Analysis of the clinical impact of *NPM1* mutant allele burden in a large cohort of younger adult patients with acute myeloid leukaemia

Running title: Clinical impact of *NPM1* mutant allele burden

Linch DC[1], Hills RK[2], AK Burnett[3], N Russell[4], Gale RE[1]

[1]Department of Haematology, UCL Cancer Institute, London, UK

[2]Nuffield Department of Population Health, Oxford, UK

[3]Department of Haematology, School of Medicine, Cardiff University, Cardiff, UK

[4]Department of Haematology, Nottingham University Hospital NHS Trust, Nottingham, UK

Corresponding author: David Linch,
Department of Haematology,
UCL Cancer Institute,
72 Huntley St,
London WC1E 6DD,
United Kingdom

e-mail: david.linch@ucl.ac.uk
Phone: (+44)-20-7679-6221
Fax: (+44)-20-7679-6222

Word count

Text:	3245
Summary:	197
Figures+Tables:	3+4
References:	27

Summary

Although an *NPM1* mutation is generally considered to be a good prognostic marker in acute myeloid leukaemia, it has recently been suggested that a higher level of *NPM1* mutant (*NPM1*^{MUT}) alleles relative to wild-type alleles is associated with poor clinical outcome. We therefore sought to confirm this finding in a larger study of 876 *NPM1*^{MUT} cases entered into UK national trials. In univariate analysis, the higher *NPM1*^{MUT} allele burden was associated with a lower complete remission (CR) rate, higher relapse rate and reduced overall survival, but this was largely attributable to the association of the higher *NPM1*^{MUT} allele burden with other known poor risk factors, particularly the presence of a concomitant *FLT3*^{ITD}. In multivariate analysis, there was no significant impact of the *NPM1*^{MUT} allele burden on CR rates, and the impact on relapse and overall survival, whilst still significant, was greatly reduced. This impact was similar in patients who did or did not receive an allogeneic transplant in first CR. We conclude that the binary presence or absence of an *NPM1* mutation, combined with minimal residual disease levels following induction therapy, should continue to be used in therapeutic management rather than stratification according to the *NPM1*^{MUT} level.

Keywords: Acute myeloid leukaemia, prognostic impact, *NPM1* mutant, variant allele frequency, therapeutic management

Introduction

Acute Myeloid Leukaemia (AML) is characterised by a limited number of recurrent driver mutations, the most frequent being in the *FLT3*, *NPM1* and *DNMT3A* genes (Cancer Genome Atlas Research Network, 2013; Papaemmanuil *et al*, 2016). At diagnosis, such mutations may be present in virtually all leukaemic cells or just in a variably sized sub-population (Gale *et al*, 2008). The presence of a given mutation in all leukaemic cells implies that this mutation either arises as an early event in leukaemogenesis or that it arises later and has a strong selective advantage over other subclones.

DNMT3A mutations generally arise at a very early stage in leukaemogenesis (Shlush *et al*, 2014; Papaemmanuil *et al*, 2016), and clones containing these mutations may even be found in elderly haematologically normal individuals (Genovese *et al*, 2014; Jaiswal *et al*, 2014; Xie *et al*, 2014). In our earlier study of AML patients with *DNMT3A* mutations (*DNMT3A*^{MUT}), the median *DNMT3A*^{MUT} allele level was 47%, with <0.5% of patients having a mutant allele level >60% and only 4% having a level <30% (Gale *et al*, 2015). This is compatible with a heterozygous mutation in all leukaemic cells, with biallelic disease being very rare. The presence of a *DNMT3A* mutation is associated with a worse prognosis in most series (Shivarov *et al*, 2013; Gale *et al*, 2015).

Conversely, the proportion of leukaemic cells bearing a heterozygous *FLT3* internal tandem duplication (*FLT3*^{ITD}) is highly variable, from a barely detectable proportion up to 100% of leukaemic cells (Gale *et al*, 2008). Those cases with more cells bearing the *FLT3*^{ITD} are associated with a worse prognosis (Gale *et al*, 2008; Schnittger *et al*, 2011; Pratcorona *et al*, 2013; Linch *et al*, 2014). Furthermore, in some patients there is biallelic disease due to uniparental disomy (Raghavan *et al*, 2008), which is associated with an even higher mutant allelic burden and still worse outcome (Gale *et al*, 2008; Linch *et al*, 2014).

Mutations in *NPM1* are also thought to arise at an early stage in leukaemogenesis, although they are not generally considered to be "founder" mutations as they occur after *DNMT3A* mutations (Shlush *et al*, 2014). The mutation is, however, usually found in virtually all the leukaemic cells (Thiede *et al*, 2006; Gale *et al*, 2008). It is usually heterozygous, with biallelic disease being rare. As a consequence, the *NPM1*^{MUT} allele level in leukaemic samples falls within a relatively narrow range, similar to the situation for *DNMT3A* mutations. In our earlier report of 345 patients with both an *NPM1* mutation and a *FLT3*^{ITD}, only 1.7% of cases were deemed to have an *NPM1*^{MUT} level >60% and 3.5% a level <30% (Gale *et al*, 2008). The presence of an *NPM1* mutation is associated with a favourable outcome (Thiede *et al*, 2006; Gale *et al*, 2008; Papaemmanuil *et al*, 2016), and the

combination of the *NPM1* and *FLT3* genotype is widely used to help determine first-line therapy (Dohner *et al*, 2017).

It is surprising therefore that Patel and colleagues have recently reported that, despite the narrow range of the mutant allele burden, the *NPM1* mutant level has important prognostic relevance (Patel *et al*, 2018). In their study of 109 patients with de novo AML and an *NPM1* mutation, they found that those in the upper quartile of *NPM1*^{MUT} allele levels had a significantly worse event-free and overall survival (OS) compared to other *NPM1*^{MUT} cases. They suggest that measurement of *NPM1*^{MUT} allele level might therefore have important implications for patient management. However, in another relatively small cohort of 147 patients, Abbas *et al* (2019) found no prognostic impact of the *NPM1*^{MUT} variant allele frequency (VAF), and in a further study by Rothenberg-Thurley *et al* (2018), although a higher VAF was associated with a shorter OS, this was not significant in multivariate analysis. To help resolve these issues we have analysed the associations and prognostic impact of the *NPM1*^{MUT} VAF in a large series of 876 cases of de novo *NPM1* mutant-positive AML entered into the UK Medical Research Council/National Cancer Research Institute (MRC/NCRI) national AML trials.

