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Flow cytometric maturity score as a novel prognostic parameter in patients with acute myeloid leukemia

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Abstract:

The European LeukemiaNet (ELN) classification is widely accepted for risk stratification of patients with acute myeloid leukemia (AML). In order to establish immunophenotypic features that predict prognosis, the expression of single AML blast cell antigens has been evaluated with partly conflicting results; however, the influence of immunophenotypic blast maturity is largely unknown.

In our study, 300 AML patients diagnosed at our institution between 01/2003 and 04/2012 were analyzed. A flow cytometric maturity score was developed in order to distinguish "mature" AML (AML-ma) from "immature" AML (AML-im) by quantitative expression levels of early progenitor cell antigens (CD34, CD117, and TdT).

AML-ma showed significantly longer relapse-free survival (RFS) and overall survival (OS) than AML-im (p<0.001). Interestingly, statistically significant differences in RFS and OS were maintained within the "intermediate risk" group according to ELN (RFS: 7.0 years (AML-ma) vs. 3.3 years (AML-im); p=0.002; OS: 5.1 years (AML-ma) vs. 3.0 years (AML-im); p=0.022).

Our novel flow cytometric score easily determines AML blast maturity and can predict clinical outcome. It remains to be clarified whether these results simply reflect an accumulation of favorable molecular phenotypes in the AML-ma subgroup or whether they rely on biological differences such as a higher proportion of leukemia stem cells and/or a higher degree of genetic instability within the AML-im subgroup.

Introduction

Acute myeloid leukemia (AML) is a malignant haematopoietic neoplasm mainly occurring in elderly patients ≥65 years of age [1]. Cytogenetic and molecular genetic abnormalities are thought to drive clonal expansion of early haematopoietic progenitor cells, which leads to rapid progressive suppression of normal bone marrow haematopoiesis. Subsequently, patients suffering from AML develop symptoms attributed to granulocytopenia, anaemia, and thrombocytopenia [2]. Besides age, cytogenetic and molecular features are the main prognostic factors that influence survival [3,4]. Hence, in 2009 the European LeukemiaNet (ELN) proposed a standardized reporting system that risk stratifies patients according to their genetic subgroup. As of today, it is well established for early prognostic assessment in AML patients [5,4,6,7,3,8-10]. Thus, patients in this cohort were primarily grouped according to these criteria [5].

In addition to conventional cytogenetics and molecular genetics, flow cytometric analysis of blast cells plays an essential role in diagnosing AML. The prognostic significance of the expression of particular antigens remains controversial with previous studies mostly focusing on single antigens [11-14]. In contrast, the influence of

immunophenotypic maturity of AML blasts on overall prognosis is largely unknown. This is the first study using a quantitative score, consisting of three routinely used early progenitor cell markers for the assessment of AML blast maturity. The study was conducted to determine the influence of blast maturity on clinical parameters such as complete/incomplete remission after induction (CR/CRi), relapse-free survival (RFS), and overall survival (OS) both in the whole cohort and within the different risk groups according to ELN.

Patients and Methods

Patients

Patients included in this study were ≥18 years and newly diagnosed with AML at the Charité University Medical Center Berlin, Campus Virchow Klinikum, from 01/2003 through 04/2012. Only patients with available flow cytometry report from our institution were included. AML-related data and patient demographics were collected for each patient. These included morphologic findings, conventional cytogenetics, molecular genetics (*FLT3, NPM1*, and *MLL*), blood count, type of treatment, date of CR/CRi as well as date of relapse, stem cell transplantation and death (Table 1).The retrospective study was performed after informed consent for bone marrow diagnostics and was in accordance with the local ethical guidelines.

Flow cytometric analysis

Of the 300 patients eligible for this study, flow cytometric analysis of bone marrow (n=284) or peripheral blood (n=16) was performed. EDTA samples were obtained and subsequently incubated with fluorochrome-labeled antibodies (FITC, PE, PC5.5, or APC) for the detection of cell surface antigens (CD1a, CD2, CD3, CD4, CD5, CD7, CD7.1/NG2, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD19, CD20, CD22, CD33, CD34, CD36, CD38, CD56, CD64, CD65, CD117, CD133, CD235a, HLA-DR) and intracytoplasmatic antigens (MPO, TdT, LF, cyCD3, cyCD22, cyCD79a) using BD FACSCalibur or BD FACSCanto™ systems (Becton, Dickinson and Company, San Jose, CA, USA). BD FACSDiva™ or BD CellQuest™ software was used for analysis of flow cytometry data. The blast cell population was identified by CD45/side scatter (SSC) gating of at least 30,000 cells. Antigen expression was considered positive when ≥20% (surface antigens) or ≥10% (intracellular antigens) gated cells were positive.

AML maturity score

To classify AML according to the degree of maturity, a score based on the quantitative expression of CD34, CD117 and TdT was developed. All three markers are clearly associated with immaturity, can easily be

quantified, and had been evaluated over the whole study period [15]. The maturity score was weighed towards CD34 and TdT, since these markers show a particularly strong correlation with immaturity. As shown in Table 2a, a score of 5 indicates maximal immaturity and a score of 0 indicates maturity. Table 2b shows the distribution of patients within the different "maturity groups". A cut-off value of <1.5 points was the strongest discriminator with regard to RFS and OS (*p*<0.001, respectively) as determined by Kaplan-Meier survival curves. The cut-off value was not determined by the standardized median-split method, as the cut-off value of <1.5 points was a stronger discriminator with regard to RFS and OS. The purpose of this classification was to achieve a factor variable with two groups that can be used to describe the influence of the immaturity on the RFS and OS within a Kaplan-Meier-Analysis based on the developed score.

Consequently, 109 patients (39.5%) were assigned to the "mature" group (0-1 points; "AML-ma") and 167 patients (60.5%) to the "immature" group (1.5-5 points, "AML-im"). The maturity score showed a good correlation with morphological maturity according to the FAB classification (data not shown).

Data collection was performed using IBM SPSS Statistics®, version 20 (IBM® 2011, Armonk, NY, USA). Clinical parameters were CR/CRi, defined as bone marrow blasts <5% with or without blood count recovery after induction therapy, RFS and OS [5]. Survival characteristics were analyzed by means of the Kaplan Meier method. Differences were determined by the Chi Square and Fisher's Exact Test (CR/CRi) and the Log rank test (RFS, OS). A p-value of *p*<0.05 was considered significant.

Results

Patients' characteristics

The baseline characteristics of the patients are given in Table 1. Median age at diagnosis was 61 years with a male predominance. Cytogenetic abnormalities were detected in n=170/273 patients (62.3%). Molecular genetic alterations (*NPM1, FLT3, MLL*) were found in n=92/256 patients (35.9%). Patients with incomplete cytogenetic results or mixed phenotype leukemias (n=53) were excluded from the analysis within the different ELN risk groups. Hence, we were able to assign 247/300 patients to an ELN risk group. The mean follow-up of all patients was 29.4 months (range 0-116.5 months). 206 of 300 (69%) patients reached CR/CRi after induction chemotherapy. In patients <60 years of age (n=141/300), the CR/CRi rate was 81.6% vs. 57.2% in patients ≥60 years (n=159/300), (*p*<0.001). Mean RFS was 69.5 months with a higher RFS in patients <60 years (76.9 months vs. 58.5 months in patients ≥60 years), (*p*=0.057, Fig 1a). Mean OS was determined to be 46.7 months, being significantly longer in patients <60 years (61.0 months) compared to patients ≥60 years (33.7 months), (*p*<0.001, Fig 1b).

