Molecular subtypes of *NPM1* mutations have different clinical profiles, specific patterns of accompanying molecular mutations and varying outcomes in intermediate risk acute myeloid leukemia

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Material and Methods

Patients

During the period from August 2005 to December 2012 a total of 2,859 adult newly diagnosed AML were analyzed at the MLL Munich Leukemia Laboratory by cytomorphology, molecular genetics, and cytogenetics in parallel. 877 (30.7%) out of these were *NPM1* mutated. Out of these 806 samples were *de novo* AML with intermediate risk karyotypes.[1] We selected 660 cases with sufficient sample material available for further molecular analyses for this study.

Patients gave informed consent to the genetic analysis and to the use of laboratory results for research. The study was approved by the Internal Review Board and adhered to the tenets of the Declaration of Helsinki.

Molecular genetics

All 2,859 cases were analyzed for *NPM1* mutations by melting curve analysis. In all 877 mutated cases the resulting product of the melting curve analysis was subsequently analyzed by Sanger sequencing.[2] Positions of *NPM1* mutations were labelled according to transcript ID ENST00000296930.

In all 660 included cases the following genes were investigated for mutations in: *CEBPA*, *CBL*, *DNMT3A*, *FLT3*-ITD, *FLT3*-TKD, *IDH1*R132 *IDH2*R140, *IDH2*R172, *MLL*-PTD, and *WT1*. Analyses were performed by a combination of gene scan analysis, melting curve analysis, quantitative real time PCR, Sanger sequencing, or next generation sequencing.[3-10] *FLT3*-ITD was evaluated as mutation per se and in addition, according to the mutation load which was calculated as the ratio of the mutation compared to the wildtype allele.[11]

Cytomorphology

Bone marrow and/or peripheral blood smears were investigated in all 660 cases by May Grünwald Giemsa staining, combined in all cases with myeloperoxidase and non-specific esterase.[12] All cases were classified according to WHO and subclassified according to the FAB classification.[13;14]

Cytogenetics

Chromosome banding analysis was performed in all 660 patients and was combined with fluorescence in situ hybridization (FISH) if needed for clarification.[15] All cases were classified according to refined MRC criteria and assigned to the intermediate risk karyotype group.[1]

Statistical Analysis

For comparison of *NPM1* types the following groups were considered: 1) type A; 2) “rare types” comprising all non-type A types, 3) types B, 4) types D, and 5) “remaining types” comprising all types non A, B, and D. Statistical analyses were performed using SPSS version 19.0.0 (IBM Corporation, Armonk, NY). All p-values reported are two-sided, accepting p=0.05 as indicating a statistically significant difference and not adjusting for multiple testing. Dichotomous variables were compared between different groups using the χ^2-test or Fisher’s exact test and continuous variables by Student’s T-test. Survival analyses were evaluated for all patients with intensive treatment regimes according to standard protocols.[16] Survival curves were calculated for overall survival (OS), event-free survival (EFS), and overall survival censoring the patients on the day of allogeneic stem cell transplantation (OS^{TXcens}) according to Kaplan-Meier and compared using the two-sided log rank test. OS was defined as the time from diagnosis to last follow-up or death. EFS was defined as the time from diagnosis to failure (persistent leukemia, relapse, death) or last follow-up, and OS^{TXcens} was calculated from diagnosis to either day of transplantation, last follow-up or death. In addition Cox regression analysis was performed for OS, EFS, and OS^{TXcens} with different parameters as covariates. Parameters which were significant in univariate analysis were included in multivariate analysis.

Results

Patient characteristics

In addition to differences in age, patients with type A showed higher WBC than the “rare types” combined (64 vs 51; p=0.043) and the “remaining types” (64 vs 45; p=0.007) in particular. Comparing type A vs type D cases showed a trend to higher WBC (64 vs 48; p=0.057) for type A cases. No other differences were seen in respect to WBC. Hemoglobin levels and platelet counts did not differ between any of the types (table 1).