Materials and methods

Patient cohort

A total of 876 younger adult patients between the ages of 16 and 59 years were identified who had cytogenetic intermediate-risk primary AML according to the MRC classification (Grimwade *et al*, 2010) and in whom a mutant *NPM1* allele had been identified and quantified at diagnosis in the UCL Leukaemia Genetics Laboratory. The patients had all been entered into UK national AML trials between 1988 and 2014: AML 10 (n=141 patients), AML 12 (n=294), AML 15 (n=219) and AML 17 (n=222). The demographics and clinical features of these patients are shown in Table 1. The median follow-up of surviving patients was 9.0 years (range, 0.4-21.8 years). Ethical approval for the trials and tissue collection for research was obtained from the Multicentre Research Ethics Committee of Wales and informed consent was provided according to the Declaration of Helsinki.

Mutant detection and quantification

DNA samples were prepared using buffy coat cells from bone marrow aspirates (n=556, 63%), peripheral blood (297, 34%) or unknown (23, 3%). *NPM1, FLT3* and *DNMT3A* mutations were

identified as described elsewhere (Gale *et al*, 2008; Gale *et al*, 2015). *NPM1*^{MUT} and *FLT3*^{ITD} VAF were quantified by capillary electrophoresis and mutant levels expressed as a percentage of total alleles.

Treatment delivered

Details of the trial protocols have been previously published (Hann *et al*, 1997; Burnett *et al*, 2010; Burnett *et al*, 2013; Burnett *et al*, 2015). The UK MRC AML12 trial is registered at http://www.controlled-trials.com under ISRCTN17833622, AML15 trial at ISRCTN17161961 and the NCRI AML17 trial at ISRCTN55675535. Of the 876 patients, 137 (16%) underwent an allogeneic transplantation for consolidation of first remission, and 49 of the *FLT3*^{ITD}-mutated patients (14%) received a FLT3 inhibitor.

Clinical end points and statistical methods

Complete remission (CR) was defined as a normocellular bone marrow containing less than 5% blasts and showing evidence of normal maturation of other marrow elements. Peripheral blood regeneration was not a requirement but 95% of cases defined as a CR achieved a neutrophil count >1x10^9/l and a platelet count >100x10^9/l. The cumulative incidence of relapse (CIR) was defined as the time from remission to relapse, with death in remission as a competing risk. OS was the time from randomisation to death.

Mantel-Haenszel and χ^2 tests were used to test for differences in demographic and clinical data. Unstratified analyses of remission were performed using the Mantel-Haenszel test. CIR was calculated using competing risks methodology. Kaplan-Meier curves were constructed for CIR and OS data and compared by means of the log-rank test, with standard tests for heterogeneity between subgroups (Early Cancer Trialists Collaborative Group, 1990). Multivariable Cox regression analysis was used to calculate cause specific hazard ratios for CIR and OS as defined within the IWG guidelines (Cheson *et al*, 2003) and used factors of prognostic significance identified in the totality of patients within the AML 10, 12, and 15 trials. Odds ratios or hazard ratios (HRs) and 95% confidence intervals are quoted for the end points. All *P* values are two-tailed.

Results

NPM1^{MUT} *allele levels*

The median level of *NPM1*^{MUT} alleles was 43% (mean, 41%; range, 6%-76%) (Fig 1), compatible with heterozygous expression of the *NPM1* mutant in nearly all leukaemic cells, with an average of 18% of

cells being non-leukaemic. The inter-quartile boundaries were <39%, 39-43%, 44-46% and >46%. Only 1% of values were >60% and 10% were <30%. Those values <30% are most likely to represent the presence of normal haematopoietic cells rather than the *NPM1* mutation being restricted to a subclone of leukaemic cells. In keeping with this, of the 87 patients with *NPM1*^{MUT} levels <30%, *DNMT3A*^{MUT} or *IDH2*^{MUT} allele burden data was available in 20 cases, with a median of 29%.

Factors correlated with high NPM1^{MUT} *level*

The level of *NPM1*^{MUT} alleles, as a continuous variable, was not related to patient age or sex (P = 0.8 and 0.7 respectively). There was a strong correlation between the level of the *NPM1*^{MUT} and the presenting white blood cell count (WBC) (P < 0.0001), and a lesser but still highly significant correlation with the performance status (PS) (P = 0.0002). Patients with a concurrent *FLT3*^{ITD} had higher levels of *NPM1*^{MUT} alleles (P < 0.0001 as a continuous variable), and patients with higher level *FLT3*^{ITD} (>50%) were concentrated in the group with higher *NPM1*^{MUT} levels. In those cases with a below median *NPM1*^{MUT} allele burden, the incidence of high level *FLT3*^{ITD} was 5% compared to 13% in those with an above median *NPM1*^{MUT} level (P < 0.0001). There was, by contrast, no relationship between *NPM1*^{MUT} levels and the presence of a concurrent *DNMT3A* mutation (P = 0.4).

Relationship between NPM1^{MUT} *level and CR, CIR and OS*

Univariate analysis

Prognostic factors for outcome identified in univariate analysis for this cohort of patients are given in Table 2. The CR rate for all patients was 93% overall but was 96% in those patients with below median levels of *NPM1*^{MUT} allele burden and 90% in those with above median levels (Hazard ratio [HR] = 2.38 [95% confidence intervals (CI) 1.38-4.11]; P = 0.002) (Table 3). If the patients were subdivided into quartiles by *NPM1*^{MUT} allele levels, then the CR rate in the lowest quartile (Q1) was 96%, and it was 95%, 92% and 87% in Q2, Q3 and Q4 respectively (P value for trend across all quartiles = 0.0003). A similar relationship was found when CR after just one course of induction therapy was considered, with an overall rate of 82%, and rates of 88%, 82%, 79% and 78% in Q1 to Q4 respectively (P = 0.007). The relapse rate was also related to the *NPM1*^{MUT} allele level. The CIR at 10 years for all patients was 42% and it was 35% and 50% for those below and above the median *NPM1*^{MUT} allele burden respectively (HR=1.67 [1.35-2.08]; P < 0.0001) (Fig 2A). By quartiles, Q1 to Q4, the CIR at 10 years was 38%, 33%, 48% and 53% respectively (Fig 2B).

In accord with the lower CR rate and higher relapse rate in the patients with the higher *NPM1*^{MUT} levels, the OS was also lower in those with higher *NPM1*^{MUT} levels. When the patients were divided into two groups, below and above the median *NPM1*^{MUT} level value of 43%, the OS at 10 years was 56% and 42% respectively (HR = 1.56 [1.29-1.89]; *P* < 0.0001) (Fig 2C). If the cohort was considered by quartiles, it was apparent that the two lowest quartiles (Q1 and Q2) had a similar OS of 56% and quartiles Q3 and Q4 had progressively poorer survival at 45% and 38% respectively (Fig 2D). For OS, the unadjusted HR for Q4 vs Q1-3 was 1.66 [1.34-2.06] (*P* < 0.0001); for Q4 vs Q1+2 it was 1.87 [1.48-2.36] (*P* < 0.0001).