Immunophenotypic maturity and clinical outcome

In 276 of 300 patients (92%) the maturity score could be determined. In 24 of 300 patients, flow cytometric analysis did not include all necessary parameters for the calculation of the novel maturity score. Regarding the CR/CRi rate, a tendency towards higher CR/CRi rates was observed in AML-ma (AML-ma: 73.4% vs. AML-im: 64.1%, p=0.115), however, this was not statistically significant, neither in the group as a whole nor in subgroups divided by age, <60 years vs. ≥60 years (data not shown).

In contrast, AML-ma showed a significantly longer mean RFS (89.4 months) when compared to AML-im (51.5 months), (p<0.001). This difference was consistently found both in the whole cohort and in the age-related subgroups: In patients <60 years, the mean RFS was 92.1 months for AML-ma patients and 58.4 months for AML-im patients) (p=0.005) (Fig 2a). In patients ≥60 years, RFS was 82.7 months for patients with AML-ma vs. 42.2 months for AML-im patients (p=0.01) (Fig 2b).

Regarding OS, patients in the AML-ma group had a longer mean OS (63.8 months) than patients in the AML-im group (32.9 months) (p<0.001). Again, this difference was consistently observed both in the whole cohort and in age-related subgroups (AML patients <60 years: p<0.001 and AML patients ≥60 years: p=0.033) (Fig 2c,d).

Influence of immunophenotypic maturity on clinical parameters within the different ELN risk groups

In order to further evaluate our immunophenotypic maturity score, the patients were categorized according to the ELN risk groups. The subdivision of the intermediate ELN risk group into "intermediate 1" and "intermediate 2" has so far been controversial. Therefore, we combined these two groups into one intermediate risk group. Thus, adequate patient numbers within the three different groups were obtained. In our AML cohort, the following risk groups according to ELN were determined: favorable risk (n=45), intermediate risk (n=132), and adverse risk (n=70). Within these different risk groups according to ELN, clinical parameters such as CR/CRi rate, RFS, and OS were consistent with the published literature [5]. In the ELN subgroups "favorable" vs. "intermediate" vs. "adverse" CR/CRi rates were 86.7% vs. 78.0% vs. 55.7% (p<0.001), RFS was 93.8 months vs. 62.5 months vs. 53.1 months (p=0.002) and OS was 77.5 months vs. 49.7 months vs. 28.0 months (p<0.001) (data not shown).

Subsequently, the influence of our novel maturity score was evaluated within the ELN subgroups with regard to clinical parameters such as CR/CRi rate, RFS, and OS. Fig 3 shows the distribution of our AML cohort within the different ELN risk groups.

Within the defined ELN risk groups, AML-ma showed higher CR/CRi rates than the AML-im patients. However, these differences did not reach statistical significance (data not shown). Regarding survival, in the

"favorable risk" group a longer RFS was observed in patients with AML-ma as compared to AML-im (102.5 months vs. 83.7 months), however, this difference was not statistically significant (*p*=0.214) (Fig 4a). In contrast, in the "intermediate risk" group, which was the largest in our cohort, a highly significant difference in RFS in favor of the AML-ma subgroup was observed (84.5 months vs. 39.7 months, *p*=0.002) (Fig 4c). In the "adverse risk" group, no difference could be observed (data not shown).

Furthermore, a longer OS in AML-ma patients as compared to AML-im patients could be observed across all ELN risk groups. However, this difference in OS reached statistical significance only in the "intermediate" risk group (AML-ma 61.4 months vs. 35.6 months, *p*=0.022) (Fig 4b).

In order to exclude that these differences were caused by an imbalance of patients who had undergone allogeneic bone marrow transplantation (n=77/276), the analyses were repeated for all patients who had not been transplanted (n=223/276). Nevertheless, the differences in RFS and OS were maintained after exclusion of patients who had undergone allogeneic stem cell transplantation.

Biological phenotype in maturity groups and ELN subgroups

In order to further characterize the subgroup of AML patients who were categorized as AML-ma and AML-im according to our maturity score, we analyzed the frequency of cytogenetic and molecular aberrations in both subgroups. In the AML-ma subgroup we found a significant accumulation of *PML-RARA* and $NPM1^{mut}$ (ELN subgroup "favorable risk") and CN-AML (ELN subgroup "intermediate risk"). In contrast, in the AML-im subgroup, there was a substantial accumulation of adverse phenotypes such as complex aberrant and monosomal karyotypes (ELN subgroup "adverse risk"). The differences are shown in Table 3.

Discussion

To the best of our knowledge, this is the first study evaluating AML blast maturity by means of a quantitative flow cytometric score in order to predict clinical outcome. We were able to show that a mature AML blast immunophenotype (AML-ma) was associated with a significantly longer RFS and OS than an immature immunophenotype (AML-im), (p<0.001). This was at least partly attributable to an accumulation of "low-risk" AML phenotypes such as $NPM1^{mut}$ and *PML-RARA* in the AML-ma group. "High-risk" aberrations (monosomal and complex aberrant karyotypes, -5 or del(5q), -7) had a higher frequency in the immature subgroup (see Table 3).

However, the differences in RFS and OS were maintained in a subgroup analysis within the different ELN risk groups. Statistical significance was only obtained in the "intermediate risk" group according to ELN with AML-

ma being superior to AML-im with regard to RFS (p=0.002) and OS (p=0.022). No statistical significance could be observed in the favorable and adverse risk groups, possibly due to lower patient numbers in these subgroups. Although there was a trend towards better CR/CRi rates within the AML-ma subgroup, these differences were not statistically significant.

The median age at first diagnosis of AML was 61 years in our patient cohort and is somewhat lower than reported in the literature (median about 67 years) [1]. This might explain, why our 5-year overall survival rate of 38.3% is higher than in previously published reports such as the US cancer registry (24.9%) [1]. A Swedish registry study showed a decrease in 5-year overall survival with every 5-years of age increase (<50 years: 51%, 50-54 years: 40%, 55-59 years: 23%, 60-64 years: 23%, 65-69 years: 13%, 70-74 years: 5%, 75-79 years: 3%, 80-84 years: 2%, ≥85 years: 0%) [16].

Additionally, our study included only patients with a comprehensive flow cytometric report, thereby indirectly excluding patients not suitable for intensive treatment since in those patients initial diagnostics are often restricted to morphologic evaluation. The monocentric design of the study at a large university center further enhances a selection bias towards younger patients in good clinical condition.