Cytomorphology

Subdividing the 660 *NPM1* mutated cases by FAB classification, this resulted in 6 M0 (1%), 239 M1 (36%), 166 M2 (25%), 197 M4 (30%), 40 M5 (6%), 11 M6 (2%), and a single case classified as M7.[14] Distribution of FAB classification subtypes did not differ between type A and “rare type” patients except for M4, which were more frequent in type A than in “rare types” (32% vs 24%; p=0.042). No differences in M4 frequencies were seen between the other *NPM1* types. In addition, frequency of M2 was higher in type B (36%) than in type A (23%; p=0.028) and “remaining types” cases (24%; p=0.030; table 1)

Cytogenetics

A normal karyotype was seen in 570 (86%) cases, whereas 90 (14%) showed aberrant karyotypes. Most frequent aberrations were trisomy 8 in 27 (4%) of the cases and trisomy 4 in 11 (2%). Loss of chromosome Y in males was more frequent in type D than in all other types (12% vs 4%, p=0.039). This effect might be due to the fact that type D patients were older than other types. No further differences were observed.

Data on *FLT3*-ITD

As it is known that not only the mutation of *FLT3*-ITD is relevant for prognosis, but the *FLT3*-ITD/wildtype ratio we evaluated our data in addition to the mutation per se according to the mutation load.[17;18] Using a threshold of 0.5 to separate patients with either no mutation or low mutation load from those patients harboring high mutation loads of *FLT3*-ITD we found 188 out of the 270 mutated patients to be in the high mutation load group. Evaluating the prognostic impact we not only confirmed all data of the mutation per se, but also showed the group of high load mutation patients being not only a significant but even highly significant worse independent prognostic parameter in multivariate Cox regression analysis (OS: p<0.001; Hazard Ratio (HR)= 1.7; EFS: p=0.002; HR=1.5; OS^{TXcens}: p<0.001; HR=1.8).

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Supplemental Tables

Supplemental Table 1:

NPM1 Subtypes, sequences, and frequencies. Positions according to transcript ID ENST00000296930

NPM1 Type	Sequence	Number of cases	Frequency (%)
A	c.863_864dupTCTG	458	69,4
B	c.863_864insCATG	72	10,9
D	c.863_864insCCTG	51	7,7
I	c.863_864insCTTG	15	2,3
J	c.863_864insCCGG	7	1,1
K	c.863_864insCCAG	7	1,1
ZE	c.863_864insTCAG	5	0,8
G	c.863_864insCAGG	3	0,5
H	c.867_868insAGGA	3	0,5
N	c.863_864insTCGG	3	0,5
R	c.863_864insTATG	3	0,5
ZA	c.863_864insTAGG	2	0,3
ZN	c.863_864insCTCG	2	0,3
ZO	c.867_868insAGAA	2	0,3
C	c.863_864insCGTG	1	0,2
O	c.863_864insTAAG	1	0,2
V	c.867_868insAGAT	1	0,2
X	c.863_864insTTCC	1	0,2
Y	c.863_864insCCGA	1	0,2
YA	c.861_863delinsTTGTCTG	1	0,2
YB	c.868_873delinsCGTCTTGGCC	1	0,2
YC	c.869_873delinsCCCTCTCCC	1	0,2
YD	c.867delinsAAGGA	1	0,2
YE	c.867_868insAAGT	1	0,2
YG	c.863_864insTCGT	1	0,2
YH	c.863_864insTCAT	1	0,2
YJ	c.863_864insTCGC	1	0,2
YR	c.861_862insATTC	1	0,2
ZB	c.863_864insTTCG	1	0,2
ZD	c.867_868insAGGC	1	0,2
ZG	c.868_871delinsCGGATGGC	1	0,2
ZI	c.863_864insTGAC	1	0,2
ZJ	c.864_866delinsCAGTAAG	1	0,2
ZK	c.863_864insCACG	1	0,2

ZL	c.868_869delinsCGCCTT	1	0,2
ZM	c.863_864insCAGA	1	0,2
ZP	c.864_865delinsCAGTCG	1	0,2
ZQ	c.863_864insTGCG	1	0,2
ZU	c.867_875delinsGGGATAGCGATGC	1	0,2
ZX	c.864_867delinsCACCACCT	1	0,2
ZY	c.863_864insTACG	1	0,2

Supplemental Table 2:

Survival analysis by Kaplan Meier method of *NPM1* mutation types and molecular mutations