We looked at the impact of the *NPM1*^{MUT} allele burden separately in those patients with and without a *FLT3*^{ITD}. The greatest impact of the *NPM1*^{MUT} level on OS was in the *FLT3*^{ITD} patients (*P* = 0.0009), and although a similar trend was apparent in the *FLT3*^{WT} group, the difference was not statistically significant (*P* = 0.09) (Fig 3). We also stratified patients according to both the *NPM1*^{MUT} (above and below median) and *FLT3*^{ITD} mutant levels. The greatest adverse impact of the higher *NPM1*^{MUT} mutant allele burden appears to be in those patients with the highest *FLT3*^{ITD} level (≥50%) (Table 4) but testing for interaction between the two *NPM1* mutational burdens revealed that the association was not statistically significant (*P* = 0.14).

Multivariate analysis

Multivariate analysis was carried out considering those prognostic factors known to be associated with outcome, that is, age, WBC, PS, the presence of a *FLT3*^{ITD} and the *FLT3*^{ITD} level (below and above 50%) and a *DNMT3A* mutation. Irrespective of whether it was considered as below and above the median or by quartiles, the *NPM1*^{MUT} allele burden had no significant impact on CR rates (Table 3). It did remain a significant factor for CIR and OS, but the significance was greatly reduced compared to the univariate analysis. For instance, the HR for the OS of Q4 compared to Q1-3 was reduced from 1.66 [1.34-2.06] (*P* < 0.0001) in the unadjusted analysis to 1.30 [1.03-1.64] (*P* = 0.03) in the adjusted analysis. For Q4 vs Q1+2 it was reduced from 1.87 [1.48-2.36] (*P* < 0.0001) to 1.39 (1.06-1.81] (*P* = 0.02).

Impact of NPM1^{MUT} *allele burden in allogeneic transplant recipients*

When OS was considered in all patients who achieved a CR and censored at the time of allogeneic transplantation in first CR, the unadjusted HR for OS by *NPM1*^{MUT} allele quartiles was 1.19 [1.07-1.32] (*P* = 0.0009). The HR adjusted for the factors described above was 1.08 [0.96-1.21] (*P* = 0.19). For those patients who received an allogeneic transplant in first CR, the impact of the *NPM1*^{MUT}

allele burden on the HR for OS was 1.21 [0.93-1.58] (P = 0.16) for the unadjusted analysis, and 1.21 [0.90-1.63] (P = 0.2) for the adjusted analysis. We found no evidence, therefore, that the impact of the *NPM1*^{MUT} allele burden was greater in transplant recipients. Furthermore, a Mantel-Byar analysis of OS from CR in those who did and did not receive an allograft found no difference in the impact of the transplant by *NPM1*^{MUT} allele level (test for heterogeneity, P = 1.0).

Impact of NPM1^{MUT} *allele burden on haematopoietic recovery*

In view of the impact of the *NPM1*^{MUT} level on outcome, we also ascertained whether the mutant level impacted on the dose intensity of the chemotherapy administered by determining the time from the administration of the first cycle of induction therapy to the second course and the time from the first to the third course of therapy as surrogates of the speed of post-chemotherapy reconstitution of normal haematopoiesis. No impact of the *NPM1*^{MUT} allele burden on either time interval was apparent (P = 0.99 and 0.3 respectively).

Discussion

This large study of younger adult patients with *NPM1*^{MUT} intermediate cytogenetic risk AML confirms the results from Patel and colleagues that a higher level of *NPM1*^{MUT} alleles is associated with a worse outcome (Patel *et al*, 2018), although the magnitude of the effect is not so great in our series. In univariate analysis, a higher *NPM1*^{MUT} allele burden was associated with a highly significant reduction in CR rate, raised CIR and reduced OS. However, the higher *NPM1*^{MUT} level was correlated with the WBC, PS, the presence of a *FLT3*^{ITD} and the presence of higher *FLT3*^{ITD} levels, all known to be adverse prognostic factors. In multivariate analysis allowing for these factors, the impact of the *NPM1*^{MUT} allele burden was markedly reduced. There was no significant impact on CR rate and the impact on OS was of borderline significance. These results are similar to those presented by Rothenberg-Thurley *et al* (2018), who found that the *NPM1*^{MUT} allele burden was not an independent predictor of survival, whereas Abbas *et al* (2019) found no prognostic impact of the *NPM1*^{MUT} allele burden. In our cohort, the impact of the *NPM1*^{MUT} allele burden was only significant in those patients where their leukaemia co-expressed mutant *NPM1* and a *FLT3*^{ITD}. This suggests that the prognostic impact of the *NPM1*^{MUT} level is largely, albeit not entirely, due to the concomitant *FLT3*^{ITD} mutations. This data is discordant with that from Patel *et al* (2018), who found no relationship between *NPM1*^{MUT} allele levels and the presence of a *FLT3*^{ITD}. The reason for this major difference is not clear but it should be noted that only 14% of the *FLT3*^{ITD}-positive patients in our

study received a FLT3 inhibitor compared to 57% and 78% in the Patel and Abbas studies respectively.

In the Patel paper, the major impact of the high *NPM1*^{MUT} allele level was in those patients who received an allograft as consolidation of first CR, but this relationship was not apparent in our series. Furthermore, we found no greater benefit from consolidation with an allograft in first CR in those patients with higher as compared to lower *NPM1*^{MUT} allele burdens. Only 16% of our patients received an allograft in first CR compared to 41% in the Patel cohort, which may have contributed to this difference, but Abbas *et al* (2019) also found no difference according to transplant status, with 43% of their patients transplanted in first CR.

The residual prognostic impact of a higher *NPM1*^{MUT} allele level, after taking into account the other associated poor prognostic factors referred to above, could have several causes. Firstly, there could be an association between higher levels of *NPM1*^{MUT} alleles and the presence of mutations in another gene which imparts the poor prognosis. We have not extensively explored this possibility, but it is noteworthy that Patel *et al* (2018) examined over 50 myeloid leukaemia-associated genes and did not find such a relationship. A second possibility is that the higher relative level of *NPM1*^{MUT} alleles reflects a lower level of residual normal haematopoietic cells that might result in slower haematological recovery post chemotherapy, and thus compromise the intended dose intensity over time. However, analysing the time between treatment cycles provided no support for this possibility. Thirdly, the higher level of *NPM1*^{MUT} alleles might be an indicator of higher tumour burden, providing information about tumour mass beyond that provided by the WBC alone. In accord with the Goldie-Coldman hypothesis (Goldie & Coldman, 1979), those cases with the greatest number of tumour cells would be expected to contain the highest number of potentially drug-resistant subclones at diagnosis, and mutations or epigenetic events giving rise to such chemo-resistance might not be readily detected prior to therapy.

Although the younger age of the patients in our cohort, all of them under the age of 60 years whereas the other three cohorts all included patients over this age, may have influenced the results, Patel *et al* (2018) reported that the impact of the *NPM1* VAF on OS was similar in patients above and below 60 years. Similarly, although there were methodological differences in the quantification of *NPM1* VAFs, with capillary electrophoresis used in our study, next generation sequencing platforms in the Patel and Rothenberg-Thurley studies and both methods used in the Abbas study, it is striking that the median value differed by only 4% between all studies (39% for the Patel study, 43% for the other three), and therefore it is unlikely that this accounts for the differences in outcome.

An important clinical issue is whether the presence of an *NPM1* mutation should be considered as a binary parameter in prognostic and therapeutic stratification, or whether the mutant allele burden should be quantified and incorporated into prognostic algorithms. There are several factors that might influence this decision. Firstly, in our study the prognostic impact after adjusting for other factors was relatively small. Secondly, because of the narrow range of *NPM1*^{MUT} allele levels, to use this parameter clinically in a multi-centre setting would require very precise measurement and quality assurance measures. For example, a 4% difference in levels due to technical issues could change a patient from the second to the fourth quartile of *NPM1*^{MUT} levels and vice-versa. Thirdly, our data suggests that there is no greater benefit of allogeneic transplantation in first CR in those with higher mutant allele burdens compared to those with lower levels. Finally, measurement of *NPM1*^{MUT} minimal residual disease levels post course 2 of therapy provides highly prognostic information that is not achieved by measurement of allelic ratios (Ivey *et al*, 2016). For these reasons, we believe that the binary presence or absence of an *NPM1* mutation should continue to be used for stratification rather than introducing quantitative thresholds.

Acknowledgments

We thank the clinical investigators who entered and managed patients in these trials. This work was supported by Bloodwise, formerly Leukaemia & Lymphoma Research, United Kingdom (grants 11005 and 14001 to DCL and REG) and the United Kingdom Medical Research Council. The work was undertaken at University College London Cancer Institute, which receives a proportion of funding from the Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme.

Authorship Contributions

DCL and REG designed the study; REG, DCL and RKH analysed the data; AKB and NR were principal trial coordinators and provided data; DCL and REG wrote the manuscript; all the authors reviewed the manuscript.

Conflict-of-interest disclosure

The authors have no competing financial interests to declare.

References

Abbas, H.A., Ravandi, F., Loghavi, S., Patel, K.P., Borthakur, G., Kadia, T.M., Jabbour, E., Takahashi, K., Cortes, J., Issa, G.C., Konopleva, M., Kantarjian, H.M. & Short, N.J. (2019) *NPM1* mutant variant allele frequency correlates with leukemia burden but does not provide prognostic information in *NPM1*-mutated acute myeloid leukemia. American Journal of Hematology, **94**, E158-E160.

Burnett, A.K., Hills, R.K., Milligan, D.W., Goldstone, A.H., Prentice, A.G., McMullin, M.F., Duncombe, A., Gibson, B. & Wheatley, K. (2010) Attempts to optimize induction and consolidation treatment in acute myeloid leukemia: Results of the MRC AML12 trial. *Journal of Clinical Oncol*ogy, **28**, 586-595.

Burnett, A.K., Russell, N.H., Hills, R.K., Hunter, A.E., Kjeldsen, L., Yin, J., Gibson, B.E., Wheatley, K. & Milligan, D. (2013) Optimization of chemotherapy for younger patients with acute myeloid leukemia: results of the Medical Research Council AML15 trial. *Journal of Clinical Oncol*ogy, **29**, 369-377.

Burnett, A.K., Russell, N.H., Hills, R.K., Kell, J., Cavenagh, J., Kjeldsen, L., McMullin, M.F., Cahalin, P., Dennis, M., Friis, L., Thomas, I.F., Milligan, D. & Clark, R.E.; UK NCRI AML Study Group. (2015) A randomized comparison of daunorubicin 90 mg/m^2 vs 60 mg/m^2 in AML induction: results from the UK NCRI AML17 trial in 1206 patients. *Blood*, **125**, 3878-3885.

Cancer Genome Atlas Research Network, Ley, T.J., Miller, C., Ding, L., Raphael, B.J., Mungall, A.J., Robertson, A., Hoadley, K., Triche, T.J. Jr, Laird, P.W., Baty, J.D., Fulton, L.L., Fulton, R., Heath, S.E., Kalicki-Veizer, J., Kandoth, C., Klco, J.M., Koboldt, D.C., Kanchi, K.L., Kulkarni, S., Lamprecht, T.L., Larson, D.E., Lin, L., Lu, C., McLellan, M.D., McMichael, J.F., Payton, J., Schmidt, H., Spencer, D.H., Tomasson, M.H., Wallis, J.W., Wartman, L.D., Watson, M.A., Welch, J., Wendl, M.C., Ally, A., Balasundaram, M., Birol, I., Butterfield, Y., Chiu, R., Chu, A., Chuah, E., Chun, H.J., Corbett, R., Dhalla, N., Guin, R., He, A., Hirst, C., Hirst, M., Holt, R.A., Jones, S., Karsan, A., Lee, D., Li, H.I., Marra, M.A., Mayo, M., Moore, R.A., Mungall, K., Parker, J., Pleasance, E., Plettner, P., Schein, J., Stoll, D., Swanson, L., Tam, A., Thiessen, N., Varhol, R., Wye, N., Zhao, Y., Gabriel, S., Getz, G., Sougnez, C., Zou, L., Leiserson, M.D., Vandin, F., Wu, H.T., Applebaum, F., Baylin, S.B., Akbani, R., Broom, B.M., Chen, K., Motter, T.C., Nguyen, K., Weinstein, J.N., Zhang, N., Ferguson, M.L., Adams, C., Black, A., Bowen, J., Gastier-Foster, J., Grossman, T., Lichtenberg, T., Wise, L., Davidsen, T., Demchok, J.A., Shaw, K.R., Sheth, M., Sofia, H.J., Yang, L., Downing, J.R. & Eley, G. (2013) Genomic and epigenetic landscapes of adult de novo acute myeloid leukemia. *New England Journal of Medicine,* **368,** 2059-2074.

Cheson, B.D., Bennett, J.M., Kopecky, K.J., Büchner, T., Willman, C.L., Estey, E.H., Schiffer, C.A., Döhner, H., Tallman, M.S., Lister, T.A., Lo-Coco, F., Willemze, R., Biondi, A., Hiddemann, W., Larson, R.A., Löwenberg, B., Sanz, M.A., Head, D.R., Ohno, R. & Bloomfield, C.D. (2003) Revised recommendations of the International Working Group for diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. *Journal of Clinical Oncology*, **21**, 4642-4649.

Döhner, H., Estey, E., Grimwade, D., Amadori, S., Appelbaum, F.R., Büchner, T., Dombret, H., Ebert, B.L., Fenaux, P., Larson, R.A., Levine, R.L., Lo-Coco, F., Naoe, T., Niederwieser, D., Ossenkoppele, G.J., Sanz, M., Sierra, J., Tallman, M.S., Tien, H.F., Wei, A.H., Löwenberg, B. & Bloomfield, C.D. (2017) Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood*, **129**, 427-447.

Early Breast Cancer Trialists Collaborative Group. (1990) Treatment of Early Breast Cancer Volume I: Worldwide Evidence 1985-90. United Kingdom Oxford University Press.

Gale, R.E., Green, C., Allen, C., Mead, A.J., Burnett, A.K., Hills, R.K. & Linch, D.C.; Medical Research Council Adult Leukaemia Working Party. (2008) The impact of *FLT3* internal tandem duplication mutant level, number, size, and interaction with *NPM1* mutations in a large cohort of young adult patients with acute myeloid leukemia. *Blood*, **111**, 2776-2284.

Gale, R.E., Lamb, K., Allen, C., El-Sharkawi, D., Stowe, C., Jenkinson, S., Tinsley, S., Dickson, G., Burnett, A.K., Hills, R.K. & Linch, D.C. (2015) Simpson's paradox and the impact of different

DNMT3A mutations on outcome in younger adults with acute myeloid leukemia. *Journal of Clinical Oncology*, **33**, 2072-2083.

Genovese, G., Kähler, A.K., Handsaker, R.E., Lindberg, J., Rose, S.A., Bakhoum, S.F., Chambert, K., Mick, E., Neale, B.M., Fromer, M., Purcell, S.M., Svantesson, O., Landén, M., Höglund, M., Lehmann, S., Gabriel, S.B., Moran, J.L., Lander, E.S., Sullivan, P.F., Sklar, P., Grönberg, H., Hultman, C.M. & McCarroll, S.A. (2014) Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *New England Journal of Medicine,* **371**, 2477–2487.

Goldie, J.H. & Coldman, A.J. (1979) A mathematical model for relating the drug sensitivity of tumours to their spontaneous mutation rate. *Cancer Treatment Reports,* **63**, 1727-1733.

Grimwade, D., Hills, R.K., Moorman, A.V., Walker, H., Chatters, S., Goldstone, A.H., Wheatley, K., Harrison, C.J, & Burnett, A.K.; National Cancer Research Institute Adult Leukaemia Working Group. (2010) Refinement of cytogenetic classification in acute myeloid leukemia: determination of prognostic significance of rare recurring chromosomal abnormalities among 5876 younger adult patients treated in the United Kingdom Medical Research Council trials. *Blood*, **116**, 354-365.

Hann, I.M., Stevens, R.F., Goldstone, A.H., Rees, J.K., Wheatley, K., Gray, R.G. & Burnett, A.K. (1997) Randomized comparison of DAT versus ADE as induction chemotherapy in children and younger adults with acute myeloid leukemia: Results of the Medical Research Council's 10th AML trial (MRC AML10). *Blood*, **89**, 2311-2318.

Ivey, A., Hills, R.K., Simpson, M.A., Jovanovic, J.V., Gilkes, A., Grech, A., Patel, Y., Bhudia, N., Farah, H., Mason, J., Wall, K., Akiki, S., Griffiths, M., Solomon, E., McCaughan, F., Linch, D.C., Gale, R.E., Vyas, P., Freeman, S.D., Russell, N., Burnett, A.K. & Grimwade, D.; UK National Cancer Research Institute AML Working Group. (2016) Assessment of minimal residual disease in standard-risk AML. *New England Journal of Medicine,* **374**, 422-433.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P.V., Mar, B.G., Lindsley, R.C., Mermel, C.H., Burtt, N., Chavez, A., Higgins, J.M., Moltchanov, V., Kuo, F.C., Kluk, M.J., Henderson, B., Kinnunen, L., Koistinen, H.A., Ladenvall, C., Getz, G., Correa, A., Banahan, B.F., Gabriel, S., Kathiresan, S., Stringham, H.M., McCarthy, M.I., Boehnke, M., Tuomilehto, J., Haiman, C., Groop, L., Atzmon, G., Wilson, J.G., Neuberg, D., Altshuler, D. & Ebert, B.L. (2014) Age-related clonal hematopoiesis associated with adverse outcomes. *New England Journal of Medicine,* **371**, 2488–2498.

Linch, D.C., Hills, R.K., Burnett, A.K., Khwaja, A. & Gale, R.E. (2014) Impact of *FLT3*^{ITD} mutant allele level on relapse risk in intermediate-risk acute myeloid leukemia. *Blood*, **124**, 273-276.

Papaemmanuil, E., Gerstung, M., Bullinger, L., Gaidzik, V.I., Paschka, P., Roberts, N.D., Potter, N.E., Heuser, M., Thol, F., Bolli, N., Gundem, G., Van Loo, P., Martincorena, I., Ganly, P., Mudie, L., McLaren, S., O'Meara, S., Raine, K., Jones, D.R., Teague, J.W., Butler, A.P., Greaves, M.F., Ganser, A., Döhner, K., Schlenk, R.F., Döhner, H. & Campbell, P.J. (2016) Genomic classification and prognosis in acute myeloid leukemia. *New England Journal of Medicine,* **374,** 2209-2221.

Pratcorona, M., Brunet, S., Nomdedéu, J., Ribera, J.M., Tormo, M., Duarte, R., Escoda, L., Guàrdia, R., Queipo de Llano, M.P., Salamero, O., Bargay, J., Pedro, C., Martí, J.M., Torrebadell, M., Díaz-Beyá, M., Camós, M., Colomer, D., Hoyos, M., Sierra, J. & Esteve, J.; Grupo Cooperativo Para el Estudio y Tratamiento de las Leucemias Agudas Mieloblásticas. (2013) Favorable outcome of patients with acute myeloid leukemia harbouring a low-allelic burden *FLT3*-ITD mutation and concomitant *NPM1* mutation: relevance to post-remission therapy. *Blood,* **121**, 2734-2738.

Patel, S.S., Kuo, F.C., Gibson, C.J., Steensma, D.P., Soiffer, R.J., Alyea, E.P. 3rd, Chen, Y.A., Fathi, A.T., Graubert, T.A., Brunner, A.M., Wadleigh, M., Stone, R.M., DeAngelo, D.J., Nardi, V., Hasserjian, R.P. & Weinberg, O.K. (2018) High *NPM1*-mutant allele burden at diagnosis predicts unfavorable outcomes in de novo AML. *Blood*, **131**, 2816-2825.

Raghavan, M., Smith, L.L., Lillington, D.M., Chaplin, T., Kakkas, I., Molloy, G., Chelala, C., Cazier, J.B., Cavenagh, J.D., Fitzgibbon, J., Lister, T.A. & Young, B.D. (2008) Segmental uniparental disomy is a commonly acquired genetic event in relapsed acute myeloid leukemia. *Blood,* **112**, 814-821.

Rothenberg-Thurley, M., Herold, T., Görlich, D., Sauerland, C., Janke, H., Prassek, V.V., Konstandin, N.P., Dufour, A.M., Schneider, S., Ksienzyk, B., Packeiser, E., Subklewe, M., von Bergwelt, M., Bohlander, S.K., Faldum, A., Braess, J., Wörmann, B.J., Berdel, W.E., Krug, U., Hiddemann, W., Spiekermann, K. & Metzeler, K.H. (2018) *NPM1* variant allele frequency and outcomes in AML. *Blood*, **132**, 1486.

Schnittger, S., Bacher, U., Kern, W., Alpermann, T., Haferlach, C. & Haferlach, T. (2011) Prognostic impact of *FLT3*-ITD load in *NPM1* mutated acute myeloid leukemia. *Leukemia*, **25,** 1297-1304.

Shivarov, V., Gueorguieva, R., Stoimenov, A. & Tui, R. (2013) *DNMT3A* mutation is a poor prognostic biomarker in AML: results of a meta-analysis of 4,500 AML patients. *Leukemia Research,* **37**, 1445-1450.

Shlush, L.I., Zandi, S., Mitchell, A., Chen, W.C., Brandwein, J.M., Gupta, V., Kennedy, J.A., Schimmer, A.D., Schuh, A.C., Yee, K.W., McLeod, J.L., Doedens, M., Medeiros, J.J., Marke, R., Kim, H.J., Lee, K., McPherson, J.D., Hudson, T.J.; HALT Pan-Leukemia Gene Panel Consortium, Brown, A.M., Yousif, F., Trinh, Q.M., Stein, L.D., Minden, M.D., Wang, J.C. & Dick, J.E. (2014) Identification of pre-leukaemic haematopoietic stem cell in acute leukaemia. *Nature*, **506**, 328-333.

Thiede, C., Koch, S., Creutzig, E., Steudel, C., Illmer, T., Schaich, M. & Ehninger, G. (2006) Prevalence and prognostic impact of *NPM1* mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood*, **107**, 4011-4020.

Xie, M., Lu, C., Wang, J., McLellan, M.D., Johnson, K.J., Wendl, M.C., McMichael, J.F., Schmidt, H.K., Yellapantula, V., Miller, C.A., Ozenberger, B.A., Welch, J.S., Link, D.C., Walter, M.J., Mardis, E.R., Dipersio, J.F., Chen, F., Wilson, R.K., Ley, T.J. & Ding, L. (2014) Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nature Medicine*, **20**(, 1472-1478.

Table 1. Demographics of the total cohort of *NPM1*^{MUT} patients studied

Parameter	Number of patients (% of group) (n = 876)
Trial	
AML10	141 (16%)
AML12	294 (34%)
AML15	219 (25%)
AML17	222 (25%)
Age (years)	
16–29	102 (12%)
30–39	163 (19%)
40-49	283 (33%)
50-59	328 (37%)
Median (range)	46 (16-59)
Sex	
Female	508 (58%)
Male	368 (42%)
WHO performance status	
0	456 (52%
1	243 (28%)
2	113 (13%)
3	64 (7%)
WBC (x10^9/l)	
0-9.9	196 (23%)
10.0-49.9	364 (42%)
50.0-99.9	165 (19%)
≥100	139 (16%)
Median (range)	29.9 (0.2-559.0)
Cytogenetics*	
Normal Karyotype	760 (87%)
Abnormal Karyotype	116 (13%)
FLT3^{ITD}	
Wild-type	518 (59%)
Mutant	358 (41%)
DNMT3A mutation	
Wild-type	314 (50%)
Mutant	316 (50%
Not known	246
Allogeneic transplant in CR1	137 (16%)

*All patients had intermediate-risk cytogenetics according to the MRC classification
Abbreviations: CR1, first complete remission; WBC, white blood cell count

Table 2. Prognostic factors identified in univariate analysis for this cohort

Variable	CR		CIR		OS	
	OR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Trial (vs AML10)		0.9		0.3		0.08
AML12	1.28 (0.55-2.96)		1.18 (0.86-1.61)		1.03 (0.79-1.35)	
AML15	1.40 (0.59-3.33)		0.89 (0.63-1.26)		0.81 (0.60-1.09)	
AML17	1.38 (0.58-3.28)		0.99 (0.70-1.40)		0.77 (0.57-1.05)	
Age (per decade)	1.69 (1.25-2.28)	0.0006	1.10 (0.99-1.22)	0.08	1.24 (1.13-1.36)	<0.0001
Male sex	0.84 (0.50-1.42)	0.5	1.08 (0.87-1.34)	0.5	1.09 (0.90-1.32)	0.4
WHO PS (per level)	1.50 (1.19-1.89)	0.0006	1.10 (0.98-1.23)	0.10	1.25 (1.13-1.37)	<0.0001
Log_{10} (WBC) – per 1 log increase	6.41 (3.44-11.95)	<0.0001	1.62 (1.32-1.99)	<.0001	1.74 (1.44-2.09)	<0.0001
Abnormal cytogenetics	1.10 (0.53-2.29)	0.8	1.14 (0.84-1.55)	0.4	0.97 (0.73-1.27)	0.8
FLT3^{ITD} mutation	2.52 (1.49-4.27)	0.0006	2.19 (1.76-2.71)	<.0001	1.88 (1.55-2.27)	<0.0001
DNMT3A mutation	1.60 (0.84-3.06)	0.16	1.46 (1.14-1.88)	0.003	1.38 (1.11-1.72)	0.004
NPM1^{MUT} allele level above/below median	2.38 (1.38-4.11)	0.002	1.67 (1.35-2.08)	<.0001	1.56 (1.29-1.89)	<0.0001

Abbreviations: CR, complete remission; CI, 95% confidence intervals; CIR, cumulative incidence of relapse; HR, hazard ratio; OR, Odds ratio; OS, overall survival: PS, performance status; WBC, white blood cell count

Table 3. Impact of *NPM1*^{MUT} allele burden on clinical outcomes

Outcome	*NPM1*^{MUT} comparison	Unadjusted		Multivariable analysis*	
		OR/HR (95% CI)	*P*	OR/HR (95% CI)	*P*
CR	Above/below median (43%)	2.38 (1.38-4.11)	0.002	1.09 (0.47-2.54)	0.8
	Per quartile	1.57 (1.22-2.02)	0.0005	1.07 (0.71-1.60)	0.8
	Q4 vs Q1-3	2.51 (1.47-4.30)	0.0008	1.28 (0.55-2.99)	0.6
CIR	Above/below median (43%)	1.67 (1.35-2.08)	<0.0001	1.56 (1.18-2.05)	0.002
	Per quartile	1.25 (1.13-1.38)	<0.0001	1.19 (1.04-1.36)	0.01
	Q4 vs Q1-3	1.56 (1.22-2.01)	0.0005	1.54 (1.13-2.11)	0.007
OS	Above/below median (43%)	1.56 (1.29-1.89)	<0.0001	1.25 (0.98-1.60)	0.07
	Per quartile	1.25 (1.14-1.36)	<0.0001	1.10 (0.97-1.23)	0.13
	Q4 vs Q1-3	1.66 (1.34-2.06)	<0.0001	1.41 (1.08-1.84)	0.01

*In the multivariable analysis, adjustments were made for factors shown to be prognostic in the totality of patients within the AML 10, 12 and 15 trials, i.e. age, performance status, white blood cell count, the presence of a *FLT3*^{ITD} mutation and the *FLT3*^{ITD} level (above and below 50%) and a *DNMT3A* mutation.

Abbreviations: CR, complete remission; CI, 95% confidence intervals; CIR, cumulative incidence of relapse; HR, hazard ratio; OR, Odds ratio; OS, overall survival; Q, quartile

Table 4. Overall survival stratified by both *NPM1*^{MUT} and *FLT3*^{ITD} mutant allele burden

NPM1^{MUT} allele level	*FLT3*^{ITD} allele level	OS at 5 years
Above median (≥44%)	<25%	47%
	25-49%	41%
	≥50%	20%
Below median (<44%)	<25%	55%
	25-49%	49%
	≥50%	52%

Figure Legends

Figure 1. *NPM1* mutant allele burden in the cohort of 876 patients.

Figure 2. Kaplan-Meier curves showing the impact of *NPM1*^{MUT} allele burden. Cumulative incidence of relapse stratified according to (A) the median mutant level and (B) quartiles. Overall survival stratified according to (C) the median mutant level and (D) quartiles.

Figure 3. Overall survival stratified according to *NPM1* mutant allele burden and the presence or absence of a *FLT3*^{ITD}. *NPM1* high and low refer to mutant level above and below the median (43%).

Figure 1

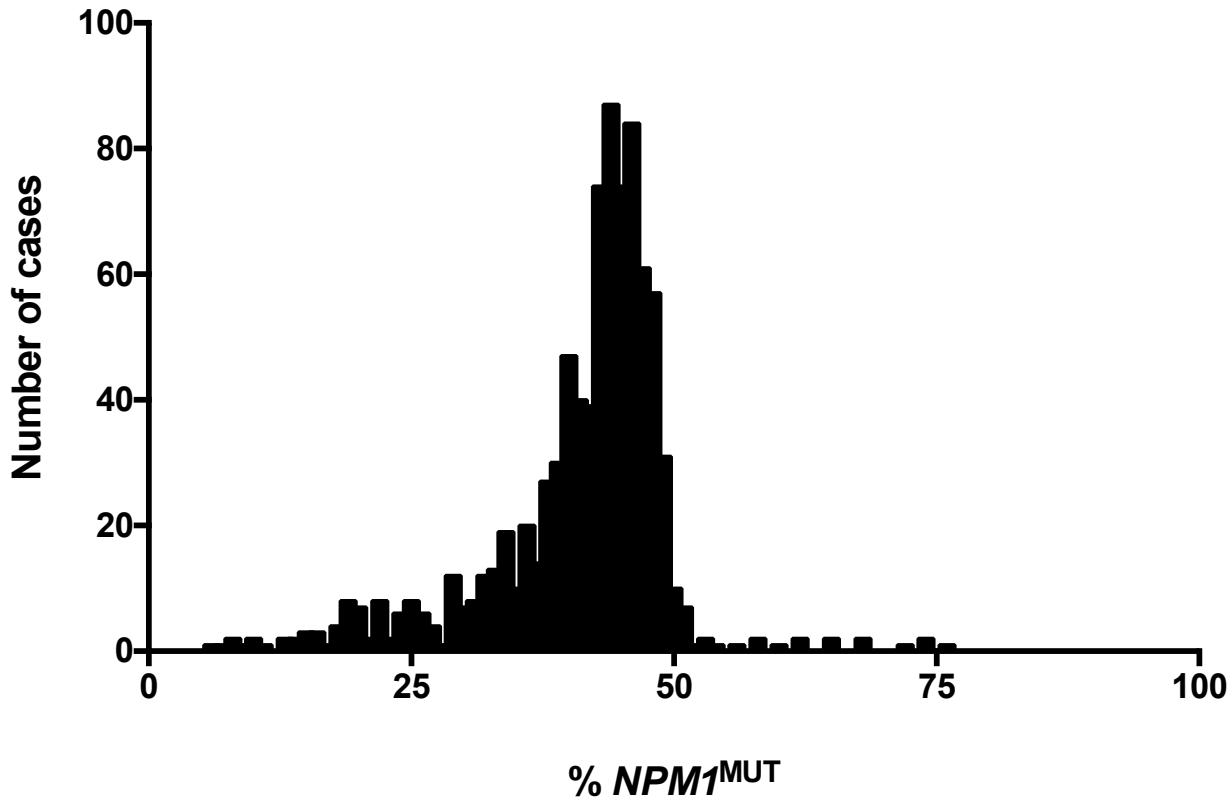


Figure 2

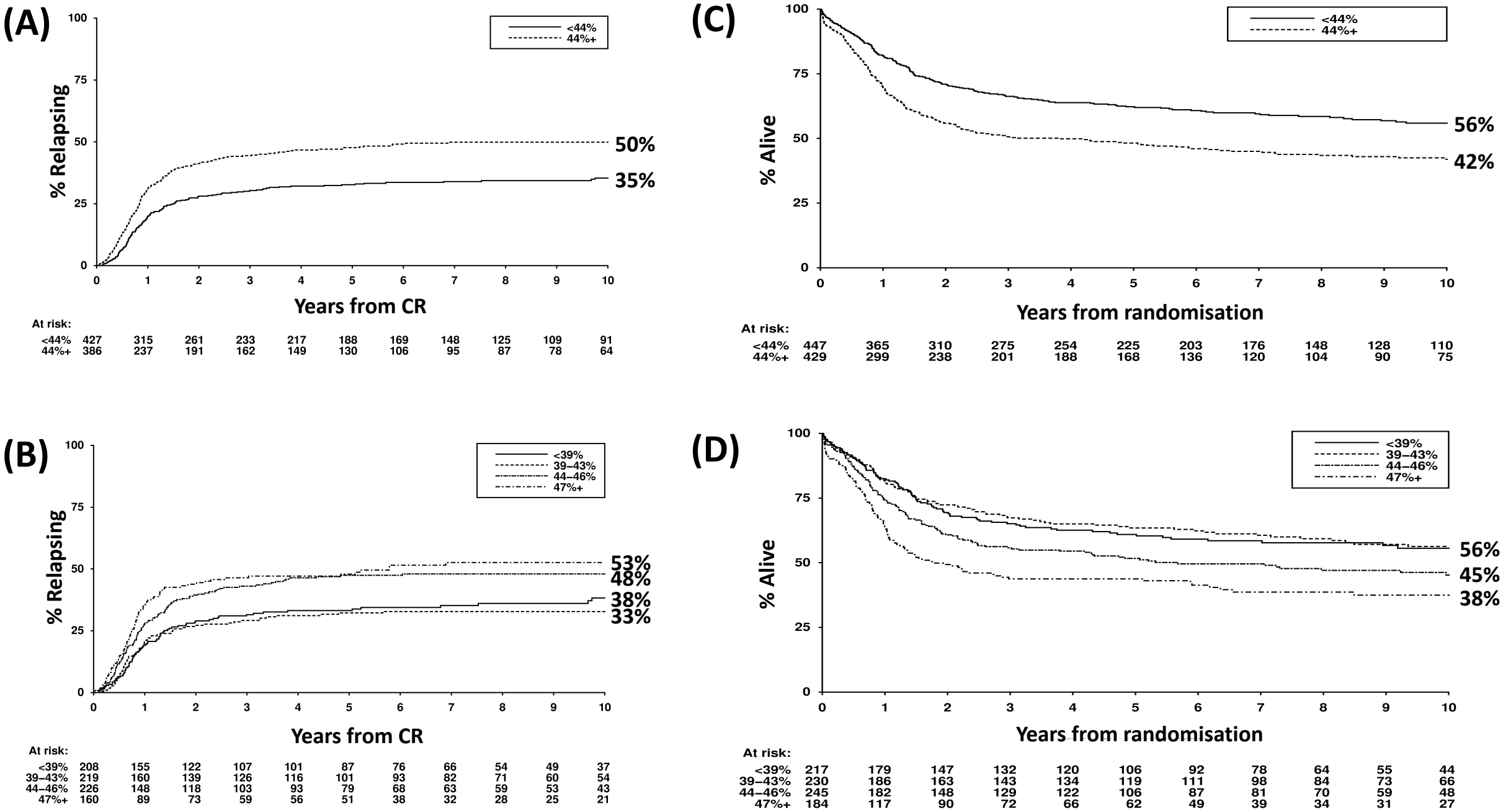
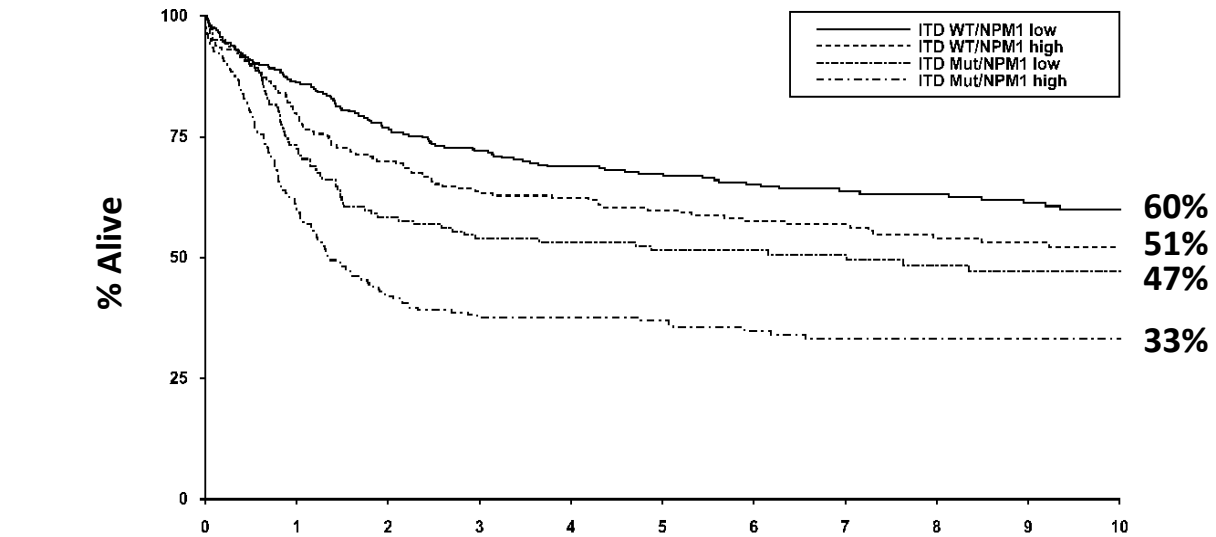

(A)
<44%
44%+
% Relapsing
100
75
50
25
0
50%
35%
0 1 2 3 4 5 6 7 8 9 10
Years from CR
At risk:
<44% 427 315 261 233 217 188 169 148 125 109 91
44%+ 386 237 191 162 149 130 106 95 87 78 64
(C)
<44%
44%+
% Alive
100
75
50
25
0
56%
42%
0 1 2 3 4 5 6 7 8 9 10
Years from randomisation
At risk:
<44% 447 365 310 275 254 225 203 176 148 128 110
44%+ 429 299 238 201 188 168 136 120 104 90 75
(B)
<39%
39–43%
44–46%
47%+
% Relapsing
100
75
50
25
0
53%
48%
38%
33%
0 1 2 3 4 5 6 7 8 9 10
Years from CR
At risk:
<39% 208 155 122 107 101 87 76 66 54 49 37
39–43% 219 160 139 126 116 101 93 82 71 60 54
44–46% 226 148 118 103 93 79 68 63 59 53 43
47%+ 160 89 73 59 56 51 38 32 28 25 21
(D)
<39%
39–43%
44–46%
47%+
% Alive
100
75
50
25
0
56%
45%
38%
0 1 2 3 4 5 6 7 8 9 10
Years from randomisation
At risk:
<39% 217 179 147 132 120 106 92 78 64 55 44
39–43% 230 186 163 143 134 119 111 98 84 73 66
44–46% 245 182 148 129 122 106 87 81 70 59 48
47%+ 184 117 90 72 66 62 49 39 34 31 27

Figure 3


ITD WT/NPM1 low
ITD WT/NPM1 high
ITD Mut/NPM1 low
ITD Mut/NPM1 high
% Alive
100
75
50
25
0
0
1
2
3
4
5
6
7
8
9
10
Years from randomisation
60%
51%
47%
33%
At risk:
ITD WT/NPM1 low 305 261 228 205 185 165 148 126 105 91 77
ITD WT/NPM1 high 213 169 148 133 127 112 92 83 69 59 48
ITD Mut/NPM1 low 142 104 82 70 69 60 55 50 43 37 33
ITD Mut/NPM1 high 216 130 90 68 61 56 44 37 35 31 27