The assignment to the different ELN risk groups was determined by cytogenetic and molecular data. During the study period, routine molecular genetic analyses performed in AML patients at initial diagnosis have become more and more comprehensive, whereas these analyses were performed with lower frequency and contained less molecular markers at the beginning of the study period. This may explain the lower frequency of molecular aberrations observed in our study as compared with the literature. Furthermore, many molecular aberrations which may impact clinical outcome (such as *c-kit, DNMT3A* and *IDH* mutations) have not been analyzed and – more importantly – are not yet integrated in the ELN risk stratification. It is very likely that the possible underestimation of molecular phenotypes in the current ELN risk classification in combination with a lack of data concerning particular molecular phenotypes has affected the risk classification within our study, particularly within the CN-AML group. However, this is an inherent limitation of AML risk stratification at a certain point in time and it certainly does not diminish our main conclusion that AML blast maturity does impact clinical outcome, irrespective of the underlying causes.

However, having said this, there is no reason to assume a systematic bias towards a better or an inferior survival which has influenced our main conclusion concerning AML maturity.

For the first time, our study shows a combined quantitative analysis of early progenitor markers in AML. The particular marker combination was chosen because it is 1) widely used, 2) allows for an easy quantification of the marker expression on AML blasts and 3) was continuously used throughout the study period. Although TdT

might be considered an unusual marker for AML, its correlation with blast immaturity is particularly strong [17]. We found that blast immaturity is correlated with particular phenotypes such as complex aberrations, monosomal karyotypes, accrual of chromosome 13 and a loss of chromosomes 5, 5q, and 7. In contrast, *PML-RARA* and *NPM1* mutations had a higher frequency in the in the AML-ma group. These results are supported by previous studies, in which AML FAB M0 was associated an accumulation the same adverse chromosomal aberrations, a higher age and consequently a worse outcome [18-20]. Furthermore, also in recently published studies, a lower frequency of favorable molecular aberrations such as *NPM1* and *CEBPA* has been described in the more immature AML subgroup AML FAB M0 [21]. Finally, the expression of CD34 in *NPM1*- and *FLT3-ITD*-mutated AML seems to be associated with a worse clinical outcome [22,23] and it was recently reported that CD34 expression has negative impact in patients with acute promyelocitic leukemia [24].

Apparently, all these results give rise to the question whether AML maturity is only a surrogate parameter for more favorable phenotypes or whether there is possibly also a causative biological relationship between maturity and a more favorable outcome.

Within a category of more biological explanations, a higher genetic instability due to higher proliferation rates in immature cells might account for a higher probability of further genetic aberrations causing “clonal evolution” towards a more aggressive genotype with increasing resistance to therapy [25-27].

Another hypothetical biological explanation is related to an increasing frequency of leukemic stem cells (LSC) within immature AML. Since these LSC – particularly in their dormant stage – may survive treatment and give rise to subsequent relapse [19,28-39]. The latter idea is supported by a recent study showing that a higher level of putative $CD34^{+}/CD38^{-}$-LSC is associated with a worse prognosis [40].

In conclusion, our novel flow cytometric AML maturity score allows for a prognostic stratification of newly diagnosed AML and gives additional prognostic information particularly in the intermediate risk group according to ELN. Within the ELN intermediate risk group, our maturity score might be an additional tool for choosing the most appropriate (risk-adapted) post remission therapy, particularly in those patients with a high overall transplantation-associated risk. Furthermore, the flow cytometric score may be helpful in cases, in which cytogenetic and/or molecular data for prognostic stratification are not available. Nevertheless, before routine use, our score should be evaluated in another large AML cohort.

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TS and AF contributed equally to this manuscript. JW, AF, and TS were the principal investigators and take primary responsibility for the paper; BD, JW, AF, TB, and AP recruited the patients; JW, IA, ST, AF, and AS performed the laboratory work for this study; JW, AF, and TS participated in the statistical analysis; JW, AF, and TS coordinated the research; JW, AF, and TS wrote the paper.

Compliance with Ethical Standards

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all patients concerning the diagnostic procedures related to AML diagnosis and treatment. For the retrospective analysis, formal consent is not required. This article does not contain any studies with animals performed by any of the authors.

Conflict of interest: The authors declare that they have no conflict of interest.

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Figure Legends

Table 1 Baseline characteristics of the 300 patients included in the study

Table 2a AML maturity score. According to quantitative antigen expression, acute myeloid leukemias were either classified as “immature” (high score) or “mature” (low score)

Table 2b Distribution of patients in the different groups of the “maturity score”

Table 3 Accumulation of cytogenetic and molecular genetic results in AML-ma and AML-im groups

Fig 1 Relapse-free survival (Fig 1a) and overall survival (Fig 1b) of all patients, <60 years and ≥60 years

Fig 2 Relapse-free survival (RFS) and overall survival (OS) in patients <60 years (Fig 2a,c) and ≥60 years (Fig 2b,d). The differences between mature AML (AML-ma) and immature AML (AML-im) are depicted

Fig 3 Subgroups according to the ELN risk stratification: each subgroup is divided into AML-ma and AML-im

Fig 4 Relapse-free survival (RFS) and Overall survival (OS) in patients in the ELN risk group “favorable” (Fig 4a) and “intermediate” (Fig 4b,c). The differences between mature AML (AML-ma) and immature AML (AML-im) are depicted

Table 1. Baseline characteristics of the 300 patients included in the study.

No. of patients	300
No. of female/male patients	139/161
Median age at diagnosis (range), in years	61 (18 – 90)
Cytogenetics (n/273)	**No. of patients (%)**
• Cytogenetically normal AML	103 (37.7%)
• Complex-aberrant karyotype	46 (16.8%)
• Monosomal karyotype	35 (12.8%)
• Trisomy 8	31 (11.4%)
• Deletion 7	30 (11.0%)
• Deletion 5q	24 (8.8%)
• Deletion 7q	15 (5.5%)
• Inv(16)	9 (3.5%)
• t(8;21)	7 (2.7%)
Molecular genetics (n/256)	
• $FLT3\text{-}ITD^{mut}$	32 (12.5%)
• $NPM1^{mut}$	28 (10.9%)
• MLL^{mut}	12 (4.7%)
• $FLT3\text{-}TKD^{mut}$	3 (1.2%)
WHO classification of AML (2008)	
• AML with recurrent genetic abnormalities	65 (21.7%)
• AML with myelodysplasia-related changes	73 (24.3%)
• Therapy-related myeloid neoplasms	5 (1.7%)
• AML, not otherwise specified	144 (48.0%)
• Acute leukemias of ambiguous lineage	13 (4.3%)

Table 2a. AML maturity score. According to quantitative antigen expression, acute myeloid leukemias were either classified as “immature” (high score, 1.5-5 points) or “mature” (low score, 0-1 points).

Antigen	Expression (%)	“Maturity Score“ (Points)
CD34	0-19 20-49 ≥50	0 1 2
CD117	0-19 20-49 ≥50	0 0.5 1
TdT	0-9 10-49 ≥50	0 1 2

Table 2b. Distribution of patients in the different groups of the “maturity score”.