Parameter		n	**Overall survival**		**Event-free survival**		**Overall survival^{TXcens}**	
			median in months	*p*	median in months	*p*	median in months	*p*
Total Cohort		562	45.8	n.a.	14.6	n.a.	48.6	n.a
Type A		410	44.0	0.052	13.8	0.048	44.9	0.044
"rare types"		152	62.6		19.4		62.6	
Type A		410	44.0	A vs B: n.s.; A vs D: 0.051; B vs D: 0.090	13.8	n.s. between types	44.9	n.s. between types
Type B		57	37.9		18.6		23.8	
Type D		38	n.r.		14.4		n.r.	
FLT3-ITD		233	19.8	<0.001	9.3	0.001	18.1	<0.001
no *FLT3*-ITD		329	62.2		18.7		62.2	
*DNMT3A*mut		304	30.4	0.001	10.7	<0.001	39.2	0.001
*DNMT3A*wt		258	62.6		25.0		62.6	
Type A	*FLT3*-ITD	170	16.9	0.001	7.3	<0.001	14.8	<0.001
	no *FLT3*-ITD	240	62.2		17.9		62.2	
	DNMT3Amut	249	23.6	0.006	10.0	<0.001	42.3	0.045
	*DNMT3A*wt	161	67.5		25.0		48.6	
Type B	*FLT3*-ITD	26	23.8	n.s.	11.7	n.s.	18.6	n.s.
	no *FLT3*-ITD	31	45.8		19.4		n.r.	
	DNMT3Amut	18	76.5	n.s.	23.2	n.s.	n.r.	n.s
	*DNMT3A*wt	39	23.8		10.6		23.8	

Type D	*FLT3*-ITD	14	n.r.	n.s.	27.9	n.s.	n.r.	n.s.
	no *FLT3*-ITD	24	49.6		14.4		49.6	
	*DNMT3A*mut	16	32.5	0.016	7.6	0.012	n.r.	<0.001
	*DNMT3A*wt	22	n.r.		31.0		14.3	

n.a.: not applicable; n.s.: not significant; mut: mutated; wt: wild type

Supplemental Table 3:

Survival analysis by Cox regression analysis of clinical parameters, cytogenetics, *NPM1* mutation types and molecular mutations (only those genes being mutated in at least 5 cases were included).

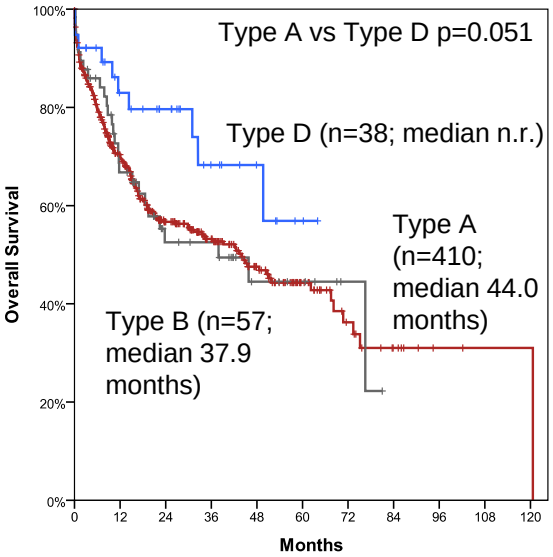
Parameters	Overall survival				Event-free survival				Overall survival^{TXcens}			
	univariable		multivariate		univariable		multivariate		univariable		multivariate	
	p	HR	p	HR	p	HR	p	HR	p	HR	p	HR
Clinical parameters												
Gender	n.s.				n.s.				n.s.			
Age	<0.001	1.41*	<0.001	1.39*	<0.001	1.28*	<0.001	1.26*	<0.001	1.59*	<0.001	1.55*
WBC x10^9/L	<0.001	1.07**	<0.001	1.05**	<0.001	1.06**	<0.001	1.04**	<0.001	1.09**	<0.001	1.07**
Hb g/dL	n.s.				n.s.				n.s.			
PLT x10^9/L	n.s.				n.s.				n.s.			
FAB classification	n.s.				n.s.				n.s.			
normal vs aberrant karyotype	n.s.				n.s.				n.s.			
***NPM1* types**												
"rare types" vs type A	0.054	0.74	n.a.		0.049	0.78	n.a.		0.045	0.70	n.a.	
A vs B vs D vs "remaining types"	0.047	0.94	n.s.		0.030	0.95	n.s.		0.052	0.93	n.s.	
A vs B vs D	0.093	0.81	n.a.		n.s.				n.s.			
Molecular mutations												
CBLmut	n.s.				n.s.				n.s.			
*CEBPA*mut	n.s.				n.s.				n.s.			
*DNMT3A*mut	0.001	1.57	0.014	1.44	<0.001	1.60	0.006	1.42	0.001	1.61	0.011	1.55
FLT3-ITD	<0.001	1.63	0.035	1.37	0.001	1.43	0.039	1.30	<0.001	2.01	0.005	1.60
FLT3-TKD	n.s.				n.s.				n.s.			
*IDH1*R132	n.s.				n.s.				n.s.			
*IDH2*R140	n.s.				0.026	0.70	0.016	0.64	n.s.			
*WT1*mut	n.s.				n.s.				n.s.			

