxia, cerebellar toxicity, or peripheral neuropathy were only reported in patients treated with PSC-833 and DNR.

Deaths resulted from common complications of chemotherapy or from progression of AML. PSC-833, which is known to increase exposure to DNR by decreasing the clearance of the drug, did not lead to increased incidence or severity of chemotherapy-related AEs.

Table 5. Adverse events that have been reported in at least 20% of the patients in at least one of the treatment arms

	DNR/Ara-C N = 211		DNR/Ara-C + PSC-833 N = 202*	
AE	**CTC 1-2, %**	**CTC 3-4, %**	**CTC 1-2, %**	**CTC 3-4, %**
Abdominal pain	21	1	31	3
Anorexia	16	4	17	3
Constipation	26	1	34	1
Coughing	23	1	25	2
Diarrhea	43	8	58	6
Dizziness	11	1	25	2
Dyspnea	18	10	22	11
Epistaxis	21	1	20	1
Febrile neutropenia/fever	51	38	51	33
Fluid overload	18	3	21	2
Headache	25	1	24	2
Herpes simplex	20	1	22	3
Hypertension	5	8	17	8
Hypokalemia	31	4	35	4
Hypotension	9	6	16	9
Infection	11	14	11	13
Injection site reaction	28	4	25	6
Mucositis n.o.s.	18	4	20	8
Nausea	49	5	47	9
Purpura	17	2	22	0
Rash	30	2	29	6
Rash erythematous	24	1	27	4
Rigors	27	1	27	2
Sepsis	12	24	20	19
Thrombocytopenia	4	19	3	17
Vomiting	43	0	48	2

Per treatment arm and per AE, the proportion of patients with a maximum grade 1-2 or 3-4 are shown. Proportions are rounded to the nearest integer. AEs were graded according to the NCIC Expanded CTC. n.o.s. indicates not otherwise specified.

*The 6 patients in the PSC-833 arm who did not receive any protocol treatment have been excluded.

Discussion

Expression of the *MDR1* gene has been associated with lower CR rates and worse OS and DFS in AML.

This randomized phase 3 study of the P-gp inhibitor PSC-833 aimed at overcoming classic MDR in patients with AML who were 60 years and older. It was designed to investigate whether the response of AML would improve by adding PSC-833 to standard induction treatment. This large study shows that PSC-833 does not improve response rate or survival in patients with AML 60 years of age and older. The overall CR rate was 51% and 5-year survival was 10% in both treatment arms, which is comparable with other published trials in this age group.[1-5] To establish the independent prognostic value of P-gp, an integrated P-gp score (IPS) of AML blasts was prospectively determined at diagnosis. In this laboratory analysis, 73% of evaluable patients were classified as P-gp positive based on demonstrated P-gp reversal in vitro, which is in accordance with previously published data in this patient group.[6] We confirmed that IPS is an independent adverse prognostic factor in older patients with AML, because CR rate and EFS, DFS, and OS decreased with increasing IPS. There was, however, no significant interaction between IPS and PSC-833 with regard to CR rate and survival end points.

Therapeutic P-gp reversal has been examined in several randomized phase 2 and phase 3 trials using first-generation P-gp modulators such as quinine and CsA.

Quinine did not show an improvement in CR rate and OS in 2 consecutive trials by the French Groupe Ouest Est Leucemies Aigues Myeloblastiques (GOELAM) mainly in patient with AML aged 15 to 65 years,[22,44] while toxicity was significantly increased.[22] In the subgroup of patients who were tested for P-gp, a higher CR rate was observed in the 29 P-gp-positive patients in the quinine arm (83% versus 48%; $P = .01$).[44]

The effect of CsA has been assessed in several trials in refractory and relapsed AML. In the UK MRC Randomised Trial for Patients with Refractory or Relapsed Acute Myeloid Leukaemia in Adults (AML-R), OS and DFS did not differ between the CsA and the standard arm, while a lower CR rate, due to increased induction deaths and resistant disease, and worse OS were observed in the subgroup of patients 60 years of age and older treated with CsA.[45] In the Southwest Oncology Group (SWOG) 9126 trial, the incidence of refractory AML was lower in CsA-treated patients, while OS and relapse-free survival (RFS) were better. The positive effect of CsA was greatest in the subgroup of patients with moderate or bright P-gp expression.[46] A phase 2 trial by HOVON in patients with relapsed/primary refractory AML failed to show improved treatment outcome in the CsA-arm.[23]

PSC-833 is a second-generation P-gp reversal agent that lacks immunosuppressive activity. Several phase 1 and phase 2 trials of PSC-833 with natural product-based (re)induction chemotherapy for AML were performed in refractory/relapse patients[29,47-50] as well as in untreated elderly patients.[28,51,52] PSC-833 significantly inhibits the hepatobiliary metabolism and excretion of cytotoxic agents, which results in increased plasma exposure and slower terminal elimination of anthracyclines. Therefore, the dosage of agents that are substrates for P-gp (DNR, mitoxantrone, etoposide) was reduced by 22% to 66% when applied concomitantly with PSC-833 to accomplish equitoxicity with the control chemotherapy regimen.[28,48,51,53] In our trial the dosage of DNR was reduced by 22% in patients treated with PSC-833. While reduction of DNR dose in PSC-833 patients may have contributed to an equitoxic plasma exposure, it was expected that the inhibition of P-gp-mediated drug efflux would compensate for that and would overcome the P-gp-mediated drug efflux in leukemic cells. While such an effect was indeed observed in the in vitro analysis of these patient samples, PSC-833 did not confer a better therapeutic effect. Other trials like the randomized phase 3 Cancer and Leukemia Group B (CALGB) 9720 trial in elderly patients with untreated AML[54] and the Eastern Cooperative Oncology Group (ECOG) 2995 trial in relapse/refractory AML and high-risk MDS[55] also failed to show a benefit of PSC-833. The first trial was prematurely closed because of excessive early mortality in the PSC-833 arm, while accrual to the second trial was discontinued early due to lack of superiority in achieving CR in patients treated with PSC-833. In these trials, there was no apparent difference in OS and DFS between the 2 arms, but the power to detect moderate differences was low due to the small patient numbers. The CALGB 19808 trial in patients with AML younger than 60 years was halted in August 2003 because PSC-833 was no longer available,[56] and results are awaited. Our study in significantly greater numbers of patients, with long follow-up and with elaborate P-gp analysis, failed to reveal a benefit for the use of PSC-833, while the independent prognostic value of P-gp at diagnosis was established.

Various reasons might be considered to explain why PSC-833 failed to overcome clinical refractory disease in older patients with AML. First, dose reduction of DNR in the presence of PSC-833 from 45 mg/m^2 to 35 mg/m^2 in the experimental arm to achieve equitoxicity may have inflicted reduced DNR exposure to AML cells. This may have caused failure of PSC-833 to achieve durable high intracellular levels of DNR in AML blasts. This issue may be clarified by the trial conducted by the MRC group (the AML 14 Trial, which will shortly close) where an additional comparison between a DNR dose of 35 mg/m^2 and 50 mg/m^2 is included.[57] Second, the clinical benefit of PSC-833 may also have been masked by the confounding contributing effect of Ara-C, which is not a P-gp substrate. In fact, Ara-C is one of the most potent anti-leukemic drugs available today.[58] Third, several alternative drug

transport mechanisms may contribute to clinical resistance, including intratumoral transmembrane transport proteins such as members of the ABC superfamily of transport proteins including MDR1, the multidrug resistance-related protein (MRP1/ABCC1), and the breast cancer resistance protein (BCRP/ABCG2), or of the lung resistance related protein (LRP), which extrude a variety of cytotoxic drugs.[21,59-61]

The study presented here shows that addition of the P-gp inhibition agent PSC-833 to standard induction chemotherapy does not improve CR rate, EFS, DFS, and OS in patients aged 60 years and older. In addition, MDR1 status at diagnosis remains an independent adverse prognostic factor, indicating the need for other strategies to overcome MDR1-mediated resistance.

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Appendix

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CHAPTER 3

Role of BCRP and its expression with MDR1 in adult AML

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Letter to the Editor

Dear editor,

With interest, we read the paper of Galimberti and co-workers which addresses the role of BCRP and its co-expression with *MDR1* in adult AML.[1] The authors report co-expression of *BCRP* and *MDR1* in their cohort of 51 AML patients like earlier has been shown in breast cancer patients. We would like to emphasize that it confirms previous published data in AML as well, where we showed that *BCRP* and *MDR1* expression was highly correlated in a cohort of 30 de novo AML patients, studied in a paired analysis at diagnosis and relapse.[2] Moreover, more recently, we showed a strong correlation between *BCRP* and *MDR1* mRNA expression in a cohort of 154 elderly AML patients using RT-PCR (Taqman analysis).[3] These findings confirmed the suggestion raised by Ross *et al*, who was the first person that reported a correlation between *BCRP* and *MDR1* expression in AML.[4]

The clinical relevance of co-expression of the drug resistance proteins may explain the lack of efficacy of MDR1 inhibition. Modulators that inhibit MDR1 only, may be less effective than modifiers that inhibit both proteins in clinical settings. In a paired analysis, we found that *BCRP* was associated with *MDR1* but not with *MRP1* and *LRP1* at diagnosis. This association was underscored by a correlation between *BCRP* mRNA expression and the functional drug efflux, which could not be inhibited by PSC-833. This correlation disappeared at relapse, where no co-expression of *BCRP* and *MDR1* was observed. Interestingly, *BCRP* was the only MDR protein expressed at a higher level at relapse as compared to diagnosis. In addition to co-expression of MDR1 and BCRP, expression of other combinations of resistance proteins is possible. In our pilot study of 30 de novo paired adult and pediatric AML patients co-expression was found exclusively between *BCRP* and *MDR1*, and not between any of the other proteins. However, in the larger cohort of elderly AML patients, co-expression was found not only between *BCRP* and *MDR1* mRNA, but also between *BCRP* and *MRP1* (*P*-values respectively < .005, < .005). These data suggest that overruling multidrug resistance using multipotent modifiers may be a better target than specific MDR proteins for clinical settings. New modifiers which target both drug resistance proteins, like compound GF120918 and Fumitremorgin C have shown superior efficacy over PSC-833 and Cyclosporin D in vitro but clinical data are not available as yet.

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CHAPTER 4

CD34 related co-expression of *MDR1* and *BCRP* indicates a clinically resistant phenotype in patients with acute myeloid leukemia (AML) of older age

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Abstract

Clinical resistance to chemotherapy in acute myeloid leukemia (AML) is associated with the expression of the multidrug resistance (MDR) proteins P-glycoprotein (P-gp), encoded by the *MDR1*/*ABCB1* gene, multidrug resistant-related protein (MRP/ABCC1), the lung resistance related protein (LRP), or major vault protein (MVP), and the breast cancer resistance protein (BCRP/ABCG2). The clinical value of *MDR1*, *MRP1*, *LRP*/*MVP* and *BCRP* mRNA expression was prospectively studied in 154 newly diagnosed AML patients ≥ 60 years, who were treated in a multicenter, randomized, phase 3 trial. Expression of *MDR1* and *BCRP* showed a negative while *MRP1* and *LRP* showed a positive correlation with high white blood cell count (respectively $P < .05$, $P < .001$, $P < .001$ and $P < .001$). Higher *BCRP* mRNA was associated with secondary AML ($P < .05$). *MDR1* and *BCRP* mRNA were highly significantly associated ($P < .001$), as were *MRP1* and *LRP* mRNA ($P < .001$). Univariate regression analyses revealed that CD34 expression, increasing *MDR1* mRNA expression, as well as *MDR1*/*BCRP* co-expression, were associated with a lower complete response (CR) rate and with worse event-free survival (EFS) and overall survival (OS). When adjusted for other prognostic actors, only CD34 related *MDR1/BCRP* co-expression remained significantly associated with a lower CR rate ($P = .03$), thereby identifying a clinically resistant subgroup of elderly AML patients.

Introduction

Clinical resistance to chemotherapy in AML is often associated with expression of (membrane) transport associated multidrug resistance (MDR) proteins. Expression of P-glycoprotein (P-gp), encoded by the *MDR1* gene, is an independent adverse prognostic factor for response and survival in *de novo* AML.[1-4] Moreover, it has been shown that besides P-gp, also the multidrug resistance related protein (MRP1/ABCC1) and the lung resistance related protein (LRP), also designated as the major vault protein (MVP), are expressed in AML. However, the prognostic significance of the latter resistance proteins has not been settled.[3,5-7] Some years ago a new drug resistant protein, i.e. the breast cancer resistance protein (BCRP/ABCG2) which is the equivalent of the mitoxantrone resistant protein and the placental ABC transporter (ABCP) was found to be expressed in AML.[8-13] The precise role of either resistance proteins among poor risk AML such as in patients of older age has not been established. This study prospectively investigated the relevance of *MDR1*, *MRP1*, *LRP* and *BCRP* mRNA expression in combination with known prognostic characteristics like CD34 expression, white blood cell (WBC) count and secondary AML as possible denominators of response and survival in patients with AML aged 60+, who were treated in the same clinical trial.

Patients and methods

Patients

A group of 154 patients with AML aged 60 years or older were included in the present study. All patients were enrolled between May 1997 and February 1999 in an international, multigroup, randomized phase 3 trial, performed under auspices of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) and the United Kingdom Medical Research Council (UK MRC).[14] In that trial, 419 eligible Caucasian patients ≥60 years with previously untreated *de novo* and secondary AML [M0-M2 and M4-M7 according to the French-American-British (FAB) classification[15]] were randomized to receive 2 cycles of induction chemotherapy consisting of daunorubicin (DNR) and cytarabine (Ara-C) with or without the P-glycoprotein (P-gp) inhibitor PSC-833 (Valspodar, Amdray®; Novartis Pharma, Basle, Switzerland). Patients in both arms in complete remission after these two cycles were to receive one consolidation consisting of Ara-C, mitoxantrone (MXT) and etoposide. Inclusion

criteria, clinical characteristics, treatment and outcome of the phase 3 trial have been previously reported.[14]

Bone marrow aspirates had been collected at diagnosis for the analysis of P-gp function and expression, as described previously.[14] Selection of patients for our study was based on availability of sufficient purified AML blast samples in our tissue bank, which was the case for 154 patients.

This study was approved by the ethics committees of the participating institutions, and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients before randomization.

Methods

Bone marrow aspirates were obtained in heparinized tubes. Mononuclear bone marrow cells (MNCs) were collected by Ficoll Hypaque density gradient centrifugation (density 1.077 g/m^3) (Pharmacia, Uppsala, Sweden). To obtain purified samples with more than 85% of blasts, T-cell depletion and adherence depletion was performed as previously described.[16] Cells were cryopreserved in Dulbecco modified Eagle medium (DMEM; Gibco, Paisley, UK), supplemented with 10% dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) and 20% fetal calf serum (FCS; Gibco) and stored in liquid nitrogen. On the day of the experiments bone marrow cells were thawed. Cells were washed and resuspended in DMEM supplemented with 10% FCS. Before RNA and DNA isolation cells were washed with phosphate-buffered saline (PBS; Gibco).

MDR1, MRP1, LRP and BCRP mRNA analysis

The drug resistance proteins were analyzed using the methods which we previously reported.[11] In brief, total RNA was isolated using the TRISOLV™ extraction as described by the manufacturer (Biotecx, Houston, TX, USA). RNA was aliquoted and stored at –80°C. RNA samples were analyzed for RNA integrity by gel electrophoresis. cDNA was synthesized by the use of the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA), diluted, aliquoted and stored at –80°C. Quantitative RT-PCR was used to measure the mRNA expression levels of *MDR1*, *MRP1*, *LRP* and *BCRP* by Taqman-chemistry on an ABI PRISM 7700 sequence detector (Applied Biosystems), using two endogenous reference genes i.e. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and porphobilinogen deaminase (PBGD).

Definition of endpoints

The clinical endpoints have been defined previously.[14] In brief, CR was defined as a normocellular BM with < 5% blasts, no Auer rods and no evidence of extramedullary involvement. Because data on peripheral blood recovery within 60 days were not always available, they were not considered as a criterion for CR. Patients who relapsed or died within 28 days after CR were considered as not having achieved a CR. EFS was calculated from the date of randomization until no CR on induction therapy, relapse after CR of death in CR, whichever came first. Patients who did not reach CR were considered failure for EFS at 1 day after randomization. Disease-free survival (DFS) was determined for all patients who achieved CR on induction therapy and was calculated from the date of CR until relapse or death, whichever came first. OS was measured from randomization until death from any cause. Patients who were still alive at the date of last contact were then censored.

Statistical analysis

The original phase 3 trial had been designed to detect with a power of 80% an increase in 2-year EFS from 9.5% in the control arm (without PSC-833) to 18% in the PSC-833 arm (2-sided significance level $\alpha = .05$), and included 419 eligible patients.

mRNA data were obtained from a subset of 154 patients with sufficient BM samples in our tissue bank available for analysis. Baseline parameters of interest were *MDR1*, *MRP1*, *LRP* and *BCRP* mRNA expression. Clinical endpoints were CR rate, EFS, DFS and OS.

Baseline characteristics of patients with or without mRNA expression data available were compared using the Fisher exact test or the Pearson chi-squared test in case of discrete variables, whichever appropriate, or the Wilcoxon rank-sum test in case of continuous variables. The association between patient baseline characteristics and mRNA expression levels was analyzed using the Pearson chi-squared test or the Spearman rank correlation test, whichever was appropriate. The prognostic value of mRNA levels with respect to CR rate was determined using logistic regression[17] while the impact of *MDR1*, *MRP1*, *LRP* and *BCRP* on EFS, DFS and OS was analyzed with Cox regression analysis.[18]

For this purpose, the natural logarithm of the mRNA expression levels of the 4 resistance genes were included in the analyses, due to the very skewed distribution of the original mRNA levels.

In addition, the outcome of patients with co-expression of *MDR1* and *BCRP* was evaluated in order to confirm the poor prognosis of AML with *MDR1*/*BCRP* co-expression reported by Benderra *et al*[19] in patients of median 52 years. These patients were defined as having mRNA

Table 1. Comparison between patients with or without data available for expression of the drug resistance genes. The results indicate that, apart from WBC count there are no differences between the 2 subgroups.

	Drug resistance genes evaluated			
	Yes	**No**	**Total**	***P***
	N (%)	N (%)	N (%)	
Number of patients	154	265	419	
Patient characteristics				
Median age, (range)	67 (60-85)	68 (58-85)	67 (58-85)	.52
Sex				.26
Male	86 (56)	163 (62)	249 (59)	
Female	68 (44)	102 (38)	170 (41)	
Secondary AML	31 (20)	73 (28)	104 (25)	.09
Median WBC count ($\times 10^9$/L) (range)	19.1 (0.1-389)	5.6 (0.5-300)	8.9 (0.1-389)	.001
N	146	243	389	
Median % $CD34^+$, (range)	32.5 (0.1-97.9)	29.7 (0.1-93.7)	30.3 (0.1-97.9)	.50
N	152	157	309	
Cytogenetic risk classification*				.12
Favorable	3 (3)	2 (1)	5 (2)	
Intermediate	90 (80)	132 (73)	222 (76)	
Unfavorable	19 (17)	47 (26)	66 (23)	
No data	42 (n.i.)	84 (n.i.)	126 (n.i.)	
Treatment arm randomized				.02
DNR/Ara-C	66 (43)	145 (55)	211 (50)	
DNR/Ara-C + PSC-833	88 (57)	120 (45)	208 (50)	

levels of these two drug resistance genes equal to or higher than the median. Their outcome was compared to the other patients with a least one of the *MDR1* and *BCRP* mRNA expression levels below the median. Logistic regression and Cox regression analyses were performed unadjusted, as well as adjusted for other prognostic factors, i.e. secondary AML, natural logarithm of WBC count, square root of percentage $CD34^+$ cells and cytogenetic risk (favorable/intermediate versus unfavorable versus unknown), as well as for treatment arm in the phase 3 trial, as about half of the patients had been randomized to receive PSC-833 in

Table 1. Comparison between patients with or without data available for expression of the drug resistance genes.

Continued

	Drug resistance genes evaluated			
	Yes	**No**	**Total**	***P***
Treatment outcome				
CR rate, % (95% CI)	52 (44-60)	50 (44-56)	51 (46-56)	.73
EFS, % (95% CI)				.72
1 year	23 (17-30)	23 (18-28)	23 (19-27)	
5 years	9 (5-14)	7 (4-11)	8 (5-11)	
DFS, % (95% CI)				.81
1 year	38 (27-48)	39 (31-48)	39 (32-45)	
5 years	17 (10-26)	14 (9-21)	15 (11-21)	
OS, % (95% CI)				.31
1 year	42 (34-50)	41 (35-46)	41 (36-46)	
5 years	14 (9-20)	8 (5-12)	10 (7-14)	

WBC indicates white blood cell; N, number of patients with data (if not available for all patients); n.i., not included when calculating percentages; DNR, daunorubicin; Ara-C, cytarabine; CR, complete response; CI, confidence interval; EFS, event-free survival; DFS, disease-free survival; and OS, overall survival.

*Classification of cytogenetic abnormalities only for 293 patients with successful cytogenetics. Favorable risk: t(8;21), inv(16) or t(16;16). Unfavorable risk: the presence of monosomies or deletions of chromosomes 5 or 7, abnormalities of the long arm of chromosome 3(q21;q26), t(6;9), abnormalities involving the long arm of chromosome 11 (11q23) or complex cytogenetic abnormalities (defined as at least 3 unrelated cytogenetic abnormalities in one clone). Patients who did not meet the criteria for favorable or unfavorable risk were classified as intermediate risk.[14]

addition to their chemotherapy. Kaplan-Meier curves[20] were generated to illustrate survival, and were compared using the log-rank test.[21] All reported *P*-values are 2-sided and, in view of the exploratory nature of these analyses, were calculated without adjustment for multiple testing. *P*-values ≤ .05 were considered statistically significant.

Table 2. Association between clinical patient characteristics and the mRNA expression of the four drug resistance genes and *MDR1*/*BCRP* co-expression. Each cell displays the Spearman rank correlation coefficient between two variables and, between brackets, the number of patients with both variables available.

	mRNA expression of				
	MDR1	***MRP1***	***LRP***	***BCRP***	***MDR1*/*BCRP* co-expression**
Characteristic					
Age	0.15	-0.01	-0.09	0.09	0.07
	(153)	(153)	(153)	(137)	(147)
Secondary AML	0.06	-0.22†	-0.21†	0.19*	0.12
	(153)	(153)	(153)	(137)	(147)
WBC count	-0.17*	0.28‡	0.36‡	-0.36‡	-0.35‡
	(145)	(145)	(145)	(131)	(139)
$CD34^+$	0.54‡	0.14	-0.08	0.17*	0.27†
	(151)	(151)	(151)	(135)	(145)
Unfavorable	0.11	-0.05	-0.23*	0.13	0.10
cytogenetic risk	(111)	(111)	(111)	(98)	(106)

WBC indicates white blood cell.

Unfavorable cytogenetic risk was defined by the presence of monosomies or deletions of chromosomes 5 or 7, abnormalities of the long arm of chromosome 3(q21;q6), t(6;9), abnormalities involving the long arm of chromosome 11 (11q23) or complex cytogenetic abnormalities (defined as at least 3 unrelated cytogenetic abnormalities in one clone).

*$P < .05$

†$P < .01$

‡$P < .001$

Results

In the phase 3 trial, a total of 419 untreated patients with AML aged 60 years and older were randomized to receive 2 induction cycles with or without PSC-833. As reported, no difference was found between both treatment arms as regards CR rate (54% in the PSC-833 arm versus 48% in the control arm, $P = .22$), 5-year EFS (7% versus 8%, $P = .53$) nor DFS (13% versus 17%, $P = .06$) and OS (10% in both arms, $P = .52$).[14] We previously reported the role of functional MDR1 expression with respect to clinical outcome in these patients.

In 154/419 of the patients sufficient BM cells were available in our tissue bank to investigate the mRNA expression level of the drug resistance genes *MDR1*, *MRP1*, *LRP* and *BCRP*. This subgroup consisted of a representative group according to age, gender, CD34 expression, cytogenetics and FAB classification (Table 1). In this test group, a higher WBC count at diagnosis was observed than in the other 265 patients, and relatively more patients had been randomized to the PSC-833 arm (57% versus 45%, $P = .02$).

There was no significant difference in the levels of *MDR1*, *MRP1*, *LRP* nor *BCRP* mRNA expression between the two treatment arms (data not shown). The CR rate and survival endpoints were also similar in both patient groups (Table 1). However, patients with mRNA data in the PSC-833 arm had a higher CR rate (61% versus 40%, $P = .02$), whereas this was 54% versus 48% ($P = .22$) in all 419 patients.

The mRNA expression levels of the resistance genes were not significantly associated with the age of the patients (Table 2). *MRP1* and *LRP* expression showed a strong positive correlation with WBC count. Negative associations of *MDR1* and *BCRP* with WBC count were observed. A significant positive association was found between CD34 and *MDR1* and also with *BCRP* mRNA expression. No significant correlation was found between *MRP1* nor *LRP*, and CD34 expression (Table 2). Interestingly, secondary AML cases had a significantly higher expression of *BCRP* ($P < .05$), and lower *MRP1* and *LRP* levels (both $P < .01$, Table 2). In the vast majority of our patients, also P-glycoprotein (P-gp) efflux and expression data were available. Function and expression data and *MDR1* mRNA expression levels were highly correlated ($P < .001$), which was published recently.[22]

In this cohort of patients of higher age with AML, *MDR1* and *BCRP* were highly associated ($P < .001$), just as were *MRP1* and *LRP* mRNA ($P < .001$) (Figure 1). A negative association was found between *BCRP* and *MRP1*, and between *BCRP* and *LRP* (both $P < .001$) (Figure 1). The 40 patients with co-expression of *BCRP* and *MDR1* had significantly higher CD34 expression [median 39.5% (range, 0.1%-97.7%) versus 25.9% (range, 0.1-97.9%) ($P = .001$)] and a lower WBC count [median 4.5 (range, 0.8-300) $\times 10^9$/L versus 28.1 (range, 0.1-389) $\times 10^9$/L ($P < .001$)]. No significant correlation of *MDR1*, *BCRP* or co-expression of *MDR1* and *BCRP* was found with unfavorable cytogenetics ($P = .4$) (Table 2).

To assess the clinical relevance of the 4 resistance genes, their expression was evaluated with regard to CR rate and survival data, respectively. The median follow up of the 25 patients still alive was 58 months (range, 1-80 months). Univariate logistic regression analysis showed that higher *MDR1* mRNA expression predicted for a lower CR rate (log(*MDR1*): odds ratio (OR) = 0.75; 95% CI, 0.61-0.93; $P = .009$), whereas *MRP1*, *LRP* and *BCRP* mRNA were not associated with CR (Table 3). *MDR1* expression was also associated with a worse EFS (log(*MDR1*): hazard ratio (HR) = 1.14; 95% CI, 1.03-1.27; $P = .01$) and OS (log(*MDR1*):

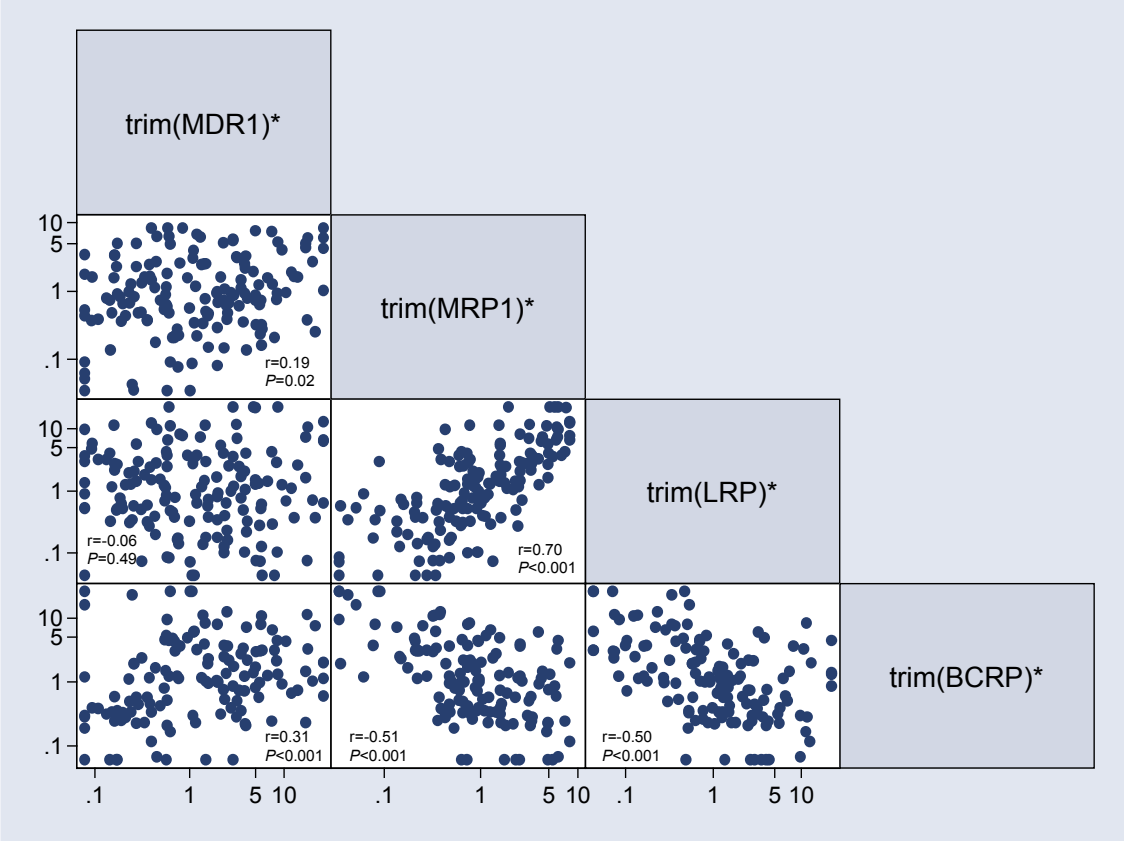

Figure 1. Association between *MDR1*, *MRP1*, *LRP* and *BCRP* mRNA expression levels.

Each dot represents the expression of two drug resistance genes in one patient. The Spearman rank correlation coefficient has been calculated, along with the corresponding *P*-value. Both the x-axis and y-axis have a logarithmic scale.

trim(X)* indicates that the 2.5% smallest and largest values of X have been shrinked; r, Spearman rank correlation coefficient; and *P*, *P*-value.

The results show a significant positive correlation between *MDR1* and *BCRP* mRNA expression as illustrated by *P*-value and correlation coefficient. In addition *MRP1* and *LRP* are highly associated. *BCRP* shows a negative correlation with *MRP1* and *LRP*.

HR = 1.16, 95% CI, 1.05-1.29; *P* = .004). Similar results were also obtained for *MDR1*/*BCRP* co-expression (Table 3, Figure 2). When the analyses were performed with adjustment for other prognostic factors, as described in the Statistical analysis paragraph, only *MDR1*/*BCRP* mRNA co-expression remained significantly associated with a lower CR rate (OR = 0.37; 95% CI, 0.15-0.91; *P* = .03), while a trend was observed for worse EFS (Table 3). On the other hand, higher CD34 expression was significantly associated with a lower CR rate (square root(CD34): OR = 0.86; 95% CI, 0.76-0.98; *P* = .02) and with worse EFS (HR = 1.12; 95% CI, 1.06-1.19; *P* < .001), DFS (HR = 1.19; 95% CI, 1.09-1.30; *P* < .001) and OS (HR = 1.17; 95% CI, 1.10-1.25; *P* < .001).

Table 3. Prognostic value of drug resistance gene expression w.r.t. CR rate, event-free survival (EFS), disease-free survival from CR (DFS) and overall survival (OS). Results of logistic (for CR rate) and Cox regression (for survival) analyses, either univariate (= unadjusted) or adjusted for treatment arm, secondary AML, WBC count (natural logarithm), % $CD34^+$ (square root) and cytogenetic risk (favorable/intermediate versus unfavorable versus unknown), are shown for each of the 4 drug resistance genes *MDR1*, *MRP1*, *LRP* and *BCRP* (natural logarithm of mRNA expression levels), and for *MDR1*/*BCRP* co-expression.

	CR rate			**EFS**			**DFS**			**OS**		
	OR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*	HR	95% CI	*P*
MDR1												
Univariate	0.75	0.61-0.93	.009	1.14	1.03-1.27	.01	1.13	0.97-1.30	.11	1.16	1.05-1.29	.004
Adjusted	0.77	0.58-1.03	.08	1.05	0.91-1.21	.48	0.95	0.77-1.18	.67	1.00	0.87-1.16	.97
MRP1												
Univariate	1.06	0.83-1.35	.63	1.02	0.90-1.15	.79	1.07	0.89-1.29	.47	1.11	0.97-1.26	.12
Adjusted	1.22	0.90-1.66	.20	1.00	0.87-1.15	.98	1.12	0.92-1.37	.26	1.05	0.91-1.21	.54
LRP												
Univariate	1.16	0.94-1.43	.16	0.95	0.86-1.06	.36	0.98	0.84-1.14	.79	0.97	0.87-1.08	.60
Adjusted	1.22	0.93-1.61	.15	0.99	0.87-1.12	.83	1.06	0.89-1.27	.52	0.98	0.86-1.12	.78
BCRP												
Univariate	0.84	0.66-1.06	.14	1.04	0.91-1.18	.58	0.95	0.77-1.16	.60	0.96	0.84-1.10	.58
Adjusted	0.79	0.59-1.06	.12	0.99	0.86-1.14	.92	0.84	0.66-1.06	.14	0.90	0.77-1.05	.19
MDR1/*BCRP*												
Univariate	0.38	0.18-0.80	.01	1.63	1.11-2.37	.01	1.65	0.90-3.01	.11	1.47	1.00-2.16	.05
Adjusted	0.37	0.15-0.92	.03	1.53	0.98-2.38	.06	1.37	0.67-2.82	.39	1.16	0.74-1.83	.51

OR indicates odds ratio; CI, confidence interval; and HR, hazard ratio

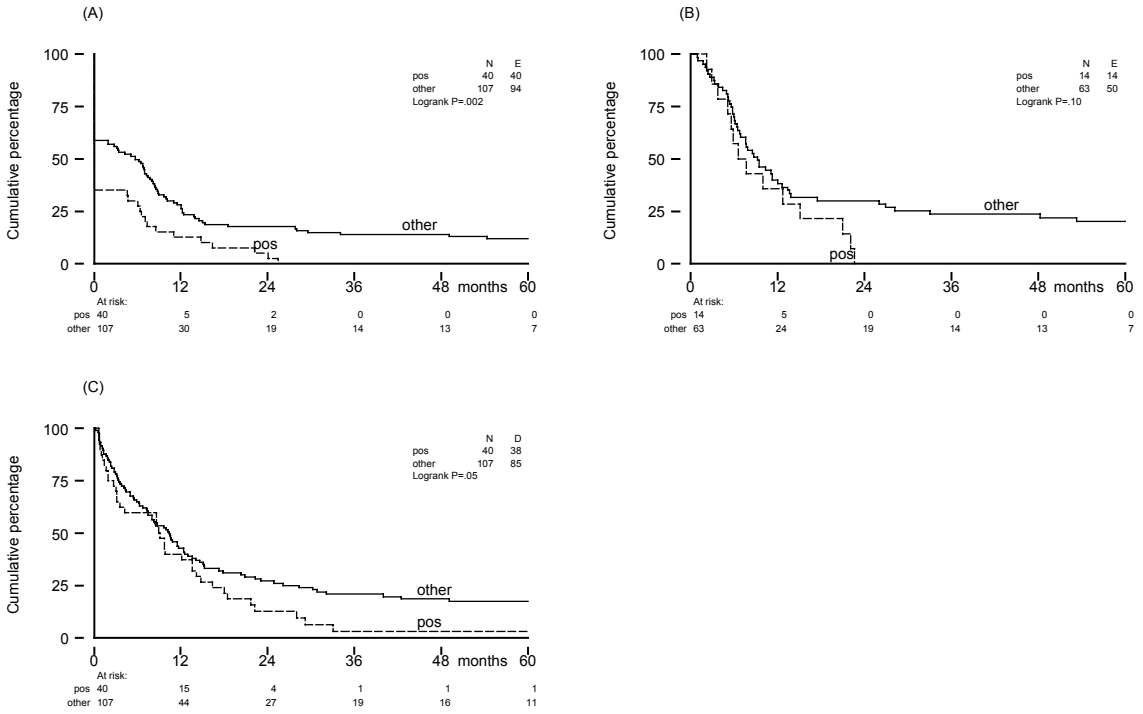

Figure 2. Survival of elderly AML patients with and without co-expression of *MDR1* and *BCRP* mRNA.
(A) Event-free survival (B) Disease-free survival (C) Overall survival.
Pos indicates patients with co-expression of *MDR1* and *BCRP*; and other, patients without co-expression

Discussion

This is the first comprehensive analysis of the effect of the major classical MDR genes in a cohort of elderly patients with AML homogeneously treated in a prospective clinical trial.[14] A wide range of expression of the various resistance genes was observed, consistent with previous studies and with comparable median values.[9-11,23] Our results show that *MRP1*, *LRP*, and *BCRP* are not associated with CR rate or survival endpoints in patients with AML aged 60 years or older, indicating that the clinical relevance of the expression of these genes is limited in this patient population. This study confirms previous reports which showed the unique prognostic role of MDR1 expression – which was however highly associated with CD34 expression – in drug resistance in elderly AML (Table 3), in contrast to the prognostic value of MRP1 expression in AML which has shown conflicting results, whereas currently, LRP is no longer thought to be important for clinical drug resistance.[4,5,7,24-27] Recently, 2 studies in respectively 40 and 31 adult AML patients showed no effect of *BCRP* gene expression on CR rate, while OS was lower in patients with the highest *BCRP* expression.[10,23]

Damiani *et al* showed, that *BCRP* expression did not influence achievement of complete remission in AML patients with a median age of 53 years and normal karyotype,[28] however *BCRP* expression was associated with higher relapse rate. In 59 children with *de novo* AML, a higher *BCRP* expression was observed in patients who did not reach CR but this was not translated in poorer survival.[29] Benderra *et al* indicated that *BCRP* gene expression was an adverse prognostic factor for CR in a group of 149 relatively younger adult AML patients, but only in patients treated with DNR and MXT and not with idarubicin.[19] In our cohort of elderly AML patients who were all treated with DNR, whereas MXT was given as consolidation therapy after reaching CR, a significant correlation of *BCRP* mRNA expression with lower CR rate could not be shown.

Our study confirms that *BCRP* and *MDR1* are co-expressed in AML patients with higher age as has been suggested previously from studies in smaller groups of relatively younger AML patients.[9-11,28] Until now, only two study have evaluated the clinical value of co-expression of *MDR1* and *BCRP* in a sufficient number, though relatively younger adult AML patients.[19,28] Benderra *et al* showed that CR rate was only 45% in the patients with co-expression of *BCRP* and *MDR1* (+/+) in comparison with 66% in the *MDR1*/*BCRP* -/+ and +/- group, and 90% in the *MDR1*/*BCRP* -/- group (P = .003).[19] Moreover, a significantly lower DFS and OS were found in the *MDR1*/*BCRP* +/+ group. Damiani *et al* found a trend towards a higher relapse rate in the small group of $BCRP^{+}/MDR1^{+}$ patients, indicating that this represents a robust resistant AML phenotype, consistent with our findings in elderly AML.[28] The recent finding that *BCRP* and *MDR1* expression was mainly found in the most resistant group of AML, using gene expression profiling, underscores the role of these drug resistance genes in AML.[30] However, this study shows, that the prominent prognostic role of CD34 expression in elderly AML should be emphasized, as higher CD34 expression was adversely associated with all clinical endpoints. *MDR1* and *BCRP*, but not *MRP1* and *LRP* gene expression were found to be associated with high CD34 expression in these elderly AML patients, which may explain why *MDR1* was no longer significant for CR rate, EFS and OS when adjusted for other prognostic variables including CD34. In the past MDR1 expression has been linked to the CD34-positive hematopoietic stem cell compartment of the leukemia subtype. In 2 other studies in younger AML patients no overexpression (on mRNA and protein level) of *BCRP* in the CD34-positive blast population of clinical AML samples was found.[13,19] In contrast, earlier studies in mice demonstrated high levels of *BCRP* and *MDR1* expression in normal hematopoietic stem cells.[31-34] Previously, *BCRP*-expression in subsets of stem cells has been reported, indicating that high *BCRP* expression may exist in $CD34^{+}/CD38^{-}$ cells or in $CD34^{+}/CD33^{-}$ cells.[12,35] The differential expression of *BCRP* and *MDR1* in specific subsets of hematopoietic stem cells is consistent with the side population phenotype as proposed by

Goodell *et al* who claim that BCRP expression can be separated from those expressing the other ABC proteins.[36] This would suggest that BCRP is expressed in even less differentiated hematopoietic stem cells than MDR1.[19] In our study in AML these immature subsets could not be separately investigated, however the unique *BCRP*/*MDR1* +/+ subgroup of patients reflects an immature leukemic cell type which has a very resistant phenotype *in vivo*, illustrated by a low CR rate and poor outcome (Table 3; Figure 2).

This is the first study in which a correlation was found between secondary AML and a high expression of *BCRP* mRNA, but not the other resistance proteins. In addition to our previous report that *BCRP* is frequently upregulated in patients with AML at relapse, we now demonstrate that expression of *BCRP* is representative of secondary AML which is especially observed in elderly patients.[11,29] Recently, Ross *et al* suggested that multidrug resistance modifiers may be of benefit for patients with multiple dysplastic features.[37] This may suggest that *BCRP* is upregulated in diseases in which exposure to xenobiotics during life plays an etiologic role.

We conclude that co-expression of CD34 related co-expression of *MDR1* and *BCRP* reflects a clinically resistant subgroup of elderly AML. In this age group only *BCRP* is correlated with secondary AML. As such, the development of new treatment strategies for elderly AML patients, may focus on modulation of drug resistance targeting both *BCRP* and *MDR1*.

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CHAPTER 5

ABCB1 gene polymorphisms are not associated with treatment outcome in elderly acute myeloid leukemia patients

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Abstract

Objectives: The classical multidrug resistance (MDR) gene *MDR1* (*ABCB1*) encodes for the drug efflux pump P-glycoprotein (P-gp). P-gp expression is an adverse prognostic factor for treatment outcome in acute myeloid leukemia (AML) and is more frequently observed in older patients. Single-nucleotide polymorphisms of the *ABCB1* gene, *C1236T*, *G2677T*, and *C3435T*, have been associated with altered drug metabolism and treatment outcome. We prospectively determined these single-nucleotide polymorphisms in AML blasts in a cohort of patients aged 60 years or older with AML and evaluated their relevance with regard to P-gp function and expression, *ABCB1* messenger ribonucleic acid (mRNA) expression, and clinical outcome.

Methods: We have analyzed purified bone marrow-derived leukemic blasts, obtained at diagnosis, in 150 patients who were treated within a multicenter, randomized, phase 3 trial of elderly patients with AML. The significance of the allelic *ABCB1* variants of *C1236T*, *G2677T*, and *C3435T* was evaluated with respect to P-gp expression and function in leukemic blasts and *ABCB1* mRNA expression levels, and these values were correlated with treatment outcome.

Results: P-gp function and expression in leukemic blasts and *ABCB1* mRNA levels in patients with AML did not vary significantly among any of the allelic variants of *ABCB1*. None of these allelic variations predicted a difference in complete response rate and survival endpoints.

Conclusions: In AML patients aged 60 years or older, allelic *ABCB1* variations of *C1236T*, *G2677T*, or *C3435T* are not associated with altered P-gp function or with *MDR1* expression at the transcriptional or translational level in leukemic blasts, and they do not significantly affect clinical prognosis.

Introduction

One of the major reasons for treatment failure in patients with acute myeloid leukemia (AML) is clinical resistance to chemotherapy, which is associated with the expression of multidrug resistance (MDR) proteins.[1] Among those, members of the family of adenosine triphosphate-binding cassette (ABC) transporter proteins play a role as drug efflux pumps in the plasma membrane.[1,2] The classical MDR phenotype results from expression of the *MDR1* gene and its 170-kd protein product, P-glycoprotein (P-gp),[3] also designated as ABC protein transporter, subfamily B, member 1 (*ABCB1*).[2] The *ABCB1* gene is expressed in the human adrenal gland, kidney, lung, liver, jejunum, colon, rectum, pancreas, and small intestine[4,5]; in human tumors derived from these organs[5]; and by endothelial cells of capillary blood vessels at the blood-brain barrier and other blood-tissue barrier sites.[6] It is localized on chromosome 7q21.1[7] and consists of 28 exons.[8]

P-gp acts as an energy-dependent drug efflux pump for chemotherapeutic drugs such as the anthracyclines and epipodophyllotoxins, commonly used in AML therapy.[9] P-gp also limits oral absorption and brain entry of human immunodeficiency virus 1 protease inhibitors, which are substrates of P-gp.[10] In AML patients increased P-gp expression and enhanced drug efflux have been reported with increasing age[11,12] and have been established as independent adverse prognostic factors for treatment outcome.[11,13-18] Mickley *et al*[19] have reported the first evidence of the presence of polymorphisms in the human *ABCB1* gene. Polymorphisms in the *ABCB1* gene have been shown to affect the pharmacokinetics of many substrate drugs, including anticancer drugs.[8,20,21] Large-scale sequencing efforts revealed the presence of at least 50 single-nucleotide polymorphisms (SNPs) in the *ABCB1* gene.[8] Population frequencies of these genetic variants vary according to racial background.[22] As determined by Cascorbi *et al*,[23] the 3 most frequent *ABCB1* gene SNPs in the Caucasian population are the synonymous SNPs *C1236T* in exon 12 and *C3435T* in exon 26, as well as the nonsynonymous SNP *G2677T* in exon 21, which results in an amino acid substitution Ser→Ala at codon 893.[24] These 3 SNPs were found to be linked and occurred in 62% of European Americans and in 13% of African Americans.[25] *G2677T* and *C3435T* were associated with altered P-gp expression and function in duodenal tissue.[26] In healthy volunteers and human immunodeficiency virus patients, allelic variants of the SNPs *G2677T* and *C1236T* have been shown to be associated with altered drug metabolism.[25,27-29] To date, clinical outcome studies in patients with known P-gp expression and function in leukemic blasts in correlation with SNPs are not available in older AML patients.

In this study, which was undertaken in a cohort of AML patients aged 60 years or older, we investigated whether *C1236T*, *G2677T*, and *C3435T* variants of the *ABCB1* gene in AML

correlate with P-gp function and expression in leukemic blasts and with *ABCB1* transcript levels. All patients were treated in a multicenter, randomized, phase 3 trial comparing standard induction treatment with or without the P-gp inhibitor PSC-833.[30] The relationship between each of these genetic polymorphisms of *ABCB1* with clinical outcome–that is, complete response rate, event-free survival (EFS), disease-free survival (DFS), and overall survival (OS)–was also assessed.

Methods

Patients

A group of 150 AML patients aged 60 years or older were included in this study. All were enrolled between May 1997 and February 1999 in an international, multicenter, randomized, phase 3 trial, conducted by the Dutch-Belgian Hemato-Oncology Cooperative Group and the United Kingdom Medical Research Council. The clinical results of that trial have been published separately.[30] In that trial 419 eligible white patients aged 60 years or older with previously untreated de novo and secondary AML (M0-M2 and M4-M7 according to the French-American-British classification[31]) were randomized to receive induction chemotherapy with or without the P-gp inhibitor PSC-833 (Amdray [valspodar]; Novartis Pharma, Basel, Switzerland). Induction chemotherapy consisted of either 2 cycles of daunorubicin at a dose of 45 mg/m^2 via a 15-minute infusion on days 1 through 3 and cytarabine at a dose of 200 mg/m^2 per 24 hours via continuous infusion on days 1 through 7 (arm A) or a similar regimen but with a lower dose of daunorubicin (35 mg/m^2) and with PSC-833, at a loading dose of 2 mg/kg over a period of 2 hours, followed by continuous intravenous infusion at a dose of 10 mg/kg per 24 hours for 72 hours (days 1-3) (arm B). Cycle 2 was given to all patients who achieved normocellular marrow with fewer than 5% blasts, no Auer rods, and no evidence of extramedullary involvement with full peripheral blood recovery (absolute neutrophil count $>1.0 \times 10^9$/L and platelet count $>80 \times 10^9$/L) within 60 days of the start of induction cycle 1. In patients with evidence of persistent AML the second cycle was administered independent of peripheral blood recovery. Patients in both arms who were in complete remission received 1 consolidation cycle without PSC-833, consisting of cytarabine, at 1 g/m^2 over a period of 6 hours via infusion on days 1 through 4; mitoxantrone, at 6 mg/m^2 as a slow intravenous bolus on days 1 through 4; and etoposide, at 80 mg/m^2 via a 1-hour infusion on days 1 through 4.

Bone marrow aspirates had been collected at diagnosis for the analysis of P-gp function and expression, as described previously.[30] Selection of patients for our study was based on availability of sufficient purified bone marrow AML blast samples in our tissue bank, which was the case for 150 patients. The study was approved by the ethics committees of the participating institutions and was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent before randomization.

Bone marrow cell preparation

Bone marrow aspirates were obtained in heparinized tubes. Mononuclear bone marrow cells were collected by Ficoll-Hypaque density gradient centrifugation (density, 1.077 g/m^3) (Pharmacia, Uppsala, Sweden). To obtain purified samples with greater than 85% blasts, adherence depletion was performed as described previously.[32] Cells were cryopreserved in Dulbecco's modified Eagle's medium (Invitrogen [Gibco], Paisley, Scotland) supplemented with 10% dimethylsulfoxide (Merck, Darmstadt, Germany) and 20% fetal calf serum (Invitrogen [Gibco]) and stored in liquid nitrogen. On the day of the experiments, bone marrow cells were thawed. For flow cytometry experiments, cells were washed and resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and gentamicin at a concentration of 4×10^6 cells/mL. Before ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) isolation, cells were washed with phosphate-buffered saline solution (Invitrogen [Gibco]).

P-gp function and expression

Measurement of the function and expression of P-gp in leukemic blast cells, as well as in $CD34^+$ cells, has been described previously.[30] In brief, functional P-gp expression was analyzed by use of a rhodamine 123 retention essay (Sigma Chemical, St Louis, MO), with PSC-833. The results are given as the ratio of the mean intracellular rhodamine 123 fluorescence of cells exposed to PSC-833 to the mean intracellular rhodamine 123 fluorescence of cells not exposed to PSC-833. P-gp expression was studied by use of the monoclonal antibodies UIC2[33] (Immunotech, Marseille, France) and MRK 16[34] (Kamiya Biomedical, Seattle, Wash). Results are given as the ratio of the mean cell-associated fluorescence of cells incubated with the anti-P-gp antibody to the mean cell-associated fluorescence of cells incubated with the control mouse immunoglobulin G2a antibody (Sigma Chemical).

ABCB1 messenger ribonucleic acid expression

Isolation of RNA. Total RNA was isolated by use of the TRISOLV extraction (Biotecx Laboratories, Houston, Tex) as described by the manufacturer. RNA was aliquoted and stored at –80°C. RNA samples were analyzed for RNA integrity by gel electrophoresis.

Complementary DNA synthesis. Complementary DNA was synthesized by use of TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, Calif). Complementary DNA was diluted, aliquoted, and stored at –80°C.

ABCB1 messenger ribonucleic acid levels. *ABCB1* messenger ribonucleic acid (mRNA) levels were measured by quantitative real-time polymerase chain reaction (PCR) based on TaqMan chemistry on an ABI Prism 7700 sequence detector (Applied Biosystems), as previously described.[35]

Genotyping

Genomic DNA was extracted from AML blasts on a MagNA Pure LC system (Roche, Mannheim, Germany) by use of the Total Nucleic Acid Extraction Kit (Roche). PCR-restriction fragment length polymorphism was performed by use of 5 ng of DNA in a PCR volume of 25 μL containing 1× buffer (10-mmol/L Tris-hydrochloric acid, pH 8.3; 1.5-mmol/L magnesium chloride; 50-mmol/L potassium chloride; and 0.001% [wt/vol] gelatin) (PerkinElmer, Wellesley, Mass), 0.2 mol/L each of the deoxynucleotide triphosphates (Roche), 1.25 U of Amplitaq Gold (PerkinElmer), and 40 pmol of each forward and reverse primer. For the *C3435T* polymorphism, forward primer 5'-CAT GCT CCC AGG CTG TTT AT-3' and reverse primer 5'-GTA ACT TGG CAG TTT CAG TG-3' were used. The PCR conditions were 7 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, with a final incubation of 7 minutes at 72°C. The 342-base pair (bp) PCR product was digested with *Dpn*II (New England Biolabs, Ipswich, Mass), resulting in 172-, 92-, and 78-bp fragments for the *3435C* allele and 264- and 78-bp fragments for the *3435T* allele. The PCR-restriction fragment length polymorphism assay for *C3435T* has been validated for wild-type, heterozygous, and homozygous mutant variants by direct sequencing. Analysis of 479 healthy Caucasian subjects by use of the described assay resulted in *CC*, *CT*, and *TT* genotypes that were in Hardy-Weinberg equilibrium ($P = .90$).[36]

For the *G2677T* polymorphism, forward primer 5'-TAG TTT GAC TCA CCT TCC CGG-3' and reverse primer 5'-GGC TAT AGG TTC CAG GCT TG-3' were used (the underlined nucleotide is a mismatch with the *ABCB1* sequence, creating a restriction site in the PCR product, which serves as an internal control for digestion efficiency). The PCR conditions

were 7 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 57°C, and 1 minute at 72°C, with a final incubation of 7 minutes at 72°C. The 218-bp PCR product was digested with *Ban*I (New England Biolabs), resulting in 194- and 24-bp fragments for the *2677G* allele and 218 bp for the *2677T* allele. For the *C1236T* polymorphism, forward primer 5'-CCT GAC TCA CCA CAC CAA TG-3' and reverse primer 5'-TAT CCT GTG TCT GTG AAT TGC C-3' were used. The PCR conditions were 7 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C, and 1 minute at 72°C, with a final incubation of 7 minutes at 72°C. The 370-bp PCR product was digested with *Hae*III (New England Biolabs), resulting in 272-, 63-, and 35-bp for the *1236C* alleles and 272- and 98-bp for the *1236T* allele. PCR digestion products were subsequently analyzed on agarose/Tris-borate-ethylenediaminetetraacetic acid gels with ethidium bromide staining.

Haplotype analysis

Additional haplotype analysis was performed with SNPs *G2677T* and *C3435T*, as described by Johne *et al*.[37] In brief, the 4 possible haplotypes were coded 11, 12, 21, and 22, where the first digit refers to *G2677T* and the second digit refers to *C3435T*. Haplotype coding was as follows: 1, identical to the reference sequence (variant *2677G* in exon 21 or variant *3435C* in exon 26); and 2, different from the reference sequence (variant *2677T* in exon 21 or variant *3435T* in exon 26). For each of the 4 *ABCB1* haplotypes deduced from these 2 SNPs, patients were classified as carriers or noncarriers.

Cytogenetic analysis

Cytogenetic analysis of bone marrow samples obtained at diagnosis was performed by standard cytogenetic techniques, as described previously.[30] Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (*ISCN 1995*).[38]

Definition of endpoints

CR was defined as normocellular bone marrow with fewer than 5% blasts, no Auer rods, and no evidence of extramedullary involvement. Data on peripheral blood recovery within 60 days after the start of a cycle were not always available, and thus peripheral blood recovery was not considered as a criterion for CR. Patients in whom AML relapsed or who died within 28 days after CR were considered as not having achieved a CR. EFS was determined from the

date of randomization until no CR with induction therapy had occurred, relapse after CR had occurred, or death had occurred, whichever came first. Patients who did not reach CR were considered failures for EFS at 1 day after randomization. DFS was determined for all patients who achieved CR and was calculated from the date of CR until relapse or death, whichever came first. OS was measured from randomization until death from any cause. Patients who were still alive at the date of last contact were censored.

Statistical analysis

The original phase 3 trial was designed to detect an increase in the 2-year EFS rate from 9.5% in the control arm (without PSC-833) to 18% in the PSC-833 arm (2-sided significance level $\alpha = .05$) with a power of 80% and included 419 eligible patients. SNPs *C1236T*, *G2677T*, and *C3435T* were determined in 150 patients with available bone marrow samples in our tissue bank. Baseline parameters of interest were functional and expression levels of P-gp and *ABCB1* mRNA levels. Clinical endpoints were CR rate, EFS, DFS, and OS.

Baseline characteristics were compared between patients with or without SNP data available by use of the Fisher exact test or the Pearson χ^2 test in the case of discrete variables, whichever was appropriate, or the Wilcoxon rank sum test in the case of continuous variables. The Hardy-Weinberg equilibrium test was performed for each of the SNPs,[39] and the linkage equilibrium between each combination of 2 SNPs was evaluated by use of the Fisher exact test. Expression and functional levels of P-gp, *ABCB1* mRNA relative expression levels, and clinical outcome were compared between the 3 allelic variants of each SNP, as well as between haplotype carriers and noncarriers. Baseline characteristics were compared between 2 groups by use of the Wilcoxon rank sum test, whereas the Kruskal-Wallis test was used for 3 groups. CR rates were calculated along with 95% confidence intervals (CIs). Odds ratios, with corresponding 95% CIs, were also determined. CR rates were compared between subgroups by use of logistic regression.[40] Survival probabilities were estimated by use of the actuarial method of Kaplan and Meier,[41] along with 95% CI. Hazard ratios, with corresponding 95% CIs, were determined. Survival endpoints were compared between subgroups by use of Cox regression analysis.[42] Logistic regression and Cox regression analyses were performed unadjusted, as well as adjusted for treatment arm, because about half of the patients had been randomized to receive PSC-833 in addition to chemotherapy. These methods were also used to investigate possible interaction between allelic variants or haplotype subgroups and treatment arm with respect to CR rate and survival endpoints. Kaplan-Meier curves were constructed to illustrate survival[41] and compared by use of the log-

rank test.[43] For illustrative purposes, we present 95% CIs. However, to adjust for multiple testing, only $P \leq .01$ was considered statistically significant, while $P \leq .05$ denoted a trend. All reported *P* values are 2-sided. All statistical analyses were performed with Stata statistical software, release 9 (StataCorp, College Station, Tex).

Results

Patients

The baseline characteristics, such as sex, age, secondary leukemia, and unfavorable cytogenetic risk, of the 150 patients with genotype data available are shown in Table 1. On the basis of the French-American-British classification, 6 patients were M0 (4%), 37 were M1 (25%), 59 were M2 (39%), 28 were M4 (19%), 12 were M5 (8%), 5 were M6 (3%), and 3 were unclassified (2%). These variables were not significantly different in comparison to those of the remaining 269 patients without genotype data who had entered into the clinical trial, with the exception of the white blood cell count, which was higher in the genotyped patients (median, 18.8×10^9/L [range, 0.8-389 $\times 10^9$/L] versus 5.7×10^9/L [range, 0.1-300 $\times 10^9$/L]; $P = .001$). In addition, there was no significant difference in CD34 expression between the genotyped and nongenotyped patients (median, 33.0% [range, 0.1%-97.9%] versus 29.2% [range, 0.1%-93.7%]; $P = .16$).

The CR rate and survival endpoints were also similar in the genotyped and nongenotyped patients. However, genotyped patients in the PSC-833 arm had a higher CR rate (61% versus 40% in the control arm, $P = .01$), contrary to the results in the remaining 269 patients (49% in the PSC-833 arm versus 52%, $P = .68$).

SNPs C1236T, G2677T, and C3435T

The distribution of the allelic variants of the 3 SNPs, *C1236T*, *G2677T*, and *C3435T*, is shown in Table 2. Each of the 3 SNPs was in Hardy-Weinberg disequilibrium ($P < .001$). For example, of the 142 patients with a known allelic variant of *G2677T*, 58 (41%) presented with the *GG* variant, 46 (32%) had the *GT* variant, and 38 (27%) had the homozygous mutant variant (*TT*). If the population were in Hardy-Weinberg equilibrium, 46 patients with *GG*, 70 with *GT*, and 26 with *TT* would have been expected. Each combination of 2 SNPs was in linkage disequilibrium ($P < .001$) (Table 2), which confirms the results of other authors, such as Kim *et al*[25] and Illmer *et al*.[44] The baseline characteristics of the patients were not significantly different between the allelic variants for each polymorphism (Table 1).

Table 1. Patient characteristics of 150 genotyped elderly AML patients

	No. (%)	Sex (%)		Age (y) [median (range)]	Treatment arm [No. (%)]	
		Male	Female		Control	PSC-833
All patients	150	57	43	67 (60-85)	68 (45)	82 (55)
C1236T (n = 115)						
CC	48 (42)	50	50	67 (60-85)	21 (44)	27 (56)
CT	35 (30)	63	37	67 (60-85)	17 (49)	18 (51)
TT	32 (28)	53	47	69 (61-83)	14 (44)	18 (56)
G2677T (n = 142)						
GG	58 (41)	59	41	67 (60-79)	24 (41)	34 (59)
GT	46 (32)	65	35	68 (60-85)	22 (48)	24 (52)
TT	38 (27)	47	53	67 (61-83)	19 (50)	19 (50)
C3435T (n = 130)						
CC	36 (28)	53	47	66 (60-74)	18 (50)	18 (50)
CT	44 (34)	61	39	69 (60-85)	18 (41)	26 (59)
TT	50 (38)	52	48	67 (61-85)	20 (40)	30 (60)
Haplotype 11 (n = 131)						
Noncarrier	58 (44)	53	47	67 (61-85)	28 (48)	30 (52)
Carrier	73 (56)	59	41	67 (60-85)	31 (42)	42 (58)
Haplotype 12 (n = 126)						
Noncarrier	91 (72)	52	48	67 (60-85)	41 (45)	50 (55)
Carrier	35 (28)	66	34	67 (60-85)	15 (43)	20 (57)
Haplotype 22 (n = 135)						
Noncarrier	68 (50)	57	43	67 (60-79)	31 (46)	37 (54)
Carrier	67 (50)	57	43	68 (61-85)†	30 (45)	37 (55)

AML, Acute myeloid leukemia; WBC, white blood cell; n, number of patients with data available for a specific single-nucleotide polymorphism or haplotype;

Haplotype 11, *2677G*/*3435C*; Haplotype 12, *2677G*/*3435T*; Haplotype 22, *2677T*/*3435T*.

†$P < .01$.

Table 1. Patient characteristics of 150 genotyped elderly AML patients, continued

	No. (%)	Secondary AML (%)	Cytogenetic data [No. (%)]	Unfavorable cytogenetic risk (%) *	WBC count ($\times 10^9$/L) [median (range)]
All patients	150	21	104 (69)	23	18.8 (0.8-389)
C1236T (n = 115)					
CC	48 (42)	19	38 (79)	29	19.1 (1.0-389)
CT	35 (30)	29	22 (63)	27	27.5 (0.8-250)
TT	32 (28)	6	22 (69)	14	11.2 (0.8-298)
G2677T (n = 142)					
GG	58 (41)	21	39 (67)	28	19.1 (1.0-389)
GT	46 (32)	26	32 (70)	25	25.4 (0.8-300)
TT	38 (27)	13	26 (68)	15	13.2 (0.8-298)
C3435T (n = 130)					
CC	36 (28)	17	28 (78)	25	23.7 (1.0-389)
CT	44 (34)	25	29 (66)	17	25.1 (0.8-300)
TT	50 (38)	16	35 (70)	23	9.8 (0.8-298)
Haplotype 11 (n = 131)					
Noncarrier	58 (44)	17	41 (71)	24	11.3 (0.8-298)
Carrier	73 (56)	22	50 (68)	22	23.5 (0.8-389)
Haplotype 12 (n = 126)					
Noncarrier	91 (72)	16	64 (70)	19	21.1 (0.8-389)
Carrier	35 (28)	26	25 (71)	28	12.5 (0.9-226)
Haplotype 22 (n = 135)					
Noncarrier	68 (50)	21	48 (71)	25	20.1 (1.0-389)
Carrier	67 (50)	19	45 (67)	18	12.4 (0.8-300)

*Restricted to patients with cytogenetic data available. Unfavorable cytogenetic risk was defined by the presence of monosomies or deletions of chromosomes 5 or 7; abnormalities of the long arm of chromosome 3; t(6;9); abnormalities involving the long arm of chromosome 11 (11q23); or complex cytogenetic abnormalities (defined as ≥ 3 unrelated cytogenetic abnormalities in 1 clone).[30]

Haplotypes

Of the 135 patients who could be assigned to haplotype 21 (*2677T*/*3435C*), only 8 (6%) were classified as carriers. Therefore haplotype 21 has been excluded from further analyses. The distribution of carriers and noncarriers according to the *ABCB1* haplotypes 11 (*2677G*/*3435C*), 12 (*2677G*/*3435T*), and 22 (*2677T*/*3435T*) is presented in Table 2. The baseline characteristics were in general not significantly different between carriers and noncarriers of each haplotype, except that haplotype 22 carriers were slightly older than noncarriers ($P < .001$).

P-gp function and expression and ABCB1 mRNA expression

P-gp efflux and expression data in purified AML blasts and in the $CD34^+$ subpopulation and the *ABCB1* mRNA expression levels in all 150 genotyped patients and in relation to each of the allelic variants of *ABCB1* are shown in Table 3. There were no statistically significant differences for any of these parameters between patients with the wild-type, heterozygous, or homozygous mutant variants for any of the 3 genetic polymorphisms or between carriers and noncarriers of each haplotype. In our 150 genotyped patients all P-gp functional and expression data and *ABCB1* mRNA expression levels were highly correlated ($P < .001$). For example, the Spearman rank correlation coefficient was 0.65 between the efflux ratio and UIC2 expression ratio, 0.83 between the MRK 16 and UIC2 expression ratio, and 0.60 between the *ABCB1* mRNA expression level and UIC2 expression ratio.

In the original phase 3 trial P-gp function or expression (or both) was available for 309 patients.[30] Therefore we also compared the PSC/rhodamine 123 ratio, MRK 16 ratio, and UIC2 ratio measured in blasts, as well as in the $CD34^+$ subpopulation, between the 150 genotyped patients (Table 3) and the approximately 160 nongenotyped patients in whom P-gp function or protein (or both) also had been measured. We did not observe a significant difference in any of these 6 P-gp-associated variables between both patient groups ($P = .22$-$.90$).

Table 2. Distribution of allelic variants of *C1236T*, *G2677T*, and *C3435T*

		G2677T				*C3435T*			
	No.	*GG*	*GT*	*TT*	N/A	*CC*	*CT*	*TT*	N/A
All patients	150	58	46	38	8	36	44	50	20
C1236T									
CC	48	36 (92)*	7 (16)	2 (7)	3	23 (85)*	17 (40)	7 (17)	1
CT	35	3 (8)	28 (65)*	2 (7)	2	4 (15)	21 (50)*	9 (21)	1
TT	32	–	8 (19)	24 (86)*	–	–	4 (10)	26 (62)*	2
N/A	35	19	3	10	3	9	2	8	16
G2677T									
GG	58					26 (79)*	15 (35)	8 (17)	9
GT	46					5 (15)	27 (63)*	12 (26)	2
TT	38					2 (6)	1 (2)	27 (57)*	8
N/A	8					3	1	3	1

The data show the number of patients with a specific combination of 2 nucleotides. The numbers in parentheses denote column percentages, based on patients with data on both single-nucleotide polymorphisms (SNPs) available. Each of the 3 SNPs is in Hardy-Weinberg disequilibrium ($P < .001$). Each combination of 2 SNPs is also in linkage disequilibrium ($P < .001$), as illustrated by the large number of patients with the same variant for each combination of 2 SNPs.

N/A, Not available.

*Patients with same variant for each combination of 2 SNPs.

Table 3. P-glycoprotein function and expression and *ABCB1* mRNA expression

	No. (%)	PSC/Rho123 ratio in blasts [median (range)]	MRK 16 ratio in blasts [median (range)]	UIC2 ratio in blasts [median (range)]	$CD34^+$ (%) [median (range)]
All patients	150	1.17 (0.83-5.43)	1.93 (0.87-8.38)	2.16 (0.85-18.8)	32.9 (0.1-97.9)
C1236T (n = 115)					
CC	48 (42)	1.18 (0.93-2.04)	1.98 (1.16-7.77)	2.08 (1.33-7.22)	40.0 (0.1-97.7)
CT	35 (30)	1.16 (0.90-2.79)	1.90 (1.40-4.67)	2.28 (0.90-7.41)	45.4 (0.1-88.4)
TT	32 (28)	1.22 (0.89-5.43)	2.01 (1.01-8.38)	2.26 (1.44-18.8)	30.5 (0.2-97.9)
G2677T (n = 142)					
GG	58 (41)	1.17 (0.90-2.04)	1.94 (0.87-7.77)	2.08 (0.85-8.14)	34.0 (0.1-97.7)
GT	46 (32)	1.16 (0.93-2.79)	1.88 (1.28-7.26)	2.15 (0.90-7.41)	37.2 (0.1-88.4)
TT	38 (27)	1.21 (0.83-5.43)	2.04 (1.01-8.38)	2.36 (1.22-18.8)	34.9 (0.2-97.9)
C3435T (n = 130)					
CC	36 (28)	1.15 (0.94-2.04)	1.82 (1.21-7.77)	2.11 (1.38-7.22)	35.0 (0.1-97.7)
CT	44 (34)	1.19 (0.93-2.79)	1.89 (1.28-7.26)	2.27 (0.90-7.41)	38.5 (0.1-92.1)
TT	50 (38)	1.19 (0.89-5.43)	1.98 (0.87-8.38)	2.23 (0.85-18.8)	32.7 (0.2-97.9)
Haplotype 11 (n = 131)					
Noncarrier	58 (44)	1.15 (0.83-5.43)	1.99 (0.87-8.38)	2.25 (0.85-18.8)	32.9 (0.2-97.9)
Carrier	73 (56)	1.18 (0.93-2.79)	1.92 (1.21-7.77)	2.20 (0.90-7.41)	41.0 (0.1-97.7)
Haplotype 12 (n = 126)					
Noncarrier	91 (72)	1.18 (0.89-5.43)	1.95 (1.01-8.38)	2.29 (0.90-18.8)	35.1 (0.1-97.9)
Carrier	35 (28)	1.16 (0.90-1.94)	1.89 (0.87-5.36)	1.94 (0.85-8.14)	37.2 (0.1-92.1)
Haplotype 22 (n = 135)					
Noncarrier	68 (50)	1.17 (0.90-2.04)	1.91 (0.87-7.77)	2.06 (0.85-8.14)	33.0 (0.1-97.7)
Carrier	67 (50)	1.20 (0.89-5.43)	2.01 (1.01-8.38)	2.32 (0.90-18.8)	35.1 (0.1-97.9)

mRNA, Messenger ribonucleic acid; Rho123, rhodamine 123.

Table 3. P-glycoprotein function and expression and *ABCB1* mRNA expression, continued

	PSC/Rho123 ratio in $CD34^+$ [median (range)]	MRK 16 ratio in $CD34^+$ [median (range)]	UIC2 ratio in $CD34^+$ [median (range)]	*ABCB1* mRNA relative expression [median (range)]
All patients	1.40 (0.77-13.2)	2.41 (0.58-17.5)	2.68 (0.44-25.6)	1.52 (0.00-74.5)
C1236T (n = 115)				
CC	1.52 (0.93-7.93)	2.58 (0.98-12.5)	2.58 (1.14-14.8)	1.95 (0.04-43.3)
CT	1.36 (1.00-7.21)	2.42 (0.91-10.1)	2.88 (0.86-13.4)	2.38 (0.08-25.8)
TT	1.39 (0.90-13.2)	2.32 (0.80-13.3)	2.43 (0.81-25.6)	1.48 (0.11-74.5)
G2677T (n = 142)				
GG	1.49 (0.96-7.93)	2.85 (1.02-12.6)	3.15 (1.14-16.8)	1.57 (0.04-43.3)
GT	1.36 (0.90-7.21)	2.19 (0.80-11.2)	2.58 (0.81-13.4)	2.14 (0.09-25.8)
TT	1.48 (0.77-13.2)	2.56 (1.06-17.5)	2.91 (1.02-25.6)	1.14 (0.00-74.5)
C3435T (n = 130)				
CC	1.45 (0.96-7.93)	2.32 (0.86-9.65)	2.58 (0.93-11.0)	1.42 (0.08-17.6)
CT	1.45 (0.90-7.21)	2.49 (0.80-12.5)	2.52 (0.81-14.8)	2.15 (0.08-43.3)
TT	1.39 (0.96-13.2)	2.37 (0.58-13.3)	2.77 (0.44-25.6)	1.48 (0.09-74.5)
Haplotype 11 (n = 131)				
Noncarrier	1.39 (0.77-13.2)	2.30 (1.02-17.5)	2.88 (1.02-25.6)	0.79 (0.00-74.5)
Carrier	1.45 (0.90-7.93)	2.50 (0.80-12.5)	2.73 (0.81-14.8)	2.28 (0.15-43.3)*
Haplotype 12 (n = 126)				
Noncarrier	1.50 (0.90-13.2)	2.36 (0.80-13.3)	2.73 (0.81-25.6)	2.33 (0.08-74.5)
Carrier	1.42 (1.00-4.07)	2.49 (1.02-12.6)	2.84 (1.20-16.8)	1.75 (0.09-43.3)
Haplotype 22 (n = 135)				
Noncarrier	1.49 (0.96-7.93)	2.58 (0.86-12.6)	2.84 (0.93-16.8)	1.49 (0.04-43.3)
Carrier	1.44 (0.90-13.2)	2.32 (0.80-13.3)	2.97 (0.81-25.6)	2.30 (0.08-74.5)

*$P < .05$.

Table 4. CR rate and 5-year OS by genotype

		CR rate			OS at 5 y		
	No. (%)	% (95% CI)	OR (95% CI)	*P* value	% (95% CI)	HR (95% CI)	*P* value
All patients	150	51 (43-60)			14 (9-20)		
C1236T (n = 115)							
CC	48 (42)	54 (39-69)	1	.56	9 (3-19)	1	.98
CT	35 (30)	60 (42-76)	1.27 (0.52-3.07)		16 (6-30)	1.03 (0.65-1.64)	
TT	32 (28)	47 (29-65)	0.75 (0.30-1.83)		9 (2-24)	1.04 (0.64-1.69)	
G2677T (n = 142)							
GG	58 (41)	52 (38-65)	1	.77	13 (6-24)	1	.68
GT	46 (32)	46 (31-61)	0.78 (0.36-1.70)		14 (6-27)	1.15 (0.75-1.75)	
GT	38 (27)	53 (36-69)	1.04 (0.46-2.35)		11 (3-25)	0.94 (0.60-1.46)	
C1236T (n = 130)							
CC	36 (28)	56 (38-72)	1	.65	12 (4-25)	1	.66
CT	44 (34)	57 (41-72)	1.05 (0.43-2.56)		14 (5-26)	1.05 (0.65-1.69)	
TT	50 (38)	48 (34-63)	0.74 (0.31-1.75)		15 (7-27)	0.86 (0.54-1.37)	
Haplotype 11 (n = 131)							
Noncarrier	58 (44)	48 (35-62)	1	.56	14 (6-25)	1	.35
Carries	73 (56)	53 (41-65)	1.23 (0.62-2.45)		13 (6-22)	1.20 (0.82-1.75)	
Haplotype 12 (n = 126)							
Noncarrier	91 (72)	55 (44-65)	1	.35	12 (6-20)	1	.60
Carries	35 (28)	46 (29-63)	0.69 (0.32-1.51)		17 (6-32)	0.89 (0.58-1.37)	
Haplotype 22 (n = 135)							
Noncarrier	68 (50)	53 (40-65)	1	.67	13 (6-22)	1	.73
Carries	67 (50)	49 (37-62)	0.86 (0.44-1.70)		13 (6-23)	1.07 (0.74-1.54)	

P values are for the comparison of the CR rate and OS between the different allelic variants of each genotype or between haplotype carriers versus noncarriers, unadjusted for treatment arm. The analyses with adjustment for treatment arm yielded very similar results and are therefore not shown.
CR, Complete response; OS, overall survival; OR, odds ration; HR, hazard ratio.

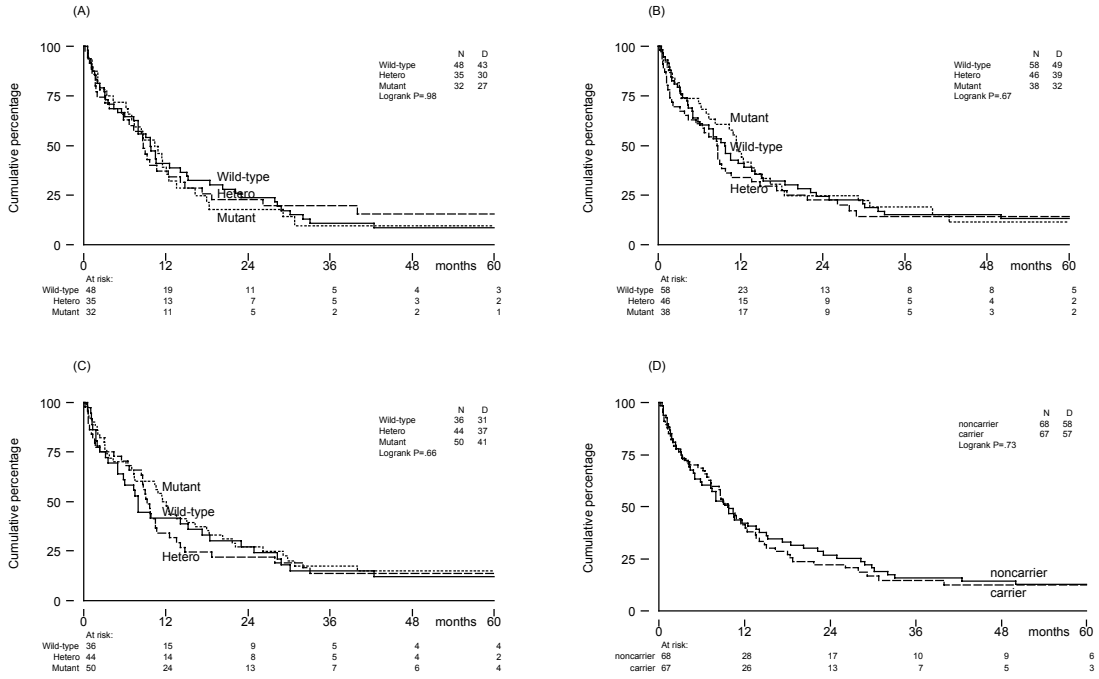

Figure 1. Overall survival in elderly acute myeloid leukemia (AML) patients by genotype or haplotype. (A) *C1236T*, (B) *G2677T*, (C) *C3435T*, and (D) haplotype 22 (*2677T*/*3435T*). Hetero, Heterozygous; N, number of patients; D, deaths.

Clinical endpoints

Survival endpoints were based on the same follow-up data as described previously.[30] The median follow-up of 24 patients who were still alive was 57 months (range, 8-81 months). Treatment results for all patients, by allelic variant of the SNPs, and by haplotype carrier versus noncarrier status, as summarized by the CR rate and OS at 5 year, are shown in Table 4. Because the analyses adjusted for treatment arm yielded similar estimates for the hazard ratios and *P* values, only the results of the unadjusted analyses have been presented.

No statistically significant differences in CR rate or OS were observed between the allelic subgroups or between the haplotype subgroups, and there was no apparent interaction between subgroups of each SNP or haplotype and treatment arm with respect to outcome, although it should be noted that the number of patients may be somewhat limited to detect such interaction. Similar analyses of EFS and DFS also did not reveal any statistically significant result and are therefore not shown. The Kaplan-Meier OS curves by allelic variant for each of the 3 SNPs, *C1236T*, *G2677T*, and *C3435T*, as well as for haplotype 22 carriers versus noncarriers, are depicted in Figure 1.

Discussion

Although some data have been published on the association of *ABCB1* SNPs with *ABCB1* mRNA expression and treatment outcome in AML patients,[44] no data are available from trials investigating allelic variants of the *ABCB1* gene in AML blasts in relation to leukemic blast P-gp function and expression, *ABCB1* mRNA expression, and treatment outcome simultaneously.

In this study in 150 previously untreated AML patients aged 60 years or older, we confirmed the linkage disequilibrium between the allelic variants of the 3 SNPs, which was also found by Kim *et al*[25] in healthy volunteers and by Illmer *et al*[44] in AML patients. A linkage disequilibrium between *G2677T* and *C3435T* was also observed in childhood acute lymphoblastic leukemia (ALL).[45]

However, in contrast to the previously mentioned reports, in our study all 3 SNPs were found to be in Hardy-Weinberg disequilibrium ($P < .001$). A possible explanation may be that we, in contrast to the other authors, extracted DNA from leukemic blasts, rather than from germline DNA. During the development of the disease, leukemic blasts tend to change chromosome contents, with a subsequent gain or loss of certain chromosomes. This might possibly have affected the observed differences in the distribution of variant alleles. In addition, it should be noted that our 150 patients were Caucasians from 12 different countries. Previously, Ameyaw *et al*[22] showed that population frequencies of genetic variants of *C3435T* vary according to racial background. A suggestion arises that subjects aged 60 years or older with a homozygous genotype are more susceptible to the development of AML than heterozygous patients, as has been shown for ALL in a Polish study among 113 children (median age, 5.1 years) and 175 healthy individuals, from which it was concluded that the mutant homozygous *3435 TT* genotype is associated with the occurrence of ALL.[46]

We did not find a significant association between any of the various allelic genotypes of *C1236T*, *G2677T*, and *C3435T* with P-gp expression, as determined with either of 2 different anti-P-gp monoclonal antibodies. Nor did P-gp function (rhodamine 123 uptake with PSC-833) reveal an association with 1 of the allelic variants of *ABCB1*, which confirms the results of Oselin *et al*,[47] who reported no impact of SNPs *G2677T* and *C3435T* on rhodamine 123 efflux in peripheral blood lymphocytes. When the transcript levels of *ABCB1* were compared, the same conclusion was evident. This conclusion was at apparent variance with the study by Illmer *et al*[44] conducted in AML patients of comparatively younger age (median, 53 years [range, 17-78 years] versus 67 years [range, 60-85 years] in our study). Their study suggested that the wild-type genotypes in exons 21 (*G2677T*) and 26 (*C3435T*) were associated with lower *ABCB1* mRNA expression. It is unlikely that our findings would

have been substantially different if we had used fresh material instead of cryopreserved material, because Broxterman *et al*[48] showed a good correlation between the values of the rhodamine 123/PSC-833 assays for fresh and thawed samples of adult acute leukemia patients.

None of the clinical endpoints, CR rate, EFS, DFS, and OS, showed a statistically significant difference between any of the 3 allelic variants of *ABCB1* or between carriers and noncarriers of the *ABCB1* haplotypes, either when unadjusted or when adjusted for treatment arm. Nor did we reveal any statistically significant interaction between the allelic variants of each of the SNPs or haplotypes and treatment arm with respect to CR rate and survival, although it should be noted that the number of patients in this analysis was not very large; therefore this study lacked the power to detect a moderate but possibly clinically relevant interaction between these 2 variables. In contrast, in 30 relatively younger patients with relapsed AML, van den Heuvel-Eibrink *et al*[49] had shown that homozygosity for *G2677T* (*GG*/*TT* versus *GT*) was associated with shorter relapse-free intervals and worse OS. Moreover, Illmer *et al*[44] found a significantly lower OS and an increased risk of relapse in patients with AML with the wild-type variant of *G2677T* and in patients with the wild-type genotype of all SNPs.

Although the CR rate and survival were the most relevant clinical endpoints, we have also briefly considered the adverse events (AEs) reported in these patients. The most frequently observed AEs in these patients have been published previously.[30] In general, there was no significant association between the allelic variants and the maximum grade of these AEs.

It should be pointed out that our randomized phase 3 trial was designed to prospectively evaluate the addition of the P-gp inhibitor PSC-833 to standard induction chemotherapy with daunorubicin and cytarabine to circumvent P-gp-mediated resistance in older patients, with a high proportion of patients with high levels of P-gp expression. Our observations suggest that P-gp function and expression in leukemic blasts are not affected by allelic variations of the *ABCB1* gene. Alternatively, the concomitant use of cytarabine may have obscured any significant impact of P-gp on outcome. Cytarabine is not a P-gp substrate and is therefore not prone to *ABCB1*-induced drug efflux, whereas it is one of the most active antileukemic drugs available today.[50]

Polymorphisms of the *ABCB1* gene have been reported to affect the pharmacokinetics of many commonly used drugs, including anticancer drugs. The homozygous T allele of the *C1236T* polymorphism was associated with significantly increased exposure to irinotecan and its active metabolite, SN-38, in 65 cancer patients.[51] Kim *et al*[25] demonstrated that, in intestinal cells in healthy subjects, the polymorphism *G2677T* was of functional importance, because plasma levels of the P-gp substrate fexofenadine were considerably reduced in *TT* patients compared with patients with the *GG* genotype. Studies investigating the influence of

the *C3435T* SNP on pharmacokinetics of the P-gp substrate digoxin[29,37] and on P-gp expression[26,52] revealed conflicting results. A meta-analysis from the relevant studies indicated that *C3435T* affected neither the pharmacokinetics of digoxin nor the expression of *ABCB1* mRNA.[53] In contrast, Wang *et al*[54] recently elegantly showed that *C3435T* was associated with lower mRNA levels as a result of decreased mRNA stability, thereby confirming the studies showing an effect of this SNP on P-gp expression and function. In our study pharmacokinetics was not measured; however, pharmacodynamic studies of in vitro P-gp function and expression and *ABCB1* mRNA expression in leukemic blasts were highly correlated, as was also reported by Oselin *et al*,[47] and the findings were not different between the allelic subgroups. This suggests that, at the cellular level, P-gp function is not affected by the investigated SNPs.

In summary, this is the first study in patients with AML where P-gp function and expression in leukemic blasts, as well as *ABCB1* mRNA levels, were measured together with an analysis of the allelic variants of 3 polymorphisms, *C1236T*, *G2677T*, and *C3435T*. We conclude that these 3 SNPs do not influence P-gp function and expression, nor do they predict clinical outcome to any significant extent, suggesting that they do not exert a major impact on drug resistance in older patients with AML.

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CHAPTER 6

Various distinctive cytogenetic abnormalities in patients with acute myeloid leukaemia aged 60 years and older express adverse prognostic value: results from a prospective clinical trial

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Abstract

Diagnostic cytogenetic abnormalities are considered important prognostic factors in patients with acute myeloid leukaemia (AML). However, the prognostic assessments have mainly been derived from patients with AML aged <60 years. Two recent studies of AML patients of 60 years and older proposed prognostic classifications with distinct discrepancies. To further study the prognostic value of cytogenetic abnormalities in this patient population, we have evaluated cytogenetic abnormalities in a series of 293 untreated patients with AML aged 60 years and older, included in a randomised phase 3 trial, also in relation to patient characteristics and clinical outcome. The most frequently observed cytogenetic abnormality was trisomy 8 (+8), in 31 (11%) patients. Abnormalities, as –5, 5q-, abn(17p) and abn(17q), were almost exclusively present in complex karyotypes. A relatively favourable outcome was only observed in five patients with core-binding factor abnormalities t(8;21) and inv(16)/del(16)/t(16;16). However, most of the other evaluated cytogenetic abnormalities, such as 5q-, –7 , +8, abn(17p), abn(17q), and complex aberrations expressed a more adverse prognosis when compared with patients with AML aged 60 years and older with a normal karyotype. Large studies to confirm the prognosis of individual cytogenetic aberrations are warranted.

Introduction

The significance of diagnostic karyotype as an independent prognostic factor in acute myeloid leukaemia (AML) was shown for the first time in a large, multicentre, prospective study in 716 newly diagnosed patients with AML at the Fourth International Workshop on Chromosomes in Leukaemia (4IWCL).[1] Follow-up studies of the 4IWCL showed that the prognostic value of the cytogenetic risk classifications was independent of the other major risk factors in AML: age, sex, French-American-British (FAB) classification and white blood cell (WBC) count, i.e. in all patients as well as in the subgroup of 305 intensively treated patients.[2,3]

Three large collaborative multicentre studies have further established the prognostic impact of cytogenetic aberrations on clinical outcome in AML,[4-6] and were reviewed by Mrozek *et al*.[7] Nowadays, cytogenetic findings at diagnosis are accepted as important prognostic factors in AML.

Generally, the presence of core-binding factor (CBF) abnormalities t(8;21) and inv(16)/del(16)/t(16;16) are associated with favourable outcome in AML. Leukaemias with abnormalities of 3q, –5, –7, t(9;22) or a complex karyotype tend to have a poor prognosis, while AMLs with a normal karyotype (i.e. no cytogenetic aberrations) or –Y were classified as intermediate risk in the three major collaborative cytogenetic studies of adult AML.[4-7] Data concerning the prognostic impact of other abnormalities, such as 5q-, 7q-, +8, abn(11q23), and more rare aberrations are conflicting,[7] indicating the need for additional studies. However, these prognostic assessments have mainly been derived from patients aged <60 years. The age distributions of the patients in the collaborative studies were [median (range)] 35 (0-55),[4] 39 (16-55)[5] and 52 (15-86) years.[6]

Two recent studies have dealt with the analysis of the prognostic value of cytogenetic abnormalities exclusively in patients with AML of older age,[8,9] and both studies proposed their own prognostic classification. Farag *et al*[8] identified AML patients with CBF abnormalities t(8;21) or inv(16)/del(16)/t(16;16) as prognostic relatively favourable, while the group with complex aberrations or `rare aberrations' had a very poor outcome. The remaining patients were designated in an intermediate risk group.[8] In contrast, Fröhling *et al*[9] included only inv(16)/del(16)/t(16;16) in the low risk group, while patients with AML with t(8;21) together with a normal karyotype, abn(11q23), +8 and +11 both within a noncomplex karyotype were classified in a standard risk group. The high-risk group consisted of all other aberrations.[9] Because of the clear discrepancies between the two classifications, obviously additional data are needed to further investigate the prognostic value of cytogenetic aberrations in patients with AML aged 60 years and older.

Here, we report the results of an analysis of cytogenetic abnormalities observed in a relatively homogeneously treated cohort of 293 patients with AML of 60 years and older. All patients were treated in a randomised, multicentre, phase 3 trial comparing daunomycin–cytarabine induction chemotherapy, with or without the P-glycoprotein (P-gp) inhibitor PSC-833.[10] Cytogenetic aberrations at diagnosis were evaluated in relation to baseline patient characteristics and with additional cytogenetic abnormalities. The relationships between cytogenetic abnormalities and clinical outcome, i.e. complete response (CR) rate, event-free survival (EFS), disease-free survival (DFS) and overall survival (OS), were also assessed. Subsequently, the results are discussed in the context of the two recent studies by Farag *et al*[8] and Fröhling *et al*.[9]

Patients, materials and methods

Patients

A group of 293 patients with AML aged 60 years and older were included in the present study. Patients were selected based on the availability of successful cytogenetic data at diagnosis. All patients were enrolled between May 1997 and February 1999 in an international, multigroup, randomised phase 3 trial, performed under the auspices of the Dutch-Belgian Haemato-Oncology Cooperative Group (HOVON) and the United Kingdom Medical Research Council.[10] In that trial, 419 eligible Caucasian patients ≥60 years with previously untreated *de novo* and secondary AML (M0-M2 and M4-M7, FAB classification[11]) were randomised to receive two cycles of induction chemotherapy consisting of daunomycin and cytarabine with or without the P-gp inhibitor PSC-833 (Valspodar, Amdray®; Novartis Pharmaceuticals, Basle, Switzerland). Patients in both arms in complete remission after these two cycles received one consolidation cycle without PSC-833 consisting of cytarabine, mitoxantrone and etoposide. Details of the treatment programme and objectives of the phase 3 trial were described previously.[10] The study was approved by the ethics committees of the participating institutions, and was conducted in accordance with the Declaration of Helsinki. All patients gave written informed consent prior to inclusion.

Cytogenetic analysis

Cytogenetic data had not been collected on the original case report forms (CRF), but were collected afterwards from the local investigators or cytogeneticists. CRFs with cytogenetic data were obtained for 362 patients, while for 57 patients no data were sent in. Of these

362 patients, cytogenetic analysis had not been performed in 46 patients, while a failure had occurred in another 23 patients. Therefore, successful cytogenetic data were available for 293 patients. Cytogenetic analysis of bone marrow (BM) or peripheral blood (PB) samples obtained at diagnosis had been performed using standard cytogenetics techniques according to local protocols. All available cytogenetic reports were reviewed by two expert cytogeneticists (HBB and EvdB). Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.[12] In particular, the following cytogenetic abnormalities were recorded: t(1;7), abn(3q), –5, 5q-, t(6;9), –7, 7q-, +8, t(8;21), 9q-, t(9;11), t(9;22), +11, abn(11q23), 12p-, 13q-, inv(16)/del(16)/t(16;16), abn(17p), abn(17q), 20q-, –21, +21, –Y, –X, and complex abnormalities (defined as at least three unrelated cytogenetic abnormalities in one clone).

Definition of endpoints

The clinical endpoints have been defined elsewhere.[10] Briefly, CR was defined as a normocellular BM with <5% blasts, no Auer rods and no evidence of extramedullary involvement. Data on PB recovery within 60 d after start of a cycle was not always available, and was not considered as a criterion for CR. Patients who relapsed or died within 28 d after CR were considered as not having achieved a CR. EFS was determined from the date of randomisation until no CR on induction therapy, relapse after CR or death in CR, whichever came first. Patients who did not reach CR were considered failure for EFS at 1 d after randomisation. DFS was determined for all patients who achieved CR and was calculated from the date of CR until relapse or death, whichever came first. OS was measured from randomisation until death from any cause. Patients still alive at the date of last contact were censored.

Statistical considerations

Patient characteristics at diagnosis were summarised for all patients, and for each cytogenetic abnormality separately. Patients with more than one aberration were therefore included more than once. Clinical endpoints were CR rate, EFS, DFS and OS.

Baseline characteristics and clinical outcome for each abnormality were compared with those of patients with a normal karyotype. Baseline characteristics were compared by using Fisher's exact test[13] in case of discrete variables, or the Wilcoxon rank sum test[14] in case of continuous variables. CR rates were calculated along with 95% confidence interval (CI), and also the odds ratio (OR) was calculated with 95% CI. The survival endpoints were based on the same data as used before.[10] The median follow-up of the 40 patients still alive was 59 months

Table 1. Cytogenetic abnormalities in 293 patients with acute myeloid leukaemia aged 60 years and older

		Age group, years			
	All patients	60-65	66-70	71-85	Median age,
Abnormality	No. (%)	No. (%)	No. (%)	No. (%)	years (range)
Total	293	108	93	92	67 (60-85)
No abnormality	158 (54)	61 (56)	52 (56)	45 (49)	67 (60-85)
Any abnormality	135 (46)	47 (44)	41 (44)	47 (51)	67 (60-79)
+8	31 (11)	11 (10)	8 (9)	12 (13)	68 (60-76)
–7	23 (8)	9 (8)	6 (6)	8 (9)	66 (61-79)
5q-	16 (5)	8 (7)	6 (6)	2 (2)	66 (60-78)
Abn(3q)	12 (4)	7 (6)	1 (1)	4 (4)	65 (60-74)
–5	11 (4)	4 (4)	2 (2)	5 (5)	67 (60-78)
Abn(17p)	11 (4)	2 (2)	4 (4)	5 (5)	70 (64-78)
Abn(17q)	10 (3)	4 (4)	4 (4)	2 (2)	67 (62-75)
+11	9 (3)	4 (4)	1 (1)	4 (4)	66 (60-77)
–Y	9 (3)	4 (4)	3 (3)	2 (2)	69 (61-72)
7q-	8 (3)	3 (3)	3 (3)	2 (2)	68 (62-78)
Abn(11q23)	6 (2)	3 (3)	2 (2)	1 (1)	66 (61-78)
12p-	6 (2)	4 (4)	2 (2)	– (-)	64 (62-70)
Complex	36 (12)	17 (16)	9 (10)	10 (11)	67 (60-78)

AML, acute myeloid leukaemia; WBC, white blood cell; and abn, abnormality.

Percentages indicate percentage of column total. Percentages do not add up to 100, because patients with more than one abnormality were counted more than once.

P-values compare type of AML between patients with a certain cytogenetic abnormality *versus* no abnormality (i.e. normal karyotype).

(range, 1-80 months). EFS, DFS and OS were estimated by the actuarial method of Kaplan and Meier,[15] while also the hazard ratios and corresponding 95% CI were determined for all three survival endpoints. Kaplan-Meier survival curves[15] were generated to illustrate survival. Only abnormalities that were observed in at least five patients were included in the analyses for prognostic factors. All reported *P*-values are two-sided. Because of the exploratory nature of this analysis, the relatively small number of patients with a certain cytogenetic aberration and the large number of significance tests, 95% CIs have been shown for illustrative purposes, but in the Results and Discussion sections, only *P*-values ≤ .01 were considered statistically significant.

Table 1. Cytogenetic abnormalities in 293 patients with acute myeloid leukaemia aged 60 years and older, continued

			Type of AML	
	Median WBC count,	Median %	*De novo*	Secondary
Abnormality	10^9/L (range)*	$CD34^+$, (range)**	No. (%)	No. (%)
Total	9.1 (0.5-300)	29.7 (0.1-95.6)	217	76
No abnormality	11.2 (0.5-300)	21.7 (0.1-95.6)	125 (58)	33 (43)
Any abnormality	8.6 (0.6-226)	36.6 (0.4-93.1)§	92 (42)	43 (57)†
+8	20.2 (0.8-226)	43.1 (0.4-93.1)†	20 (9)	11 (14)
–7	6.2 (0.9-62.4)	43.3 (0.4-87.9)	15 (7)	8 (11)
5q-	3.6 (1.9-62.4)	44.0 (8.4-73.5)†	10 (5)	6 (8)
Abn(3q)	26.7 (1.4-59.8)	44.5 (1.0-78.8)†	8 (4)	4 (5)
–5	2.8 (1.1-24.0)	23.1 (2.6-65.2)	7 (3)	4 (5)
Abn(17p)	25.0 (2.8-90.2)	34.7 (8.4-65.2)	7 (3)	4 (5)
Abn(17q)	3.6 (1.1-90.2)	53.8 (3.7-70.3)†	9 (4)	1 (1)
+11	12.8 (1.6-42.2)	57.7 (24.2-92.4)‡	6 (3)	3 (4)
–Y	4.0 (1.1-11.5)	40.7 (11.6-83.0)†	7 (3)	2 (3)
7q-	3.1 (0.9-65.5)	31.6 (6.3-57.7)	5 (2)	3 (4)
Abn(11q23)	10.6 (1.9-97.0)	6.6 (5.0-46.4)	2 (1)	4 (5)†
12p-	21.2 (2.8-39.6)	35.2 (28.9-57.7)	5 (2)	1 (1)
Complex	3.6 (0.6-90.2)	40.0 (2.6-83.0)‡	20 (9)	16 (21)‡

*WBC count was not available for 25 patients.

**% $CD34^+$ was not available for 67 patients.

†$P < .05$.

‡$P < .01$.

§$P < .001$.

Results

Patient characteristics

Patient baseline characteristics, including age, WBC count, CD34 positivity and secondary leukaemia of the 293 patients are shown in Table 1. One hundred and eighty-one patients were male (62%), and 112 (38%) were female. According to the FAB classification, 20 patients had an AML type M0 (7%), 62 M1 (21%), 109 M2 (37%), 47 M4 (16%), 20 M5 (7%), 18 M6 (6%), 2 M7 (1%), and 15 unclassified (5%). The patients were randomised

between the two treatment arms as follows: 146 (50%) in the control arm *versus* 147 (50%) in the PSC-833 arm.

Incidence of specific cytogenetic aberrations in patients with AML aged 60 years and older

A total of 158 of the 293 patients (54%) presented with a normal karyotype, while cytogenetic aberrations were present in 135 patients (46%). Cytogenetic abnormalities observed in one to four patients were: t(6;9) [n = 2], t(8;21) [n = 2], 9q- [n = 2], t(9;22) [n = 1], inv(16)/del(16)/t(16;16) [n = 3], 20q- [n = 3], –21 [n = 4], +21 [n = 4] and –X [n = 2].
The frequency of cytogenetic abnormalities observed in at least five patients and their associated baseline characteristics are displayed in Table 1. The most frequently observed abnormality was +8 in 31 patients (11%). No significant variation was observed in the percentages of particular cytogenetic abnormalities across the age range of patients entered into the trial, neither was there an apparent difference in WBC count in patients with a specific aberration compared with patients with a normal karyotype. A significantly higher CD34 positivity ($P < .01$) was observed in AML with +11 and in patients with a complex karyotype. Complex cytogenetics was also associated with secondary leukaemia: 9% of cases with *de novo* AML presented with a complex karyotype when compared with 21% of patients with secondary AML ($P < .01$).

Frequency of additional cytogenetic abnormalities

Of the 135 patients with cytogenetic abnormalities, 92 (68%) had multiple chromosomal abnormalities. To further characterise the cytogenetic features of these patients with AML of older age, the frequency of patients with a combination of two chromosomal aberrations was also determined (Table 2). +8 was present as a sole abnormality in 13/31 patients (42%) and as part of a complex karyotype in 9/31 (29%). On the other hand, abnormalities –5, 5q-, abn(17p) and abn(17q) were almost exclusively noted as part of a complex karyotype, i.e. in 10/11 (91%), 14/16 (88%), 9/11 (82%) and 10/10 (100%) patients, respectively.

Response to chemotherapy

The CR rate of the 293 patients was 54% (95% CI, 48%-59%), while this was 61% (95% CI, 53%-68%) in the 158 patients with a normal karyotype. Compared with the group of patients with a normal karyotype, a significantly lower CR rate (7/23 = 30%, $P < .01$) was observed in patients with –7, while none of 11 patients with abn(17p) achieved a CR ($P < .001$). There was a suggestion that there may possibly be a lower CR rate in patients with 5q- or complex abnormalities ($P < .05$, Table 3).

Table 2. Characterisation of additional cytogenetic abnormalities associated with primary chromosomal aberrations

	Total	+8	–7	5q-	Abn(3q)	–5	Abn(17p)	Abn(17q)	+11	–Y	7q-	Abn(11q23)	12p-	Complex
Total	293	31	23	16	12	11	11	10	9	9	8	6	6	36
Sole abnormality	43	13 (42%)	8 (35%)	1 (6%)	6 (50%)	0	0	0	6 (67%)	3 (33%)	2 (25%)	3 (50%)	1 (17%)	–
With the following:														
+8	31	–	5	4	1	2	3	2	1	0	2	1	2	9‡
–7	23		–	8§	3	5§	3†	5§	1	1	0	2	1	13§
5q-	16			–	1	2	5§	4‡	0	2	2	1	2†	14§
Abn(3q)	12				–	0	0	2	1	0	0	1	0	3
–5	11					–	2	3‡	0	1	1	1	1	10§
Abn(17p)	11						–	3‡	0	0	2†	0	0	9§
Abn(17q)	10							–	1	1	1	1	1	10§
+11	9								–	0	0	1	0	1
–Y	9									–	0	0	1	4†
7q-	8										–	1	1	5§
Abn(11q23)	6											–	0	3†
12p-	6												–	5§
Complex	36													–

Abn, abnormality.

Fisher's exact test was used to calculate *P*-values for comparison of abnormality A *versus* not-abnormality A with abnormality B *versus* not-abnormality B.

†$P < .05$.

‡$P < .01$.

§$P < .001$.

Event-free survival

The median EFS was 3 months (95% CI, 0-6 months), with a 5-year EFS of 9% (95% CI, 6%-13%). When restricted to the 158 patients with a normal karyotype, the EFS was median 6 months (95% CI, 3-9 month), and 13% (95% CI, 8%-19%) at 5 years. Because of a CR rate of <50% for most of the cytogenetic abnormalities (Table 3), by definition, the median EFS was 0 months for these subgroups of patients.

Disease-free survival

The median DFS of the 157 patients who achieved a CR was 10 months, (95% CI, 8-13 months), with a 5-year DFS of 17% (95% CI, 11%-23%). When restricted to the 96 patients with a normal karyotype, the DFS was median 13 months (95% CI, 9-17 months), and 21% (95% CI, 14%-30%) at 5 years. An adverse outcome was seen especially for 5q-, –7, +8, abn(11q23) and abn(17q) (all $P < .01$).

Overall survival

The median OS was 10 months (95% CI, 8-12 months), with a 5-year OS of 12% (95% CI, 8%-16%). When restricted to the 158 patients with a normal karyotype, the OS was median 14 months (95% CI, 10-18 months), and 18% (95% CI, 12%-24%) at 5 years. A relatively favourable outcome was only observed in the five patients with CBF AML, of whom three were still alive after 53-63 months from randomisation, although reliability was limited due to the small sample size.

Almost all other cytogenetic aberrations were associated with a worse outcome compared with patients with a normal karyotype, with the exception of 7q- or –Y patients, who had a median OS of 30 and 11 months, respectively. The worst outcome was observed in patients with an abnormality of chromosome 17: the 11 patients with abn(17p) and 10 patients with abn(17q) all died within 10 months ($P < .001$). For illustrative purposes only, Kaplan-Meier survival curves were generated for patients with a favourable prognosis [t(8;21) or inv(16)/del(16)t(16;16)], an intermediate prognosis (normal karyotype, 7q- or –Y) and a poor prognosis [(abn(3q), –5, 5q-, –7, +8, +11, abn(11q23), 12p-, abn(17p), abn(17q) or complex abnormalities], respectively (Figure 1).

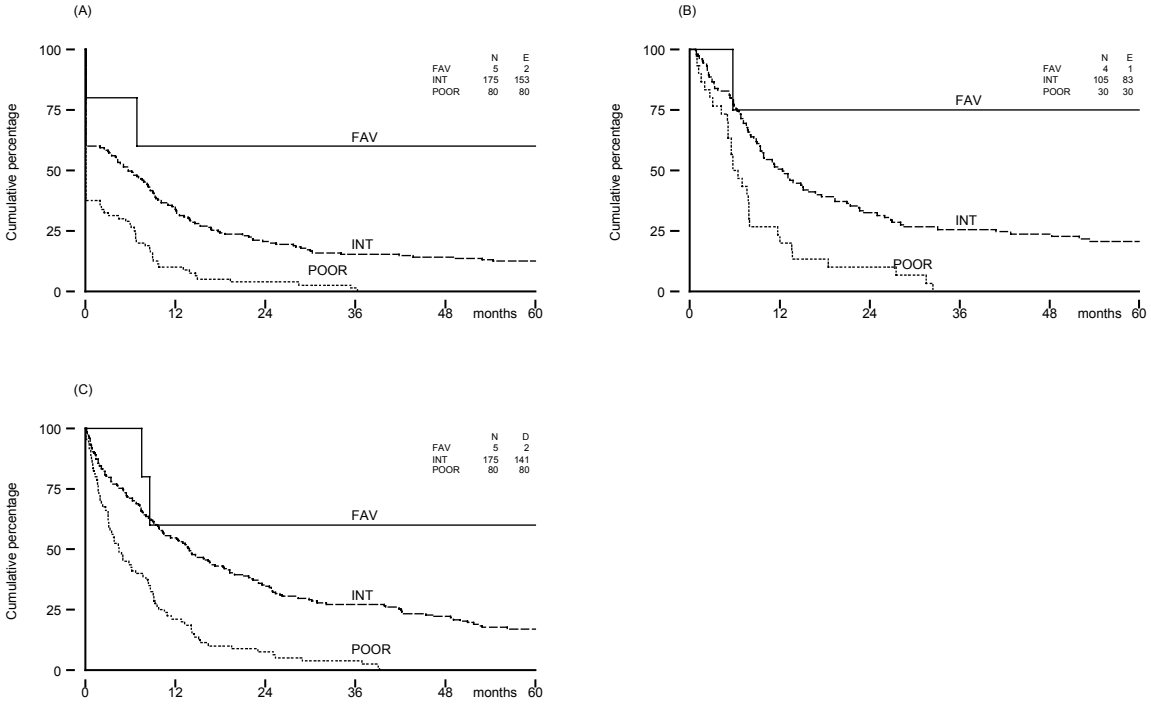

Figure 1. Kaplan-Meier survival curves of elderly patients with acute myeloid leukaemia by prognosis based on the cytogenetic abnormalities.

Patients were classified as favourable [t(8;21) or inv(16)/del(16)/t(16;16))], intermediate [normal karyotype, 7q- or –Y] or poor prognosis [abn(3q), -5, 5q-, –7, +8, +11, abn(11q23), 12p-, abn(17p), abn(17q) or complex abnormalities]. (A) event-free survival. (B) disease-free survival from CR. (C) overall survival. N, number of patients; E, number of events; D, number of patients that have died.

Table 3. CR rate, disease-free survival (DFS) from CR, and overall survival (OS), by cytogenetic aberration

			CR rate			DFS			OS		
Abnormality	No.	(%)	No.	% (95% CI)	OR (95% CI)	rel/d1	med	HR (95% CI)	Dead	med	HR (95% CI)
All patients	293		157	54 (48-59)		117/13	10		253	10	
No abnormality	158	(54)	96	61 (53-68)	1	66/9	13	1	126	14	1
Any abnormality	135	(46)	61	45 (37-54)	0.53 (0.33-0.85)‡	51/4	8	1.55 (1.09-2.21)†	127	8	1.80 (1.40-2.31)§
+8	31	(11)	13	42 (25-61)	0.47 (0.21-1.02)	11/2	6	2.56 (1.41-4.68)‡	31	5	2.76 (1.83-4.16)§
Non-complex	22	(8)	9	41 (21-64)	0.45 (0.18-1.11)	8/1	6	2.29 (1.14-4.60)†	22	3	2.78 (1.75-4.42)§
Complex	9	(3)	4	44 (14-79)	0.52 (0.13-2.00)	3/1	8	3.60 (1.26-10.2)†	9	8	2.62 (1.30-5.25)§
–7	23	(8)	7	30 (13-53)	0.28 (0.11-0.73)‡	5/2	4	2.99 (1.36-6.59)‡	23	4	2.90 (1.82-4.60)§
5q-	16	(5)	5	31 (11-59)	0.29 (0.10-0.89)†	4/1	5	5.02 (1.88-13.4)‡	16	8	2.83 (1.65-4.85)§
Abn(3q)	12	(4)	5	42 (15-72)	0.46 (0.14-1.51)	5/0	6	1.87 (0.75-4.64)	12	9	1.99 (1.09-3.62)†
–5	11	(4)	5	45 (17-77)	0.54 (0.16-1.84)	5/0	8	2.00 (0.80-4.98)	11	8	2.36 (1.26-4.41)‡
Abn(17p)	11	(4)	0	0 (0-28)	0 (0-0.27)§	n.a.			11	2	5.19 (2.70-9.95)§
Abn(17q)	10	(3)	4	40 (12-74)	0.43 (0.12-1.59)	3/1	5	6.10 (2.07-17.7)‡	10	3	4.31 (2.19-8.47)§
+11	9	(3)	4	44 (14-79)	0.52 (0.13-2.00)	4/0	12	2.01 (0.73-5.57)	9	4	2.01 (1.02-3.96)†
–Y	9	(3)	5	56 (21-86)	0.81 (0.21-3.12)	5/0	7	1.91 (0.77-4.75)	9	11	1.69 (0.86-3.34)
7q-	8	(3)	4	50 (16-84)	0.65 (0.16-2.68)	3/0	41	0.84 (0.27-2.71)	6	30	0.80 (0.35-1.81)
Abn(11q23)	6	(2)	3	50 (12-88)	0.65 (0.13-3.30)	2/1	5	5.19 (1.54-17.6)‡	6	6	3.20 (1.39-7.42)‡
12p-	6	(2)	3	50 (12-88)	0.65 (0.13-3.30)	3/0	6	2.69 (0.83-8.71)	6	8	2.61 (1.13-5.99)†
CBF AML*	5	(2)	4	80 (28-99)	2.58 (0.28-23.7)	1/0	>61	0.21 (0.03-1.56)	2	>63	0.34 (0.08-1.37)
Complex	36	(12)	14	39 (23-57)	0.41 (0.20-0.86)†	11/2	7	1.96 (1.08-3.56)†	35	5	2.40 (1.63-3.53)§

CI, confidence interval; OR, odds ratio; rel/d1, number of patients with a relapse after CR, resp. who died in 1st CR; med, median in months; HR, hazard ratio; n.a., not applicable; and CBF, core-binding factor.

*CBF AML comprises t(8;21) and inv(16)/del(16)/t(16;16).

P-values are for comparing outcome between patients with a certain cytogenetic abnormality *versus* no abnormality (i.e. normal karyotype).

†$P < .05$. ‡$P < .01$. §$P < .001$.

Discussion

Cytogenetic abnormalities confer profound prognostic impact in adults with AML with regard to treatment outcome. However, the prognostic effect of cytogenetic aberrations has mainly been studied in patients aged <60 years, and has been reviewed by Mrozek *et al*.[7] In a few reports of AML patients older than 60 years, risk group classifications were used that had been validated in younger patients, ignoring the age-specific characteristics of AML in elderly patients.[16-18] Only recently, two studies in AML patients of older age evaluated the impact of individual cytogenetic aberrations, but reported classifications based on cytogenetic abnormalities with distinct discrepancies.[8,9] Therefore, we report the results of a study that addressed, in an unbiased approach, the question of the prognostic value of cytogenetic abnormalities in patients with AML aged 60 years and above.

These patients of older age with AML had a low response to standard induction chemotherapy (CR rate of 54%) and poor survival (median OS of 10 months),[10] comparable with the results in previous studies.[19-23] In younger patients, the CBF abnormalities t(8;21) and inv(16)/del(16)/t(16;16) have been associated with a favourable outcome.[4-6] Although there were only five such patients (2%) in the study presented here, their good prognosis was illustrated by a CR rate of 80%, with three patients still being in continuous CR at 53, 56 and 63 months from randomisation.

The patients without cytogenetic abnormalities (158 patients; 54%) were considered as a reference risk group, similar to the reports by Grimwade *et al*.[4,16] In our study, these patients had a CR rate of 61%, while their median OS was 14 months. Almost all patient groups with cytogenetic aberrations [–5, 5q-, –7, +8, abn(11q23), abn(17p), abn(17q) and complex abnormalities] had either a statistically significant poorer OS ($P < .01$) when compared to those with a normal karyotype, or there was a suggestion of adverse OS in AML patients with abn(3q), +11 or 12p- ($P < .05$). Patients with –Y or 7q- had an OS, which was not significantly different from the AML patients with a normal karyotype.

The cytogenetic aberrations abn(3q), –5, 5q-, –7, abn(17) and complex abnormalities have previously been associated with an adverse outcome among patients of mainly younger age.[4-6,24] The prognostic impact of the cytogenetic abnormalities abn(11q23), +11 and 12p- is less clear. Although the present study suggests a negative prognostic impact of those aberrations, the number of patients represented in this cohort is small (<10 patients of each cytogenetic subgroup).

Table 4. Overall survival (OS) at 3 and/or 5 years, including 95% CI, according to cytogenetic abnormalities of three studies in patients with AML aged 60 years and older (Farag *et al*;[8] Fröhling *et al*;[9] this study)

	Farag *et al**				Fröhling *et al*				This study						
			5-year OS				3-year OS				3-year OS		5-year OS		Proposed
Abnormality	No.	(%)	%	(95% CI)*	No.	(%)	%	(95% CI)	No.	(%)	%	(95% CI)	%	(95% CI)	prognosis
All patients	635		7	(5-9)	361		15	(11-19)	293		19	(15-24)	12	(8-16)	
Normal	287	(45)	8	(5-12)	161	(45)	19	(13-26)	158	(54)	28	(22-36)	18	(12-24)	Int
Any abnormality	348	(55)	–		200	(55)	–		135	(46)	9	(5-15)§	5	(2-10)§	Poor
CBF AML	31	(5)	19	(8-35)	–		–		5	(2)	60	(13-88)	60	(13-88)	Fav
t(8;21)	15	(2)	20	(5-42)	12	(3)	10	(2-65)	2	(1)	100		100		Fav/Int
idt(16)	16	(3)	19	(5-40)	14	(4)	26	(10-66)	3	(1)	33	(1-77)	nr		Fav
Abn(3q)	–		–		14	(4)	0§		12	(4)	0†		0†		Poor
+4	–		–		5	(1)	20	(3-100)	–		–		–		Int
–5/5q-	86	(14)	0		57	(16)	0§		25	(8)	4	(0.3-17)§	0§		Poor
–5	–		–		16	(4)	–		11	(4)	9	(1-33)‡	0‡		Poor
5q-	–		–		41	(11)	–		16	(5)	0§		0§		Poor
–7/7q-	97	(15)	2	(0.4-7)	52	(14)	0§		31	(11)	13	(4-27)‡	6	(1-19)‡	Poor
–7	–		–		23	(6)	–		23	(8)	4	(0.3-18)§	0§		Poor
7q-	–		–		29	(8)	–		8	(3)	38	(9-67)	25	(4-56)	Int
+8	88	(14)	7	(3-13)	47	(13)	16	(7-33)	31	(11)	3	(0.2-14)§	0§		Int/Poor
Non-complex	–		–		22	(6)	26	(12-56)	22	(8)	5	(0.3-19)§	0§		Int/Poor
Complex	–		–		25	(7)	5	(1-35)§	9	(3)	0§		0§		Poor
–9/9q-	17	(3)	6	(0.4-24)	–		–		–		–		–		Int
–11/11q-	28	(4)	4	(0.3-15)	–		–		–		–		–		Int

+11	24	(4)	0		20	(6)	7	(1-41)†	9	(3)	11	(1-39)†	0†		Poor
Non-complex	–		–		6	(2)	20	(3-100)	8	(3)	13	(1-42)	0		Int
Complex	–		–		14	(4)	0§		1	(0)	–		–		Poor
Abn(11q23)	17	(3)	12	(2-31)	11	(3)	18	(5-64)	6	(2)	0‡		0‡		Int/Poor
–12/12q-	38	(6)	0		–		–		–		–		–		Poor
12p-	–		–		–		–		6	(2)	0†		0†		Poor
Abn(12p)	–		–		25	(7)	5	(1-31)§	–		–		–		Poor
–13/13q-	17	(3)	6	(0.4-24)	–		–		–		–		–		Int
13q-	–		–		15	(4)	0§		0		–		–		Poor
+13	20	(3)	5	(0.3-21)	17	(5)	6	(1-39)§	–		–		–		Int/Poor
+14	–		–		9	(2)	11	(2-70)	–		–		–		Int
–17/17p-	58	(9)	2	(0.1-8)	25	(7)	0§		–		–		–		Poor
Abn(17p)	–		–		–		–		11	(4)	0§		0§		Poor
Abn(17q)	–		–		–		–		10	(3)	0§		0§		Poor
–18	29	(5)	0		9	(2)	0§		–		–		–		Poor
–20/20q-	28	(4)	0		23	(6)	0‡		–		–		–		Poor
+21	20	(3)	0		17	(5)	0§		4	(1)	0		0		Poor
+22	17	(3)	6	(0.4-24)	17	(5)	10	(2-59)†	–		–		–		Poor
–Y	28	(4)	11	(3-25)	8	(2)	–		9	(3)	0		0		Int
Complex	122	(19)	2	(0.3-5)	61	(17)	4	(1-15)§	36	(12)	6	(1-16)§	3	(0.2-12)§	Poor

idt(16) indicates inv(16)/del(16)/t(16;16); –, not available; nr, not reached; fav, favourable; and int, intermediate.

P-values compared outcome between patients with a certain cytogenetic abnormality *versus* no abnormality (i.e. normal karyotype).

*N.B. *P*-values were not reported for separate cytogenetic abnormalities in Farag *et al*.[8]

†$P < .05$. ‡$P < .01$. §$P < .001$.

In the current HOVON trials for AML, 7q- is still considered as unfavourable cytogenetic risk, although others suggested that patients with 7q- without co-existing abnormalities of chromosome 5 may have prolonged survival.[25,26] In our study, the two patients with 7q-, who were still alive after 64 and 73 months, did not have –5 or 5q-.

Interestingly, 31 patients in this study had +8, which was associated with an unfavourable prognosis (Table 3, Figure 1). Thirteen patients (42%) showed a sole +8, while +8 was part of a complex karyotype in only 9/31 (29%), suggesting that this abnormality is independently associated with a poor prognosis in older patients with AML. The prognostic value of +8, the most common trisomy in AML, has been the subject of many reports with inconsistent results. While some studies assigned an intermediate prognosis to patients with AML +8,[4,5,16,26-29] others found that the prognosis of these patients was poor.[30-32] Two important hypotheses can be considered in explaining this discrepancy in outcome. Firstly, the intensity of treatment may influence the outcome of AML patients with +8. Byrd *et al*[33] concluded that the outcome of +8 patients was poor when treated with short-term cytarabine-based chemotherapy, while Farag *et al*[8] reported a better outcome in patients with AML and following stem cell transplantation. The second explanation for the variable prognostic impact of +8 could relate to the presence of additional poor prognostic cytogenetic aberrations.[6,34,35] However, in our study, the outcome of the 18 patients with +8 and accompanying abnormalities was not markedly different from 13 patients with isolated +8, while also the results of +8 patients with or without complex aberrations were very similar (Table 3), although the limited numbers of patients preclude definite conclusions.

Two recent studies have especially addressed the prognostic impact of cytogenetic aberrations in patients with AML aged 60 years or older.[8,9] The two studies agreed in a favourable prognostic impact on OS of AML patients with inv(16)/del(16)/t(16;16). However, there was no agreement concerning AML with t(8;21). Farag *et al*[8] reported a favourable outcome, while Fröhling *et al*[9] observed an intermediate risk. In our study, only five AML patients with CBF abnormalities [three patients with inv(16)/del(16)/t(16;16) and 2 with t(8;21)] were present, with a relatively good prognosis. Obviously, a larger cohort of AML patients of 60 years and older with t(8;21) is needed to be conclusive. All three studies (Farag *et al*,[8] Fröhling *et al*,[9] this study) agreed in assigning the AML patients with a normal karyotype to the intermediate risk group and the complex cytogenetic abnormalities to the poor risk group. The prognostic value of other cytogenetic abnormalities is less clear. Fröhling *et al*[9] included AML with +8 or +11 within a non-complex karyotype and abn(11q23) in the intermediate risk group. The intermediate risk prognosis of +8 and abn(11q23) was also found by Farag *et al*,[8] but these patients had a poor outcome in our study. In contrast with Fröhling *et al*,[9] we found an adverse prognosis of AML with +11 without complex aberrations. Most other cytogenetic

aberrations were included in the intermediate risk group by Farag *et al*[8] with the exception of a residual poor prognostic group with 'rare aberrations', while Fröhling *et al*[9] assigned all other cytogenetic abnormalities to the poor risk group. Because of these inconclusive data, and in an attempt to give more insight in the prognostic value of the cytogenetic abnormalities in patients with AML of 60 years and older, the OS results of all cytogenetic subgroups from the studies by Farag *et al*,[8] Fröhling *et al*[9] and our study were summarised in Table 4. It shows that, with the exception of AML with CBF abnormalities, no favourable risk group could be identified, while many distinct cytogenetic aberrations were associated with a more adverse prognosis when compared with AML patients with a normal karyotype.

The prognostic value of infrequent individual cytogenetic abnormalities remains to be established in future trials for patients with AML of older age, e.g. in the recently closed HOVON 43 AML trial, in which more than 850 patients of 61 years and older have been included. This would be important to optimise the cytogenetic classification in terms of prognostic value as a guide in the clinical management of patients with AML of older age. Any such classification would, however, have to be validated in new patient cohorts.

In conclusion, only the very small subgroup of AML patients of older age with CBF abnormalities have a relatively favourable outcome, while various distinctive cytogenetic aberrations express a more adverse prognosis when compared with the AML patient group with a normal karyotype. To further elucidate the predictive impact of more rare occurring cytogenetic abnormalities, analyses of additional prospective studies are needed.

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CHAPTER 7

General discussion

General discussion

Significance of chemotherapy resistance in AML at older age

The overall outcome of treatment with conventional chemotherapy of patients of older age with AML has remained highly unsatisfactory. During the past 15 years, CR rates between 45-60% have been reported, while the median DFS of patients who achieved a CR was less than one year, resulting in an OS at 4-5 years of about 10%.

The way to improve treatment outcome for a specific disease is through research to better treatment modalities, e.g. new drugs, dose increase or dose intensification, or addition of other treatment (modalities) to the current standard therapy. The only reliable basis for evaluating new treatment modalities with respect to feasibility and efficacy is via properly conducted prospective clinical trial. Clinical trials are conducted in phases. The trials at each phase have a different purpose and help scientists answer different questions. The primary aim of a phase I trial is determining the maximum tolerated dose (MTD) of a new agent. In a phase II trial, the effectiveness and safety are investigated, i.e. if the new treatment induces clinical responses with acceptable toxicity in a sufficient proportion of patients, and to decide which agents should be tested further. Phase III trials are designed to compare a new treatment modality – after it has been shown to be reasonably effective in a phase II trial – to the current standard treatment(s) for the same disease. This is done by assigning eligible patients randomly to one of the treatment arms in the phase III trial. In these clinical trials the category of patients which are eligible are defined by inclusion and exclusion criteria.[1,2]

A major problem in the treatment of AML is the high rate of failure to respond to standard chemotherapy regimens. This condition has been called multidrug resistance (MDR), and refers to resistance to a broad range of structurally and functionally unrelated cytotoxic agents. The classical form of multidrug resistance, MDR1 (ABCB1), is related to high expression of the (membrane) P-glycoprotein (P-gp). High expression of ABCB1 on leukemia cells will extrude cytostatic agents out of the cells, thus causing a diminished accumulation of chemotherapy in leukemic cells. In retrospective studies ABCB1/P-gp expression was associated with lower CR rates and decreased DFS and OS in AML, which resulted in a rationale for ABCB1 modulation as a therapeutic approach (for an extensive review, see Sonneveld *et al*[3]). MDR in patients with AML increases with age.[4,5]

Therapeutic abrogation of chemotherapy resistance

Therapeutic drugs have been developed that inhibit ABCB1 function aiming at an enhanced intracellular concentration of these agents, with the purpose of overcoming drug resistance. Because of the high frequency of ABCB1 in AML, clinical trials have been performed with first-generation P-gp inhibitors such as Quinine, Verapamil and Cyclosporin A (CsA). These trials showed moderate therapeutic effects and/or the clinical application of these agents was hampered by unexpected interactions with the pharmacokinetics of the cytostatic agents. In a phase III trial by the French GOELAM group, Quinine was added to induction chemotherapy with mitoxantrone and cytarabine in 315 patients aged 16-65 years with acute leukemias. While CR rates were not significantly different between the two treatment arms, Quinine induced a significant increase in the incidences of cardiac toxicity, which was also seen with Verapamil, while also death in aplasia was more frequent in the Quinine treatment arm.[6] A higher CR rate was not observed either in another GOELAM phase III trial in 425 patients with AML aged 15-60 years, when Quinine was added to idarubicin and cytarabine.[7] A phase III trial performed by the MRC in 235 relapsed or refractory AML patients of median age 48 years (range, 4-75 years) evaluated the addition of CsA to reinduction chemotherapy with cytarabine, daunorubicin and etoposide. No statistically significant differences were observed in CR rates and DFS between patients treated with or without CsA. Neither did CsA affect OS, except in patients ≥ 60 years who fared worse on CsA.[8] A randomized, phase II HOVON trial which investigated the benefit of CsA added to mitoxantrone and etoposide in 80 patients of median age 47 years (range, 19-77) with refractory or relapsing AML, also failed to show improved treatment outcome in the CsA arm, while liver toxicity was observed in a large number of CsA patients, which was inherent to the activity of CsA on the biliary excretory system.[9] On the other hand, the SWOG 9126 trial, in which 226 patients aged 18-70 years with AML refractory to induction chemotherapy or in relapse were randomized to receive cytarabine and daunorubicin with or without CsA, showed a benefit of CsA. A lower incidence of refractory AML in the CsA treated patients was observed, while OS and relapse free survival were improved. The effect of CsA on survival was greatest in patients with moderate or bright P-gp expression.[10]

The second-generation P-gp inhibitor PSC-833 (Valspodar) was developed in order to exclude pharmacokinetic interactions. Initial phase I and phase II trials had shown promising results,[11,12] which warranted further evaluation in a randomized phase III trial. The phase III trial and its outcome, as well as several side studies, are the subject of this thesis.

The phase III trial investigated in patients with AML aged 60 years or older whether the addition of PSC-833 to induction chemotherapy with daunorubicin and cytarabine would

improve outcome. The primary endpoints was EFS, defined as time from randomization until induction failure, relapse or death whatever the cause, whichever came first. Secondary endpoints were CR rate, DFS, OS, safety and tolerability, as well as the association between expression of P-gp by AML blasts at diagnosis and response to treatment in general and outcome with PSC-833 in particular. Our randomized phase III trial showed that the addition of PSC-833 to standard induction chemotherapy did not result in improved outcome. We did not detect a statistically significant improvement in EFS (HR = 1.07; 95% CI, 0.87-1.30; *P* = .53), resulting from similar CR rates in the two treatment arms (54% vs. 48%; OR = 1.27; 95% CI, 0.87-1.87, *P* = .22), and no improvement of DFS in the PSC-833 arm of the patients who actually achieved a CR (HR = 1.33; 95% CI, 0.99-1.78; *P* = .06). In addition, 5-year OS was 10% in both arms (HR = 1.07; 95% CI, 0.87-1.32; *P* = .52), which was similar to other phase III trials in patients with AML aged 60 years and older.[13,14] Adverse events more often observed in the PSC-833 arm concerned the central and peripheral nervous system, while also a higher incidence of liver AEs, i.e. bilirubinemia, was reported in the experimental arm despite a 22% dose-reduction in daunorubicin in the PSC-833 arm. In this trial restricted to patients with AML of older age, a high proportion of the patients had intrinsic multidrug resistance. A total of 73% of evaluable patients were classified as P-gp positive, based on drug efflux if available or on P-gp expression otherwise, in line with data presented by Leith *et al*.[4] We also observed a negative association between P-gp and treatment outcome; patients with the highest P-gp score, showed the lowest CR rates and worst survival outcomes, which confirms results of numerous retrospective trials.[3] In order to evaluate whether the benefit of PSC-833 was related to the degree of ABCB1, we also performed logistic and Cox regression analysis with a treatment arm by P-gp interaction term. No significant interaction was detected, suggesting no major difference in benefit of adding PSC-833 to daunorubicin and cytarabine between ABCB1-based subgroups. However, it should be kept in mind that this trial was not designed, and therefore lacking the power, to detect a moderate but possibly clinically relevant interaction a priori; moreover, for only 309 of the 419 eligible patients the P-gp status at diagnosis was available.

Two other phase III trials by the CALGB[15] and the ECOG[16] in patients with AML or MDS also failed to show a benefit of PSC-833. Accrual to both trials was discontinued early, due to unexpected excessive early mortality and lack of superiority in achieving CR in patients treated with PSC-833, respectively. The lack of efficacy led to the decision to halt further trials with PSC-833 in AML and the early closure in 2003 of another phase III trial by the CALGB.[17] The results of these phase III trials stress the importance to confirm favorable phase II results in independent phase III trials, before they are adopted as a new standard treatment.

Zosuquidar is a highly selective and potent third generation P-gp inhibitor which demonstrated minimal pharmacokinetic interaction with anthracyclines in phase I trials.[18,19] In the ECOG 3999 phase III trial, 442 eligible patients > 60 years of age, median 69, with newly diagnosed AML or high-risk MDS were randomized to receive daunorubicin (45 mg/m^2/d, D1-3) and cytarabine (100 mg/m^2/d, D1-7) and either Zosuquidar (550 mg/m^2) or placebo.[18] In this trial, Zosuquidar was given as a 6-hour infusion starting 1 hour prior to daunorubicin. The median OS was 7.7 months in the Zosuquidar arm versus 9.4 months in the placebo arm (P = .45), while in the subset of patients with high P-gp status these numbers were 4.6 and 8.3 months, respectively (P = .2). The lack of benefit of Zosuquidar in this trial may be due to an insufficient duration of inhibition of P-gp in leukemic cells, since Zosuquidar has a short half-life.[18] Accordingly, the ECOG initiated a phase I/II trial in newly diagnosed patients with AML 55-75 years of age, in which a 72-hour continuous infusion schedule of Zosuquidar was added to the same daunorubicin–cytarabine regimen as administered in the ECOG 3999 trial.[19] The phase I trial established 700 mg/d as the recommended dose of Zosuquidar for the phase II trial. Preliminary phase II data of 20 evaluable P-gp-positive patients revealed a 50% CR rate (including CR with incomplete platelet recovery). Accrual to the phase II trial is ongoing.[19]

Predicting treatment outcome in AML at older age

As mentioned before, MDR1 status at diagnosis is an important prognostic factor for clinical outcome. Besides ABCB1, other drug transport proteins, such as ABCC1, LRP and ABCG2, have also been reported to be expressed in AML. We have evaluated these 4 MDR proteins, and have investigated the expression and prognostic impact. ABCB1 was highly associated with ABCG2, while there was also a significant negative association between MRP1 and LRP. It was observed that ABCB1 was associated with a poor clinical outcome, while also the subgroup of patients with ABCB1/ABCG2 co-expression had a poor prognosis. The poor prognosis associated with mutual ABCB1 and ABCG2 expression was also found by Benderra *et al*[20] in 149 patients with newly diagnosed AML; patients with ABCB1/ABCG2 co-expression had a worse outcome than patients with expression of only ABCB1 or ABCG2, while patients without ABCB1 or ABCG2 expression had the best prognosis. This would suggest a rationale for modifying agents like GF120918 (Elacridar), also known as GG918, a modulator which blocks both ABCB1 and ABCG2,[21] but randomized clinical trials which evaluated its efficacy in AML have not been published. However, when we repeated our analyses with adjustment for other prognostic factors, neither ABCB1 nor ABCB1/ABCG2 co-expression were any longer statistically significantly associated with survival, while only

the latter resulted in a significantly lower CR rate. This might be partly explained by the highly significant association between ABCB1 expression and CD34, the latter being an important adverse prognostic factor for clinical outcome in AML.[22]

More recently, the presence of single nucleotide polymorphisms (SNPs) in the *ABCB1* gene has been described. These are mutations at one position of the gene. If a SNP results in an amino-acid substitution, it is called a non-synonymous SNP, otherwise it is called a synonymous SNP. The 3 most common SNPs in the *ABCB1* gene are the synonymous SNPs C1236T and C3435T, and the non-synonymous SNP G2677T. In our trial among 150 patients with SNP-data available, the clinical results between the allelic variants of the 3 SNPs, and between carriers and non-carriers of the 4 haplotypes based on the SNPs G2677T and C3435T were not significantly different. This was in contrast with results from a study in 405 relatively younger patients with AML by Illmer *et al,*[23] who showed that subgroups of patients with an unfavorable prognosis could be identified based on SNP-data only; an increased risk of relapse and a significantly lower OS was observed in patients with AML with the wild-type variant of G2677T. We did however confirm the linkage disequilibrium between each combination of two SNPs, which has been described previously. In contrast to many other publications, we also found that the 3 SNPs were in Hardy-Weinberg disequilibrium. This might be the result of the large number of countries that participated in this trial, and the variation in genetic variants of SNPs according to racial background,[24] although an alternative explanation might however be that subjects of 60 years or older with a homozygous genotype are more susceptible to the development of AML than those with a heterozygous genotype. Nevertheless we also showed that P-gp function and expression were not significantly different between the allelic variants, which suggests that the allelic variants of these 3 polymorphisms do not exert a major impact in this patient population.

Besides e.g. higher age and CD34 expression, specific cytogenetic abnormalities are important prognostic factors in AML. Until recently the association between karyotype and outcome was derived mainly from trials of younger patients. Karyotype analysis is performed at diagnosis for the vast majority of patients with AML. Because collection of these data was not part of the protocol, the local physicians and cytogeneticists were asked to provide the cytogenetic data, and for more than 85% of the patients, the available data were actually sent in. We observed a favorable prognosis only in the few patients with CBF abnormalities t(8;21) and inv(16)/del(16)/t(16;16), while most of the other evaluated cytogenetic aberrations like –5, 5q-, –7, +8, and(17p), abn(17q) and complex abnormalities, were associated with an unfavorable prognosis as compared to patients with a normal karyotype. The favorable prognosis of patients with CBF abnormalities and the unfavorable prognosis of abnormalities associated to chromosomes 5 and 17 and complex abnormalities were also observed in two

recently published papers in patients with AML aged 60 years and older by Farag *et al*[25] and Fröhling *et al*,[26] and earlier described in mainly younger patients with AML and reviewed by Mrozek *et al*.[27] However, while we observed a poor outcome of patients with +8, this was not confirmed in the other studies in elderly AML, while also in younger AML patients its prognosis has been reported both as intermediate or poor. Discordant results in elderly AML were also observed for abn(11q23). Accordingly, there is still no universally accepted prognostic index for patients with AML based on cytogenetic abnormalities, which stresses the need to karyotype all patients with AML treated in clinical trials, e.g. in the ongoing HOVON trials.

Today, mutations in particular genes and specific gene expression patterns in AML are associated with novel AML subclasses, and be used as predictors of clinical outcome.[28,29] These genetic explorations may also lead the way to the identification of new targets for therapy with the aim of circumventing chemotherapy resistance. New drugs may be designed with the deliberate objective of affecting a known molecular lesion or signaling pathway in the neoplastic cell, thus critically inhibiting leukemia cell survival.[30] These therapeutic compounds may tackle distinct molecular subsets of leukemia. It is anticipated that their greater specificity will allow for application with enhanced effect and reduced toxicity. Another class of drugs is being developed through redesign of the chemical structure of currently available chemotherapeutic agents in an effort to accomplish a better efficacy profile of the drug. The merits of these new drugs will need to be clarified in subsequent trials, which will need to be done in the context of combination chemotherapy, which may ultimately advance treatment development in older patients with AML.[30]

In conclusion, there can be no question about the urgent need for intense further research which hopefully will precipitate better treatment strategies for the older patient population. In fact, the National Comprehensive Cancer Network, a consortium of prominent American cancer centers, considers “clinical trials” as the preferred option in patients with untreated AML age 60 and above.[31] Better clinical outcome of (subgroups of) elderly patients with AML remains to be achieved through improvement of CR rates and prolongation of the remission duration, with the eventual aim to prolong survival and improve survival quality.[30] The value of the new treatment modalities will ultimately have to be established in independent, prospective, clinical trials.

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CHAPTER 8

Summary / samenvatting

Summary

Acute myeloid leukemia (AML) is a clonal disease, characterized by an increase in the number of myeloid cells in the bone marrow and a maturation arrest during the differentiation to mature blood cells. When untreated, the disease is generally fatal in weeks to months.
AML is a disease that is most prevalent in older people, with a median age in the order of 65 years. The common way of treating older patients with AML involves induction and consolidation chemotherapy, with the aim of attaining a complete hematological remission and with the subsequent aim to maintain the response. The outcome of patients aged 60 years or older is, however, rather dismal with complete response (CR) rates of about 50% and 5-year overall survival (OS) of about 10%.
A major problem in the treatment of AML is the high rate of failure to respond to the chemotherapy that may involve various cytotoxic agents. This phenomenon of multidrug resistance (MDR), refers to the condition that cancer cells are resistant to a broad range of structurally and functionally unrelated cytotoxic agents. The classical form of multidrug resistance, i.e. MDR1 (ABCB1), relates to the (membrane) P-glycoprotein (P-gp), a functional transporter that extrudes cytotoxic agents out of the cell. High expression of ABCB1 on leukemia cells is associated with a diminished accumulation of various types of chemotherapeutic drugs in leukemic cells. The prevalence of the MDR phenotype in patients with AML increases with higher age. Therapeutic drugs have been developed that inhibit ABCB1 function with the purpose of overcoming drug resistance. Initial phase I and phase II trials with the P-gp inhibitor PSC-833 (Valspodar) had shown promising but not definitely conclusive results. This resulted in the launch of an international, randomized phase III trial under auspices of the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON) and the United Kingdom Medical Research Council (UK MRC), addressing the value of the addition of PSC-833 in induction chemotherapy in newly diagnosed patients with AML of 60 years or older. Between May 1997 and February 1999, 419 eligible patients were randomized to receive induction chemotherapy with daunorubicin and cytarabine with or without PSC-833, to investigate whether the addition of PSC-833 would improve clinical outcome. The trial and its outcome, as well as several side studies, are the subject of this thesis.
In **chapter 2** the results of the phase III trial are presented. Patients of 60 years or older with untreated, newly diagnosed AML were randomized to receive standard induction chemotherapy consisting of cytarabine (200 mg/m^2/day, D1-7) and daunorubicin (45 mg/m^2/day, D1-3), or the same regimen with the addition of PSC-833 (2 mg/kg in a 2-hour loading dose followed by 10 mg/kg/day, D1-3), but with daunorubicin in a dose of

35 mg/m^2/day, which according to the pilot study was the maximum tolerated dose when combined with PSC-833.

We demonstrate in **chapter 2** that combining PSC-833 with remission-induction chemotherapy consisting of cytarabine and daunorubicin does not result in significantly higher CR rates nor in better event-free survival (EFS), disease-free survival (DFS) and OS. Previous studies had indicated that an increased expression of multidrug resistance markers, as determined by P-gp efflux and expression, was associated with worse clinical outcome. These results are confirmed in the phase III trial in previously untreated patients with AML of older age, who indeed have a high frequency of the multidrug resistance marker ABCB1.

Apart from P-gp efflux and expression, classical MDR can also be assessed by measuring *ABCB1* mRNA levels. Other MDR proteins have also been investigated in patients with AML, e.g., the transmembrane proteins multidrug resistance-associated protein (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2), and the lung resistance related protein (LRP). In **chapter 3** and **chapter 4**, the association between these four MDR proteins was investigated, as well as their impact on clinical outcome. We noticed that frequencies of high expression of ABCB1 and ABCG2 were highly associated, and also a highly significant association between ABCC1 and LRP expression was observed. The impact of higher mRNA levels on clinical outcome was however limited. We showed that neither ABCC1, LRP nor ABCG2 were significantly associated with CR rate, nor with the survival endpoints EFS, DFS and OS. Higher *ABCB1* mRNA levels predicted poor clinical outcome when evaluated in a univariate analysis, while also ABCB1/ABCG2 co-expression was associated with a lower CR rate and decreased EFS and OS. However, when adjusted for other confirmed prognostic factors in AML, like previous MDS, cytogenetic risk classification and CD34 expression, only ABCB1/ABCG2 co-expression remained associated with a lower CR rate but not with survival, while the *ABCB1* mRNA level appeared no longer a statistically significant prognostic determinant at all. In fact, we showed that ABCB1 and CD34 were highly correlated, which may partly explain the latter result.

The *ABCB1* gene is located on chromosome 7. Mutations at one position of a gene are called single nucleotide polymorphisms (SNPs). In **chapter 5** we investigated whether SNPs at positions 1236, 2677 and 3435 of the *ABCB1* gene were associated with each other. We also examined a possible relationship of these genetic polymorphisms with clinical outcome. In this population of older, mainly Caucasian patients with AML, we found that each of the three SNPs was in Hardy-Weinberg disequilibrium, an observation that was contradictory to many other publications. In fact we observed less heterozygous patients than expected. Each combination of two SNPs was also in linkage disequilibrium, i.e. the wild-type, heterozygous respectively homozygous mutant allelic variants of two SNPs were highly correlated, which

has been reported previously. There was however no major impact of the three SNPs on clinical outcome, neither for each of the individual SNPs separately nor when the haplotypes based on the SNPs G2677T and C3435T were considered.

AML subgroups had been identified which reflect subsets with variable prognosis, i.e., favorable or unfavorable. Characterization of unfavorable subgroups is in part determined by (higher) age and certain cytogenetic abnormalities in leukemic blasts. The impact of the specific cytogenetic aberrations on prognosis had however been established mainly in patients with AML of younger age. In **chapter 6** AML-specific cytogenetic abnormalities were evaluated in relation to the clinical outcome parameters in the study series of AML patients aged 60 years and older. In 293/419 patients who were included in the phase III trial, the cytogenetics data were obtained. The leukemias with distinct particular AML-specific cytogenetic abnormalities were each represented in relatively low numbers in this series of limited size due to the low incidence of these abnormalities. Nevertheless, the data may seem consistent with a comparatively good prognosis of patients with AML with core-binding factor (CBF) abnormalities t(8;21) and inv(16)/del(16)/t(16;16) in this older patient population, an observation which had previously been found in younger patients. Various other cytogenetic aberrations were associated with a very poor prognosis as compared to AML with a normal karyotype, including abnormalities of chromosomes 5 and 17, –7, +8 and complex abnormalities. Our results and those of two other recently published trials in elderly patient populations, suggest that cytogenetic abnormalities in older patients with AML in general confer a poor prognosis. Nevertheless, larger series of patients are required to confirm these results and to be able to classify patients with the rare abnormalities, and to come up with a universal, validated risk score for patients aged 60 years and older with AML.

The results of this thesis are discussed in **chapter 7**. The overall outcome of the randomized phase III trial are similar to other published reports in this population of patients with AML of 60 years and older, with a CR rate of 51% and a 5-year OS of 10%. However, the addition of PSC-833 to standard induction chemotherapy did not result in favorable outcome in the experimental arm. As the outcome of patients treated with cytarabine and daunorubicin remains disappointing, investigations for better treatment modalities remain warranted. Suggestions for further clinical research have been presented. The side studies attached to clinical trials may reveal subgroups of patients with a poor prognosis, who are candidates for specific experimental therapy. The safety and effectiveness of these new treatment regimens in specific subgroups of patients must ultimately be evaluated through prospective clinical trials.

Samenvatting

Acute myeloïde leukemie (AML) is een vorm van kanker, die zich kenmerkt door een toename in het beenmerg van onrijpe cellen (blasten) die niet uitrijpen tot normaal functionerende bloedcellen. Wanneer de ziekte niet wordt behandeld, zal de patiënt in het algemeen binnen enkele weken tot maanden overlijden.
AML is een ziekte die voornamelijk voorkomt bij ouderen, met een mediane leeftijd van ongeveer 65 jaar. De behandeling van oudere patiënten met AML bestaat gewoonlijk uit inductie- en consolidatie chemotherapie, met als doel het bereiken van een complete hematologische remissie en vervolgens die response te behouden. De resultaten van de behandeling van patiënten van 60 jaar en ouder zijn echter teleurstellend, met complete response (CR) percentages van ongeveer 50% en een 5-jaars overleving (OS) van ongeveer 10%.
Een groot probleem bij de behandeling van AML is het hoge percentage patiënten dat niet reageert op de chemotherapie die uit verschillende cytotoxische (celdodende) middelen kan bestaan. Dit fenomeen, multidrug resistentie (MDR) genaamd, verwijst ernaar dat kankercellen resistent zijn voor een breed spectrum aan cytotoxische middelen die onderling verschillen qua structuur en werkzaamheid. De klassieke vorm van multidrug resistentie, MDR1 (ABCB1), heeft betrekking op het (membraan) eiwit P-glycoprotein (P-gp), een functionele transporter die cytotoxische middelen uit de cel pompt. Een hoge expressie van ABCB1 op leukemiecellen is geassocieerd met een verminderde opname van verschillende typen chemotherapeutische middelen in leukemiecellen. Het MDR fenotype komt vaker voor naarmate patiënten met AML ouder zijn. Er zijn therapeutische middelen ontwikkeld die de functie van ABCB1 hinderen om zo de multidrug resistentie te overwinnen. Initiële fase I en fase II studies met de P-gp remmer PSC-833 (Valspodar) hadden wel veelbelovende, maar nog geen afdoende resultaten laten zien. Dit leidde tot een internationale, gerandomiseerde fase III studie onder leiding van de HOVON (Stichting Hemato-Oncologie voor Volwassen Nederland) en de Engelse Medical Research Council (MRC), naar de waarde van de toevoeging van PSC-833 aan inductie chemotherapie bij nieuw gediagnosticeerde patiënten met AML van 60 jaar en ouder. Tussen mei 1997 en februari 1999 werden 419 eligible patiënten gerandomiseerd tussen inductie chemotherapie bestaande uit daunorubicine en cytarabine met of zonder PSC-833, om te onderzoeken of toevoeging van PSC-833 de klinische resultaten zou verbeteren. Dit proefschrift gaat over deze fase III studie en de uitkomsten daarvan, alsmede over enkele nevenstudies.
In **hoofdstuk 2** worden de resultaten van de fase III studie beschreven. Patiënten van 60 jaar en ouder met onbehandelde, nieuw gediagnosticeerde AML werden gerandomiseerd tussen

een behandeling met standaard inductie chemotherapie bestaande uit cytarabine (200 mg/m^2/dag, D1-7) en daunorubicine (45 mg/m^2/dag, D1-3), of hetzelfde schema met PSC-833 (inloopschema van 2 mg/kg in 2 uur gevolgd door 10 mg/kg/dag, D1-3) daaraan toegevoegd, maar daunorubicine in een dosering van 35 mg/m^2/dag, wat volgens de pilot studie de maximaal getolereerde dosis (MTD) was wanneer het werd gegeven in combinatie met PSC-833.

We laten in **hoofdstuk 2** zien dat de combinatie van PSC-833 met remissie inductie chemotherapie bestaande uit cytarabine en daunorubicine niet leidt tot significant hogere CR percentages, en ook niet tot een verbetering van event-vrije overleving (EFS), ziekte-vrije overleving (DFS) en OS. Voorgaande studies hadden aangetoond dat een verhoogde expressie van multidrug resistentie merkers, vastgesteld door P-gp functie en expressie, geassocieerd was met slechtere klinische resultaten. Dit wordt bevestigd door de fase III studie in onbehandelde oudere patiënten met AML, die zich sowieso vaak presenteren met de multidrug resistentie merker ABCB1.

Naast P-gp uitscheiding en expressie, kan klassieke MDR ook bepaald worden door het meten van het *ABCB1* mRNA niveau. Er zijn ook andere MDR eiwitten onderzocht in patiënten met AML, bijvoorbeeld de transmembraan eiwitten multidrug resistance-associated protein (MRP1/ABCC1) en breast cancer resistance protein (BCRP/ABCG2), en het lung resistance related protein (LRP). In **hoofdstuk 3** en **hoofdstuk 4**, wordt de associatie tussen deze 4 MDR eiwitten onderzocht, alsmede hun invloed op de klinische uitkomst. We vonden dat ABCB1 en ABCG2 expressie sterk gecorreleerd waren, alsmede een significante associatie tussen ABCC1 en LRP expressie. De invloed van hogere mRNA niveaus op de klinische resultaten was echter beperkt. We hebben vastgesteld dat noch ABCC1 en LRP, noch ABCG2 significant geassocieerd waren met het CR percentage, of met de overlevingseindpunten EFS, DFS en OS. Hogere *ABCB1* mRNA niveaus lieten wel slechtere klinische resultaten zien in een univariate analyse, terwijl ook ABCB1/ABCG2 co-expressie geassocieerd was met een lager CR percentage en een slechtere EFS en OS. Echter, wanneer de analyse werd gedaan, geadjusteerd voor andere erkende prognostische factoren in AML zoals voorafgaande MDS, cytogenetische risicoclassificatie en CD34 expressie, dan was ABCB1/ABCG2 co-expressie alleen nog geassocieerd met een lager CR percentage maar niet met overleving, terwijl het mRNA niveau van *ABCB1* in het geheel niet meer een statistisch significante factor was. Wel vonden we dat ABCB1 en CD34 zeer significant gecorreleerd waren, wat wellicht deels een verklaring kan zijn voor voorgaande bevinding.

Het *ABCB1* gen ligt op chromosoom 7. Mutaties op één positie van een gen worden single-nucleotide polymorfismen (SNP) genoemd. In **hoofdstuk 5** hebben we onderzocht of SNPs op posities 1236, 2677 en 3435 van het *ABCB1* gen met elkaar geassocieerd zijn. We hebben

ook gekeken of er een mogelijk verband is tussen deze genetische polymorfismen en de klinische resultaten. In deze populatie van oudere, voornamelijk Kaukasische patiënten met AML, zagen we dat elk van de drie SNPs in Hardy-Weinberg disequilibrium was, wat niet in overeenstemming was met vele andere publicaties. In feite zagen we minder heterozygote patiënten dan verwacht. Elke combinatie van twee SNPs was ook in linkage disequilibrium, dat wil zeggen dat de wild-type, heterozygote, respectievelijk homozygoot mutante allelische varianten van twee SNPs sterk gecorreleerd waren, iets wat al eerder gepubliceerd was. Er was echter geen grote invloed van de drie SNPs op de klinische uitkomsten, noch voor elk van de SNPs apart, noch wanneer werd gekeken naar de haplotypes gebaseerd op de SNPs G2677T en C3435T.

Er zijn verschillende subgroepen patiënten met AML bekend met elk hun eigen prognose, hetzij gunstig, hetzij ongunstig. Ongunstige subgroepen worden deels bepaald door (hogere) leeftijd en bepaalde cytogenetische afwijkingen in leukemie blasten. De impact van de specifieke cytogenetische afwijkingen op de prognose was echter vooral bepaald bij jongere patiënten met AML. In **hoofdstuk 6** zijn AML-specifieke cytogenetische afwijkingen geëvalueerd in relatie tot de klinische uitkomsten in de patiënten met AML van 60 jaar en ouder die in de fase III studie waren geïncludeerd. Van 293 van de 419 patiënten werden de cytogenetische data verkregen. De verschillende AML-specifieke cytogenetische afwijkingen kwamen elk maar voor in betrekkelijk weinig patiënten wegens het beperkte aantal patiënten in deze studie en de geringe incidentie van deze afwijkingen. Desalniettemin lijken de resultaten de betrekkelijk goede prognose te bevestigen van patiënten met AML met core-binding factor (CBF) afwijkingen t(8;21) en inv(16)/del(16)/t(16;16) in deze oudere patiënten populatie, wat eerder al aangetoond was in jongere patiënten Diverse andere cytogenetische afwijkingen werden geassocieerd met een zeer slecht prognose vergeleken met patiënten met AML met een normaal karyotype, zoals afwijkingen van chromosomen 5 en 17, –7, +8 en complexe afwijkingen. Onze resultaten en die van twee andere recent gepubliceerde studies in oudere patiënten populaties, suggereren dat cytogenetische afwijkingen in oudere patiënten met AML over het algemeen geassocieerd zijn met een slechte prognose. Desalniettemin zijn grotere series patiënten nodig om deze resultaten te bevestigen en om patiënten met zeldzame afwijkingen te kunnen classificeren, en om tot een universele gevalideerde risicoscore voor patiënten van 60 jaar en ouder met AML te komen.

De resultaten van deze studie worden besproken in **hoofdstuk 7**. De uitkomsten van de gerandomiseerde fase III studie komen overeen met andere trial publicaties over patiënten met AML van 60 jaar en ouder, met een CR percentage van 51% en een 5-jaars overleving van 10%. Helaas leidde de toevoeging PSC-833 aan standaard inductie chemotherapie niet tot betere resultaten in de experimentele behandelarm. Aangezien de uitkomsten van patiënten

behandeld met cytarabine en daunorubicine teleurstellend blijven, is verder onderzoek naar betere behandelingen noodzakelijk. Er zijn enkele suggesties voor verder klinisch onderzoek gedaan. De nevenstudies die aan klinische studies hangen kunnen mogelijk subgroepen van patiënten identificeren met een slechte prognose, die kandidaat zijn voor een specifieke experimentele behandeling. De veiligheid en effectiviteit van deze nieuwe behandelschema's in specifieke subgroepen patiënten moeten uiteindelijk geëvalueerd worden in prospectieve klinische studies.

Abbreviations

4IWCL	Fourth International Workshop on Chromosomes in Leukemia
ABC	ATP-binding cassette
ABCB1	ABC protein transporter, subfamily B, member 1
ABCC1	ABC protein transporter, subfamily C, member 1
ABCG2	ABC protein transporter, subfamily G, member 2
abn	abnormality
AE	adverse event
Ala	alanine
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANC	absolute neutrophil count
Ara-C	cytarabine
ATP	adenosine triphosphate
AUC	area under the curve
BCRP	breast cancer resistance protein
BM	bone marrow
bp	base pair
CALGB	Cancer and Leukemia Group B
CBF	core-binding factor
cDNA	complementary DNA
CI	confidence interval
CNS	central nervous system
CR	complete response
CRF	case report form
CsA	Cyclosporin A
CTC	Common Toxicity Criteria
del	deletion
DFS	disease-free survival
DMEM	Dulbecco modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNR	daunorubicin
ECOG	Eastern Cooperative Oncology Group
EFS	event-free survival
ETO	etoposide

FAB	French-American-British
GOELAM	Groupe Ouest Est Leucemies Aigues Myeloblastiques
HIV	human immunodeficiency virus
HOVON	Dutch-Belgian Hemato-Oncology Cooperative Group
HR	hazard ratio
inv	inversion
IPS	integrated P-gp score
ISCN	International System for Human Cytogenetic Nomenclature
IULN	institutional upper limit of normal
LRP	lung resistance related protein
mAb	monoclonal antibody
MDR	multidrug resistance
MDS	myelodysplastic syndrome
mIg	monoclonal immunoglobulin
MRC	Medical Research Council
mRNA	messenger RNA
MRP1	multidrug resistance-related protein
MVP	major vault protein
MXT	mitoxantrone
NCI	National Cancer Institute
NCIC	National Cancer Institute of Canada
OR	odds ratio
OS	overall survival
PB	peripheral blood
PCR	polymerase chain reaction
P-gp	P-glycoprotein
Rho123	rhodamine 123
RNA	ribonucleic acid
RR	relative risk
RT-PCR	reverse-transcriptase PCR
SD	standard deviation
Ser	serine
SNP	single-nucleotide polymorphism
SWOG	Southwest Oncology Group
t	translocation
WBC	white blood cell
WHO	World Health Organization

Dankwoord

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Curriculum vitae

De auteur van dit proefschrift werd op 5 februari 1969 geboren in Roden. Hij behaalde in 1987 zijn VWO-diploma aan het Nienoordcollege te Leek. Daarna ging hij Wiskunde studeren aan de Rijksuniversiteit Groningen, en in 1993 studeerde hij af in de Statistiek en Stochastiek, bij Dr. H.G. Dehling. Tijdens het laatste half jaar van zijn studie deed hij een aanvullende stage Medische Statistiek in de Dr. Daniel den Hoed Kliniek (DDHK) in Rotterdam, begeleid door Drs. W.L.J. van Putten.
Na zijn afstuderen werd hij opgeroepen om zijn dienstplicht vervullen.
Op 1 mei 1994 trad hij in dienst als statisticus bij de afdeling Trialbureau, Medische Registratie & Statistiek (TMS) van de DDHK. Sinds de fusie van de DDHK met het Dijkzigt Ziekenhuis, het Sophia Kinderziekenhuis en de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam, is hij werkzaam als biostatisticus in het Erasmus MC-Daniel den Hoed, op de afdeling Trials & Statistiek-HOVON Data Centrum (hoofd Drs. W.L.J. van Putten).
Sinds zijn indiensttreding is hij betrokken bij vele (inter)nationale klinische studies op het gebied van de hematologie, welke uitgevoerd worden door de Stichting Hemato-Oncologie voor Volwassenen Nederland (HOVON). Zijn aandachtsgebieden omvatten momenteel vooral acute lymfatische en myeloïde leukemie, AL amyloïdose, chronische myeloïde leukemie, multipel myeloom, non-Hodgkin lymfoom en stamceltransplantaties.
Daarnaast omvatten zijn werkzaamheden statistische consultaties voor artsen en onderzoekers van verschillende afdelingen in het Erasmus MC, o.a. (in alfabetische volgorde) chirurgie, hematologie, hyperthermie, immunologie, neurologie, psychiatrie, radiotherapie en virologie.
Sinds juni 2001 is hij geregistreerd als Biostatisticus - VVS.
Eind 2001 raakte hij betrokken bij het onderzoek dat in dit proefschrift is beschreven. Na een periode van ruim anderhalf jaar, waarin nog veel aanvullende data verkregen werden, heeft hij sinds september 2003 gewerkt aan de analyse van de gegevens en de publicatie van de resultaten, onder begeleiding van Prof.dr. P. Sonneveld en Prof.dr. B. Löwenberg van de afdeling hematologie van het Erasmus MC.
Hij is getrouwd met Fonny Wilkens, en samen hebben ze een dochter, Kirsten.

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