“Maturity Score“ (Points)	No. of Patients	Percentage (%)
0	35	11.7
0.5	20	6.7
1	54	18.0
1.5	36	12.0
2	24	8.0
2.5	11	3.7
3	73	24.3
3.5	3	1.0
4	12	4.0
4.5	2	0.7
5	6	2.0
Not fully available	24	8.0
Total	300	

Table 3. Accumulation of cytogenetic and molecular genetic results in AML-ma and AML-im groups.

AML-ma (n=109)	**AML-im (n=167)**
NPM1^{mut} $p<0.001$	Complex-aberrant karyotype $p<0.001$
t(15;17)/*PML-RARA* $p<0.001$	Monosomal karyotype $p<0.001$
CN-AML $p<0.001$	Deletion 5q $p=0.001$
	Monosomy 7 $p<0.001$

A

N = 300

RFS

1.0
0.8
0.6
0.4
0.2
0.0

< 60 y

≥ 60 y

$p = 0.057$

0 20 40 60 80 100 120

Months

B

N = 300

OS

1.0
0.8
0.6
0.4
0.2
0.0

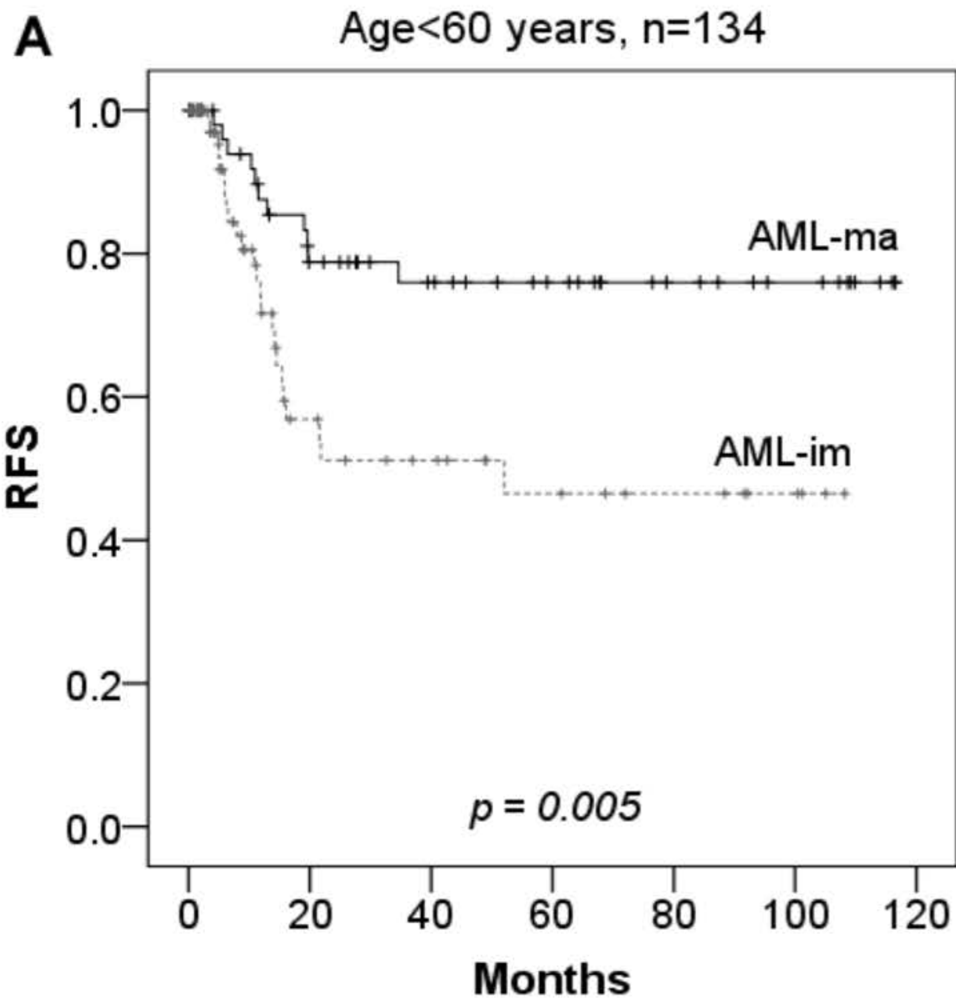
< 60 y

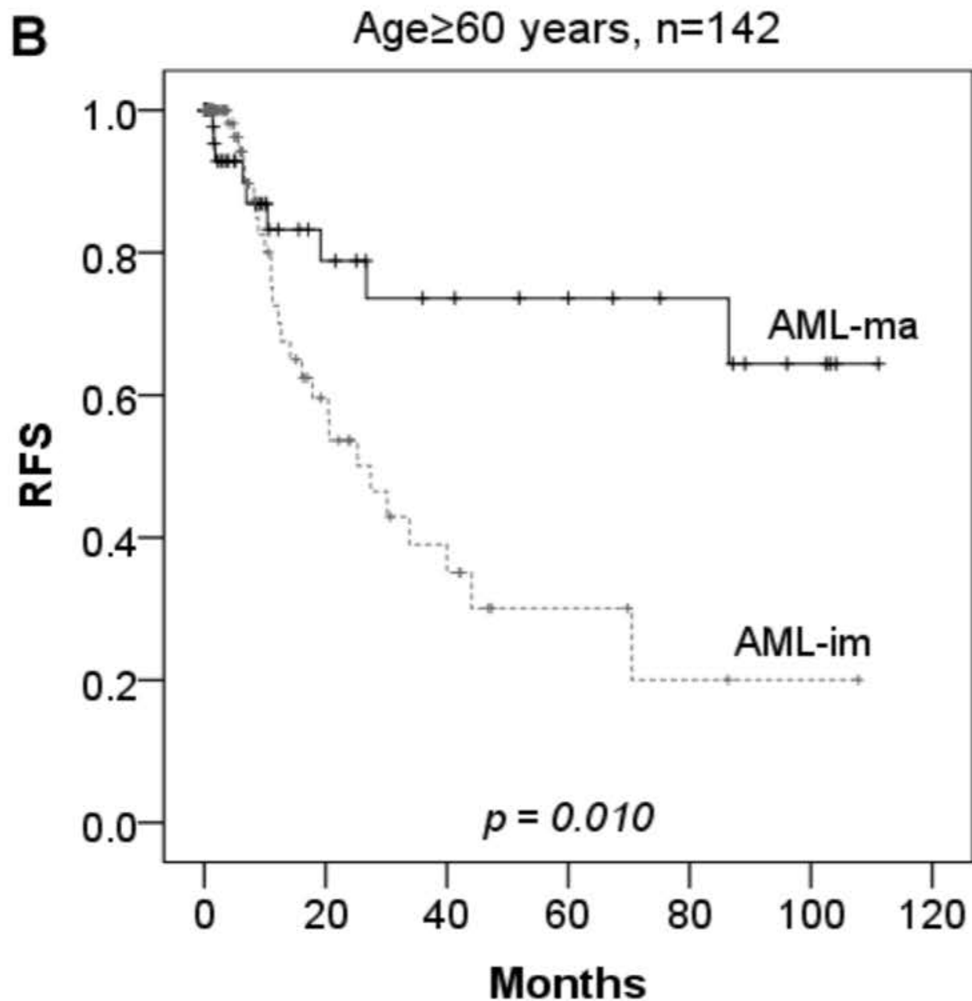
≥ 60 y

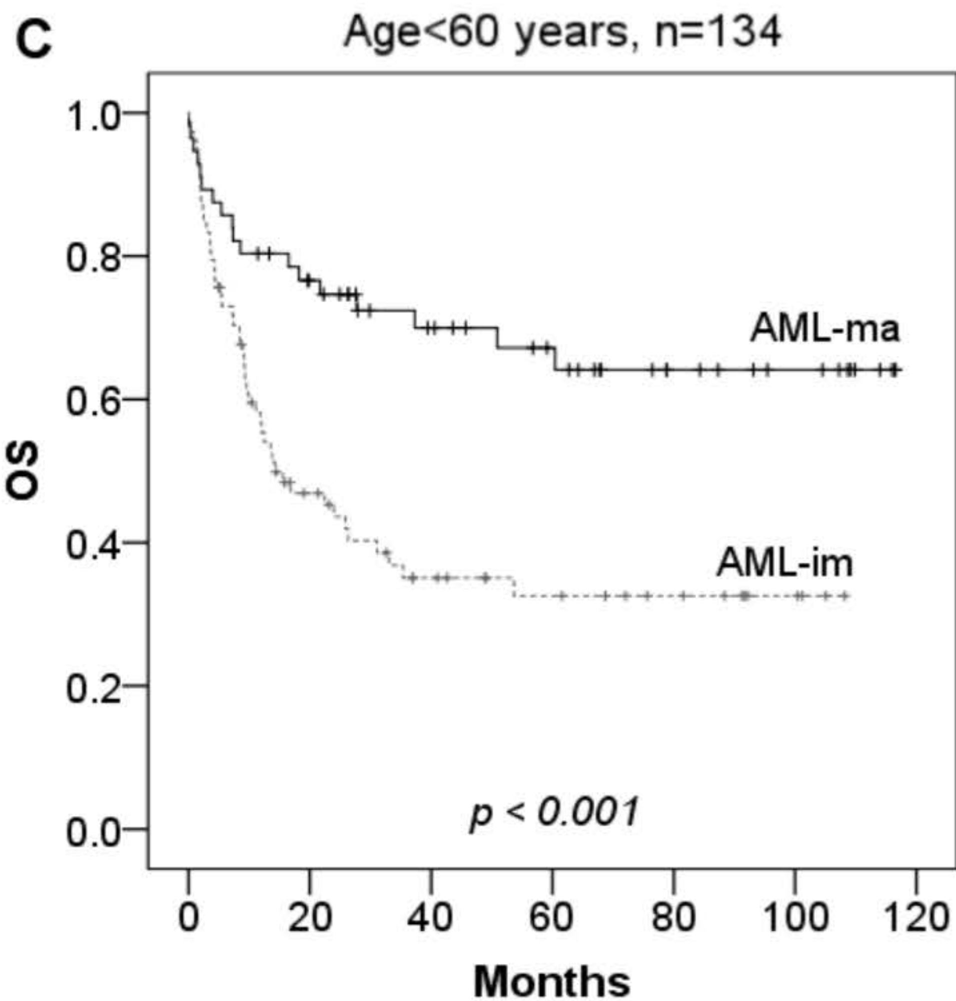
$p < 0.001$

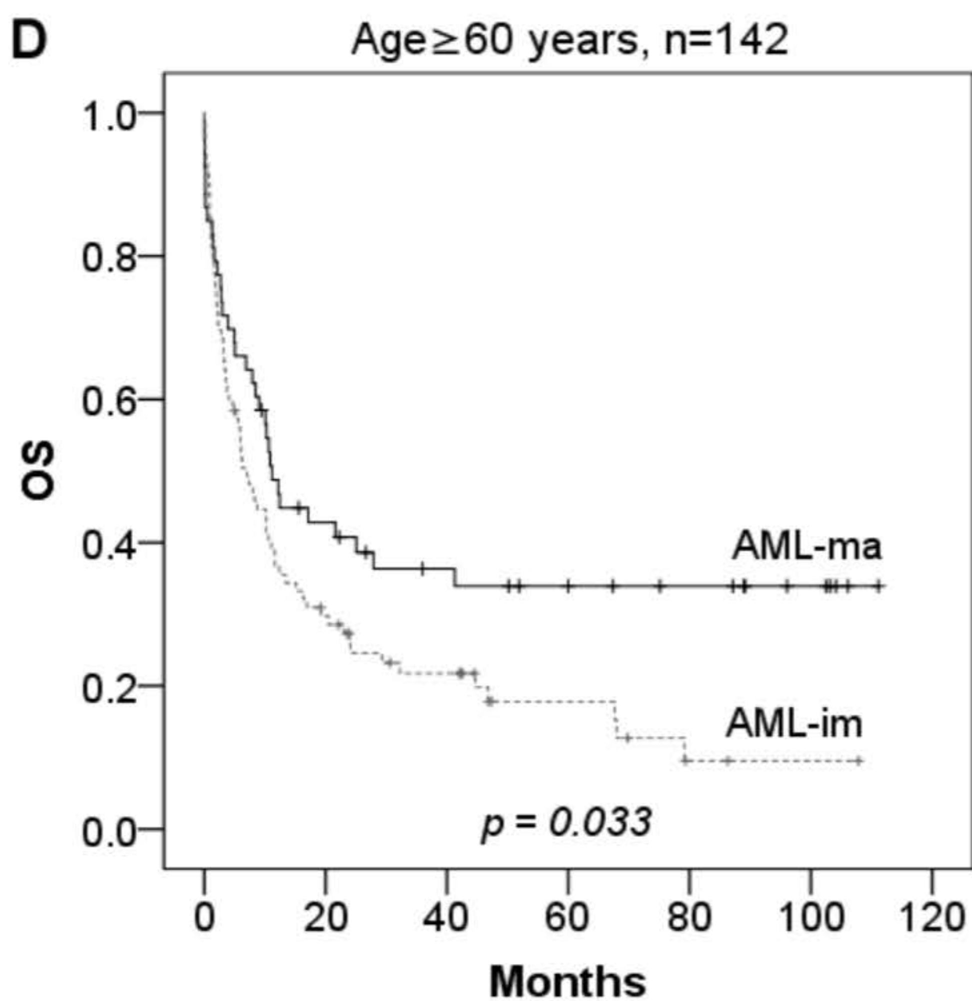
0 20 40 60 80 100 120

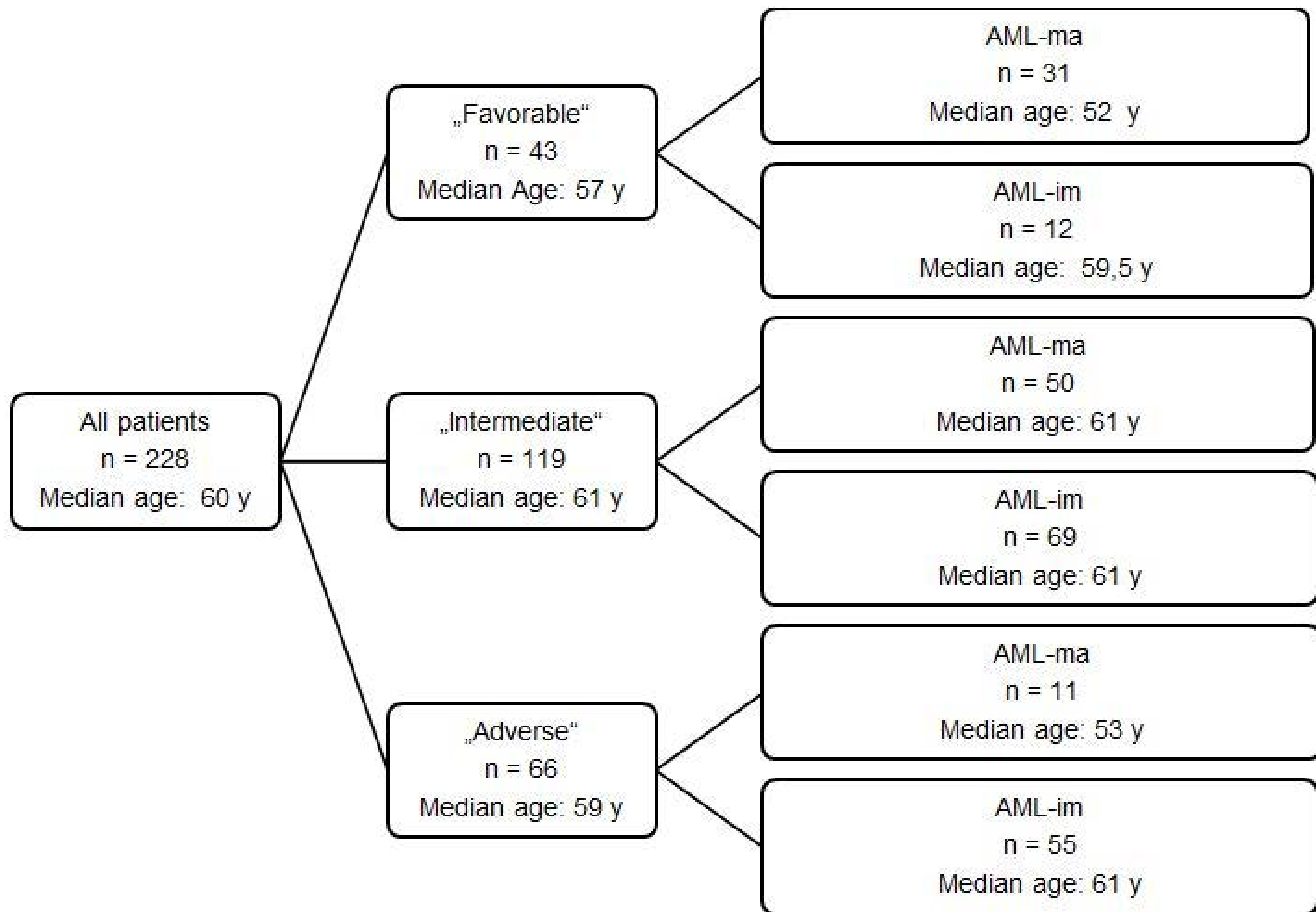
Months

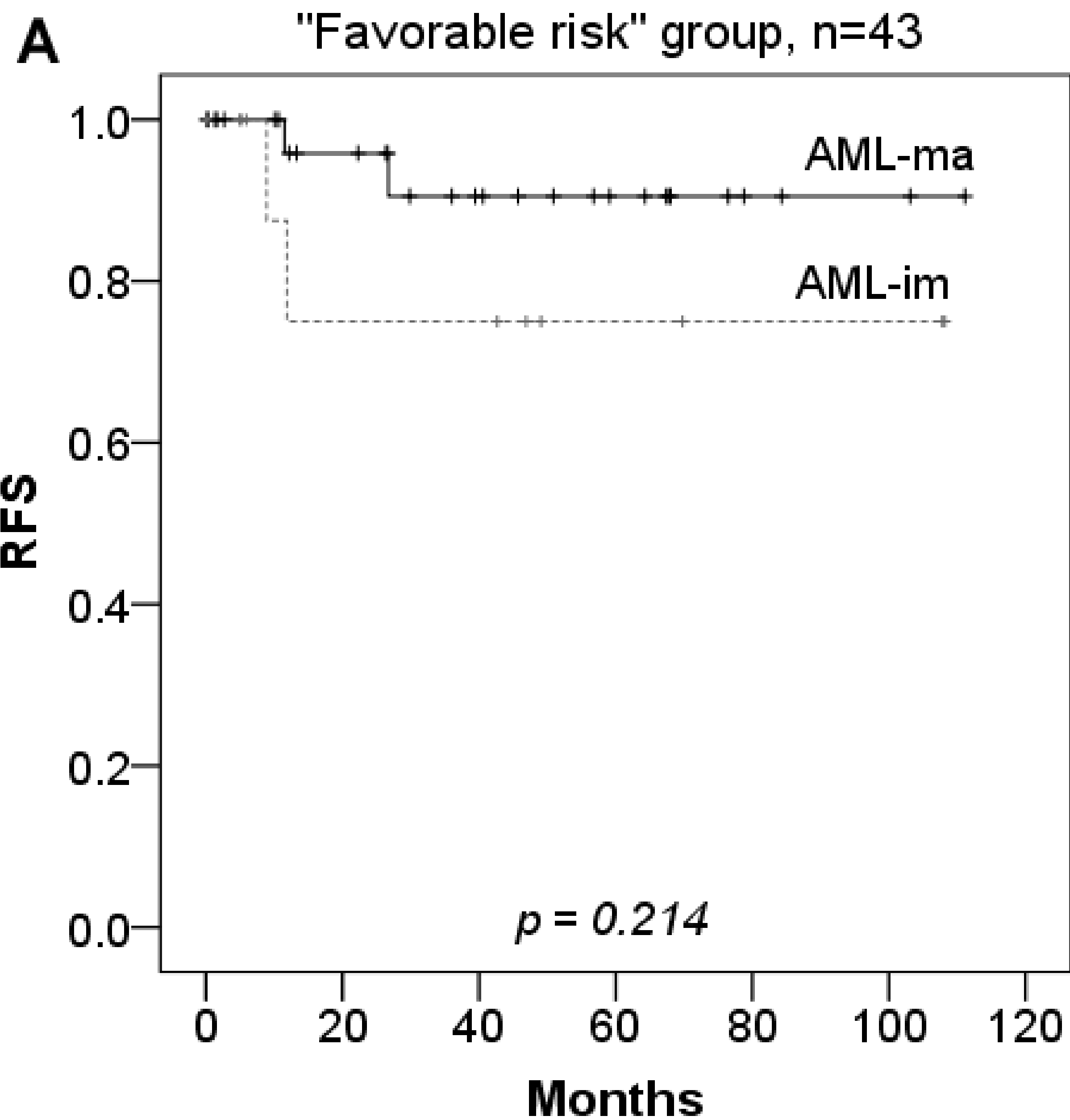

A
Age<60 years, n=134
1.0
0.8
0.6
0.4
0.2
0.0
RFS
AML-ma
AML-im
p = 0.005
0
20
40
60
80
100
120
Months

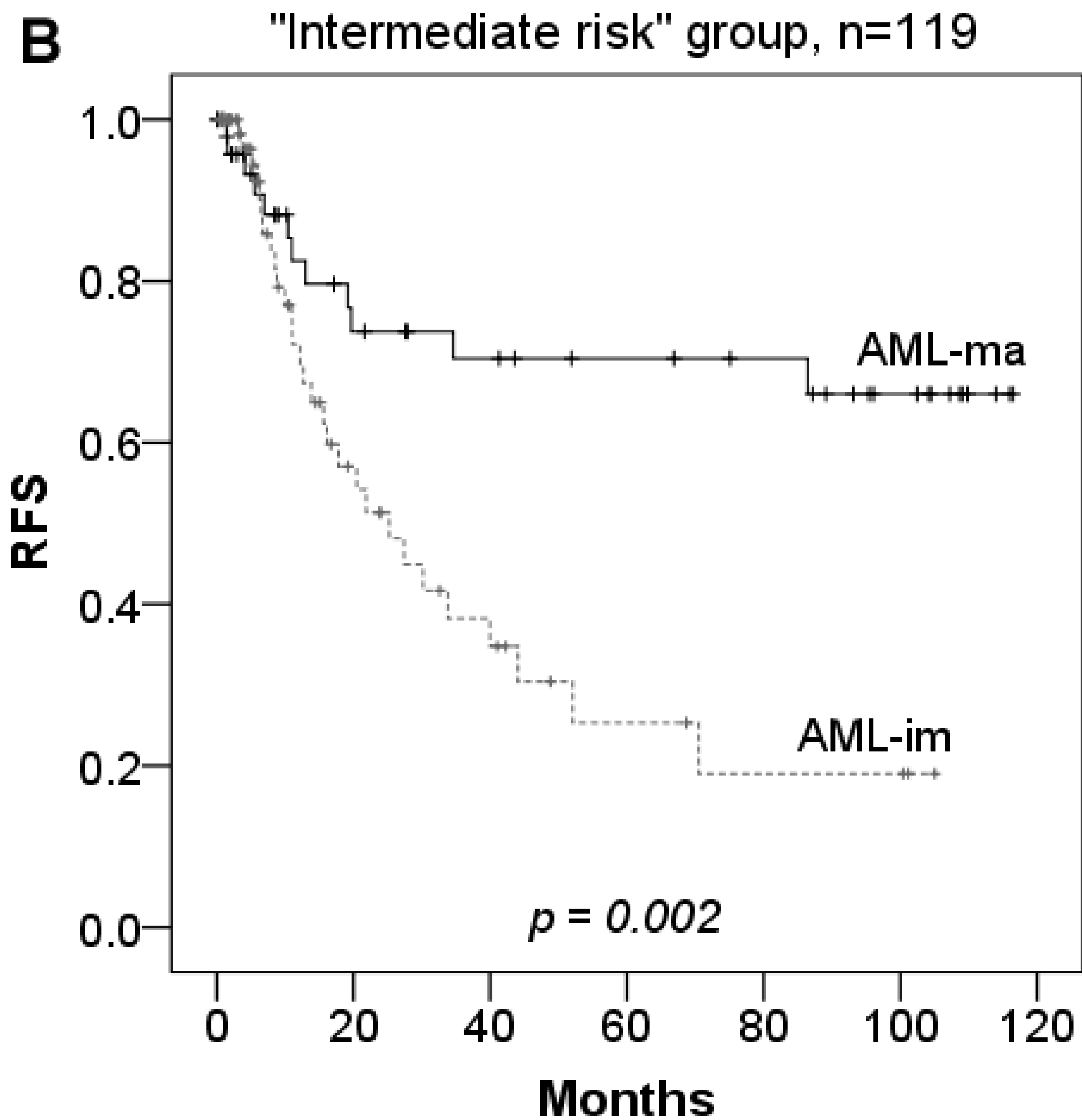

B
Age≥60 years, n=142
1.0
0.8
0.6
0.4
0.2
0.0
RFS
AML-ma
AML-im
p = 0.010
0
20
40
60
80
100
120
Months

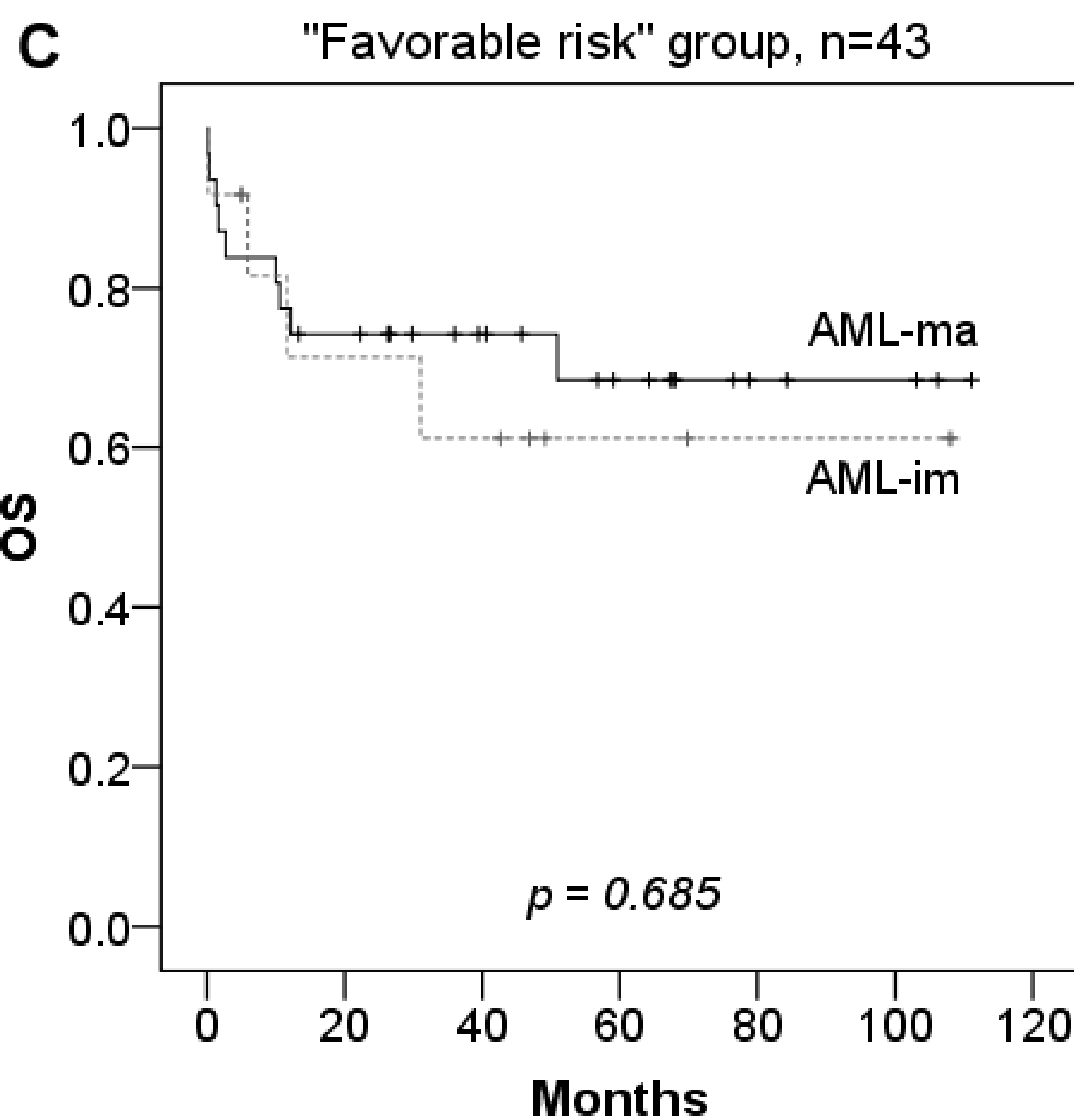

C
Age<60 years, n=134
1.0
0.8
0.6
0.4
0.2
0.0
OS
AML-ma
AML-im
p < 0.001
0
20
40
60
80
100
120
Months

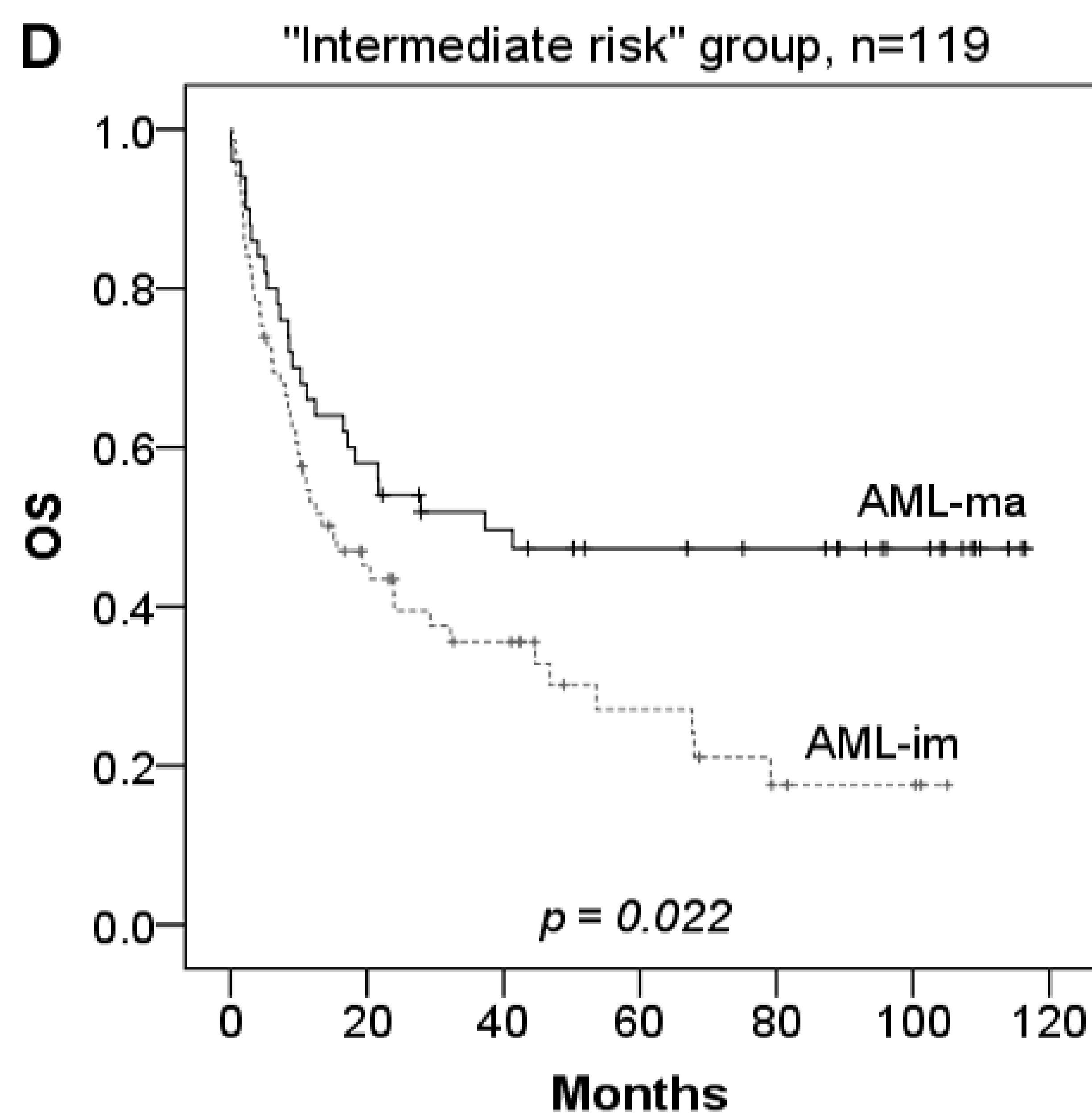

D
Age≥60 years, n=142
1.0
0.8
0.6
0.4
0.2
0.0
OS
AML-ma
AML-im
p = 0.033
0
20
40
60
80
100
120
Months


All patients
n = 228
Median age: 60 y
„Favorable“
n = 43
Median Age: 57 y
„Intermediate“
n = 119
Median age: 61 y
„Adverse“
n = 66
Median age: 59 y
AML-ma
n = 31
Median age: 52 y
AML-im
n = 12
Median age: 59,5 y
AML-ma
n = 50
Median age: 61 y
AML-im
n = 69
Median age: 61 y
AML-ma
n = 11
Median age: 53 y
AML-im
n = 55
Median age: 61 y


A
"Favorable risk" group, n=43
1.0
0.8
0.6
0.4
0.2
0.0
RFS
AML-ma
AML-im
p = 0.214
0
20
40
60
80
100
120
Months


B
"Intermediate risk" group, n=119
1.0
0.8
0.6
0.4
0.2
0.0
RFS
AML-ma
AML-im
p = 0.002
0
20
40
60
80
100
120
Months


C
"Favorable risk" group, n=43
1.0
0.8
0.6
0.4
0.2
0.0
OS
AML-ma
AML-im
p = 0.685
0
20
40
60
80
100
120
Months


D
"Intermediate risk" group, n=119
1.0
0.8
0.6
0.4
0.2
0.0
OS
AML-ma
AML-im
p = 0.022
0
20
40
60
80
100
120
Months