n.s.: not significant; *per 10 years of increase; **per 10x10^9/L; n.a.: not applicable

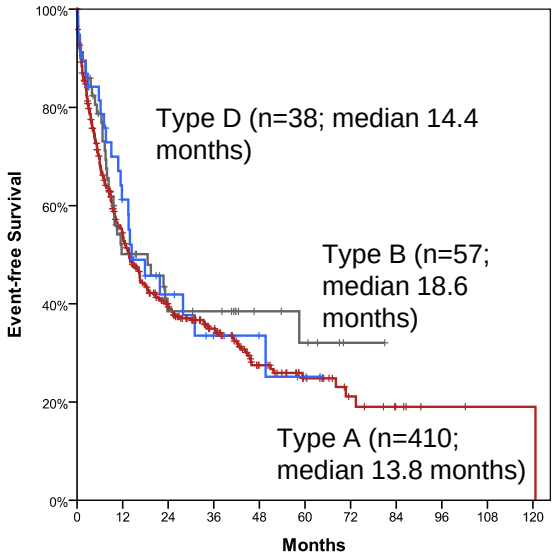
Supplemental Figure 1 A-C:

Kaplan Meier analyses on **A:** Overall survival, **B:** Event-free survival, and **C:** Overall survival censored at the day of allogeneic stem cell transplantation comparing type A (n=410), type B (n=57), and type D (n=38) patients.

A:



B:



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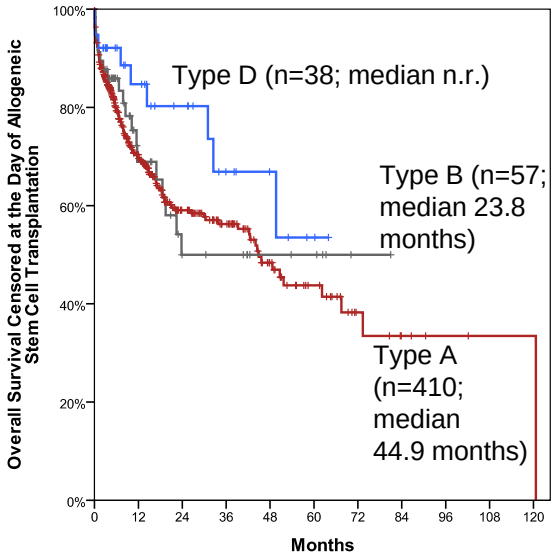
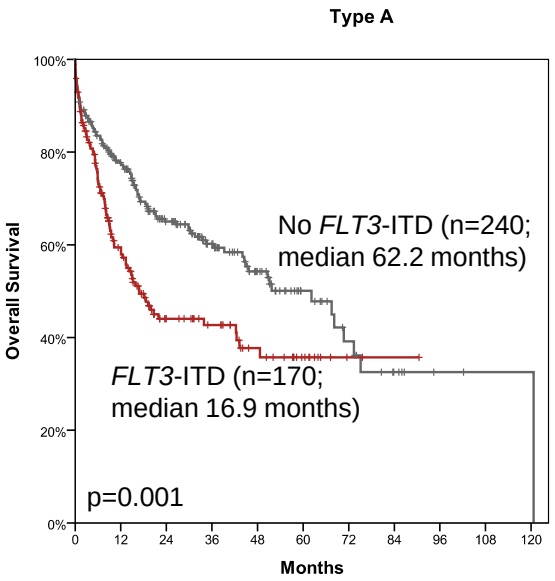


Supplemental Figure 2 A-C:

Kaplan Meier analyses on prognostic impact of *FLT3*-ITD on overall survival within **A:** Type A, **B:** Type B, and **C:** Type D patients

A:



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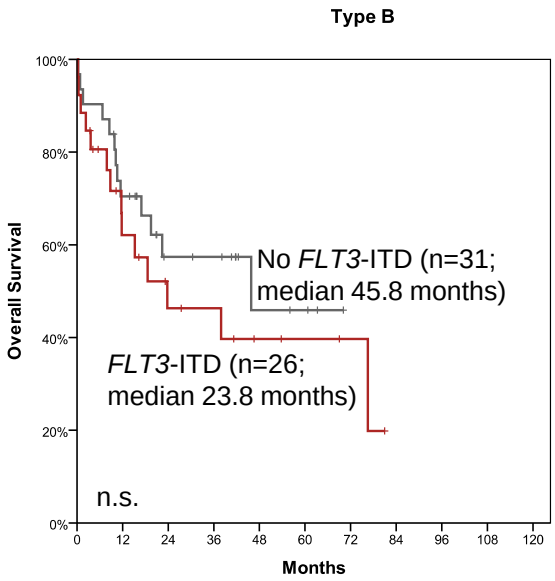
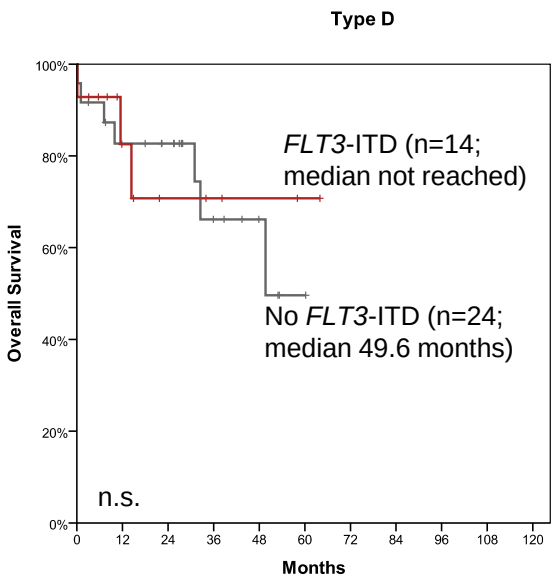


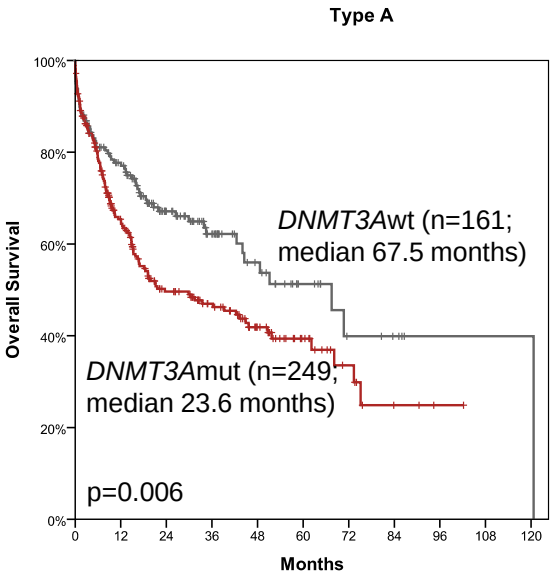
C:



Supplemental Figure 3 A-C:

Kaplan Meier analyses on prognostic impact of *DNMT3A* mutations on overall survival within **A:** Type A, **B:** Type B, and **C:** Type D patients

A:



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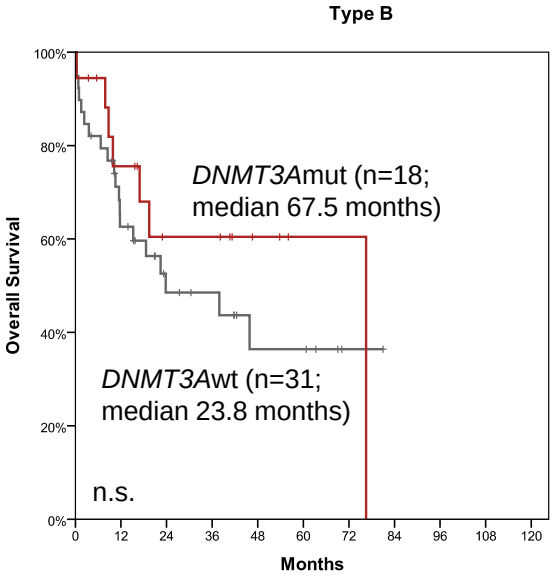

C: