



Published in final edited form as:

Nat Med. 2018 July ; 24(7): 1015–1023. doi:10.1038/s41591-018-0081-z.

Somatic mutations precede acute myeloid leukemia years before diagnosis

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Abstract

The pattern of somatic mutations observed at diagnosis of acute myeloid leukemia (AML) has been well-characterized. However, the premalignant mutational landscape of AML and its impact on risk and time to diagnosis is unknown. Here we identified 212 women from the Women's Health Initiative who were healthy at study baseline, but eventually developed AML during follow-up (median time: 9.6 years). Deep sequencing was performed on peripheral blood DNA of these cases and compared to age-matched controls that did not develop AML. We discovered that mutations in *IDH1*, *IDH2*, *TP53*, *DNMT3A*, *TET2* and spliceosome genes significantly increased the odds of developing AML. All subjects with *TP53* mutations ($n = 21$ out of 21 patients) and *IDH1* and *IDH2* ($n = 15$ out of 15 patients) mutations eventually developed AML in our study. The presence of detectable mutations years before diagnosis suggests that there is a period of latency

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Author contributions

P.D., M.S.S., M.L.G., G.J.R. and D.C.H. designed and supervised the study; P.D., N.M.-T., M.L.G., G.J.R. and D.C.H. wrote the manuscript; P.D. compiled epidemiological data; N.M.-T. performed experiments; P.D., N.M.-T., M.L.G. and D.C. H. analyzed data; P.D., N.M.-T., O.S., D.C.H. and K.V.B. performed and/or supervised statistical studies; G.C. performed experiments; S.L., M.S. and E.K.R. reviewed and interpreted data.

Competing interests

The authors declares no competing interests.

Supplementary information is available for this paper at <https://doi.org/10.1038/s41591-018-0081-z>.

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that precedes AML during which early detection, monitoring and interventional studies should be considered.

The pathogenesis of AML is characterized by serial acquisition of somatic mutations and several genes are recurrently mutated in AML^{1–3}. However, it is not known when such mutations appear prior to the development of overt disease, how they evolve and the specific risk associated with each one. Furthermore, the acquisition of AML-associated mutations has also been found in normal aging, and approximately 10% of people older than 65 years of age^{4–7} have clonal hematopoiesis. The presence of clonal hematopoiesis is associated with an increased risk of hematologic malignancies and cardiovascular disease. However, studies of clonal hematopoiesis to date have included very few subjects who subsequently developed AML. Whole-exome sequencing on peripheral blood samples from 17,182 individuals was performed in a previous study⁵. Mutations in genes that are associated with hematologic cancers were found in 5.6% of subjects aged 60–69 years and in up to 18.4% of those aged >90 years. Only 16 hematologic cancers were reported in this group, of which 6 were cases with AML. In another study⁴, DNA sequencing was performed on peripheral blood from 12,380 Swedish individuals (mean age, 55 years), with clonal mutations detectable most commonly in *DNMT3A*, *ASXL1* and *TET2*. Clonal hematopoiesis was strongly associated with increased risk of hematologic cancer (hazard ratio, 12.9; 95% confidence interval, 5.8–28.7), but there were only 12 cases with AML reported in the study⁴. The objective of the present work was to establish a genomically defined premalignant state of AML and ascertain the premalignant mutational landscape of AML years before the diagnosis of the disease. We further sought to investigate whether specific mutations, allele burdens or patterns of coexisting mutations would affect the risk and time-to-diagnosis of AML. To this end, we performed deep sequencing of serially collected peripheral blood samples obtained from 212 women a median of 9.6 years before their diagnosis of AML, along with 212 age-matched controls.

Results

Study population and samples.

The Women's Health Initiative (WHI) was a large, prospective clinical trial and observational study of more than 160,000 women initiated by the US National Institutes of Health (NIH) in 1991 and consisted of three clinical trials and an observational cohort designed to assess the impact of hormonal therapy on postmenopausal health issues in women with an average follow-up of 10.8 years (s.d., 3.3 years)^{8,9}. Detailed clinical data regarding medical history, medications and complete blood counts were available at baseline assessment. New diagnoses were updated in follow-up, with central confirmation of all new diagnoses of cancer. The participants in the clinical trials were followed at baseline and three, six and nine years after study entry, whereas the participants of the observational cohort were followed at baseline, one and three years after study entry. In addition, all participants of the observational cohort filled out yearly questionnaires for the first eight years of follow-up, updating any new medical diagnoses during follow-up.

Identification of cases.

In the WHI cohort, 212 study participants eventually developed pathologically confirmed AML. Of these, baseline peripheral blood DNA at study entry was available and passed quality control in 188 participants; these were identified as cases to be included in the final analyses. Additional follow-up samples for 130 cases were available at one and/or three years after baseline, all prior to the diagnosis of AML. Exclusion criteria for cases included known diagnosis of any hematological disorder (myeloid and lymphoid), including AML, prior to WHI baseline evaluation.

Controls.

Age-matched controls ($n = 212$) were selected from participants who were confirmed not to have a diagnosis of AML while being followed during the WHI study. Exclusion criteria included concurrent or history of prior hematologic disorder (myeloid and lymphoid), including AML, at WHI baseline. Controls were matched to cases by age at baseline within two years, history of non-hematologic cancers at baseline and type and timing of any cancers that occurred in cases after WHI baseline, but before the diagnosis of AML as well as the exact timing of blood sample collection and follow up in the WHI. Matching was done in a time forward manner to ensure that each control had as much control time as its matched case¹⁰. Of the 212 controls, peripheral blood was available and passed quality standard in 181 controls at WHI baseline and these were included in the analyses. Additional follow up samples for 126 controls were available at one and/or three years after baseline.

Landscape of mutations in blood before AML diagnosis

With a median of 9.6 years before diagnosis of AML, cases were more likely to harbor mutations than controls (odds ratio, 4.86; 95% confidence interval, 3.07–7.77; $P = 3.8 \times 10^{-13}$). The most common mutations identified above 1% variant allele fraction (VAF) included *DNMT3A* (36.7% of cases compared to 18.8% of controls), *TET2* (25.0% of cases compared to 5.5% of controls), *TP53* (11.2% of cases compared to 0% of controls), *SRSF2* (6.9% of cases compared to 0% of controls), *IDH2* (6.4% of cases compared to 0% of controls), *SF3B1* (5.9% of cases compared to 1.1% of controls), *JAK2* (5.3% of cases compared to 0.6% of controls) and *ASXL1* (3.2% of cases compared to 3.3% of controls; Fig. 1a, Supplementary Fig. 2 and Supplementary Table 2). Spliceosome mutations in *SF3B1*, *SRSF2* and *U2AF1* were identified as a group in 13.8% of cases ($n = 26$ out of 188) compared to 1.1% of controls ($n = 2$ out of 181). Similarly, IDH mutations as a group (both *IDH1* and *IDH2*) were identified in 8% of cases ($n = 15$ out of 188) compared to 0% of controls. There was no association between the presence of any mutation and abnormal hemoglobin, white-blood cell count and/or platelet level (Supplementary Table 1). We were not able to evaluate the relationship between red cell distribution width (RDW), absolute neutrophil count and mean corpuscular volume (MCV) with mutations as the WHI did not collect these data.

Overall, cases with AML demonstrated greater clonal complexity than controls, and 46.8% of the cases with AML showed comutations, compared to 5.5% of controls (odds ratio, 9.01; 95% confidence interval, 4.1–21.4; $P = 9.7 \times 10^{-10}$). As shown in Fig. 1b, the most common

comutations present in cases with AML were *DNMT3A* with *TET2*, *DNMT3A* with *SRSF2*, *TET2* with *SRSF2*, and *IDH2* with *SRSF2*. Among controls, mutations were generally present individually. When tested for mutual exclusivity and co-occurrence patterns, mutations in *DNMT3A*, *TP53*, *TET2* and *ASXL1* had a tendency toward mutual exclusivity. Conversely, co-occurrence was noted for *IDH2* and *SRSF2* as well as *RUNX1* and *PHF6*. Among controls, no common co-occurrence pattern was identified and *DNMT3A* with *TET2* was found to be mutually exclusive (Supplementary Fig. 3). Overall, cases with AML had a median of one mutated gene (median, 1; range, 0–8), whereas controls had median of no mutated genes (median, 0; range, 0–2) ($P < 2.2 \times 10^{-16}$). Older individuals (≥ 65 years) had more mutated genes in both cases and controls. The same held true for mutations categorized as pathogenic versus variant of unknown significance (Fig. 1c). We also found that the incremental increase in the number of mutations increased age-adjusted odds of AML per mutation (odds ratio, 3.27; 95% confidence interval, 2.47–4.45).

Presence of mutations at baseline increases risk of AML

Having a mutation at baseline assessment was associated with statistically increased odds of developing AML (odds ratio, 4.86; 95% confidence interval, 3.07–7.77; $P = 3.8 \times 10^{-13}$) (Table 1). This finding was independent of age: odds ratio of 4.39 (2.08–9.61) for cases aged <65 years; odds ratio of 6.19 (3.25–12.14) for cases aged ≥ 65 years. Overall, 68.62% ($n = 129$) of cases and only 30.9% of controls ($n = 56$) were found to have mutations. Mutations were found in 53.75% of cases and 20.78% of controls <65 years old, and in 79.63% of cases and 38.46% of controls who were ≥ 65 years old. This rate of clonal hematopoiesis in the control group is generally higher than previous reports^{4,5,11}, which were performed using low-coverage whole-exome sequencing and a higher VAF cutoff. We therefore also determined the clonal hematopoiesis rate using previous variant classification and VAF cutoff criteria (Supplementary Fig. 4 and Supplementary Table 4). When performing this adjustment, the observed rate of clonal hematopoiesis in controls was more in line with previous studies at around 7% (Supplementary Fig. 4 and Supplementary Table 4).

Among the recurrently mutated genes, some mutations demonstrated increased specificity and penetrance for the development of AML. All participants with mutations in *TP53* ($n = 21$ out of 21), *IDH1* or *IDH2* ($n = 15$ out of 15), or *RUNX1* with *PHF6* ($n = 3$ out of 3) in our cohort eventually developed AML. Multivariable analysis was performed to evaluate potential associations between individual mutated genes and the development of AML, adjusting for confounders including comutated genes and age (Fig. 2a). This analysis found that *TP53* (odds ratio, 47.2; 95% confidence interval, 2.5–879.1), IDH (including *IDH1* and *IDH2*) (odds ratio, 28.5; 95% confidence interval, 1.4–562.8), spliceosome genes (including *SF3B1*, *SRSF2* and *U2AF1*) (odds ratio, 7.4; 95% confidence interval, 1.7–32.2), *TET2* (odds ratio, 5.8; 95% confidence interval, 2.6–12.9) and *DNMT3A* (odds ratio, 2.6; 95% confidence interval, 1.5–4.5) were associated with significantly increased odds of developing AML relative to controls and will be referred to as ‘high-risk genes’ hereafter. Similar results were obtained when considering only those variants flagged as ‘likely pathogenic’ (Supplementary Fig. 16). Study participants who had more than one variant in *DNMT3A* or *TET2* demonstrated significantly increased odds of AML (Fig. 2b): two or more *DNMT3A* variants (odds ratio, 12.6; 95% confidence interval, 3.0–52.9), one *DNMT3A* variant (odds

ratio, 2.1; 95% confidence interval, 1.2–3.8); two or more *TET2* variants (odds ratio, 69.3; 95% confidence interval, 3.8–1,280.7), one *TET2* variant (odds ratio, 3.29; 95% confidence interval, 1.4–7.8). Because single mutations in *DNMT3A* and *TET2* were the most commonly found mutations in controls, this finding distinguishes the pattern of *DNMT3A* and *TET2* mutations in cases compared to controls. Mutations in *IDH1* and *IDH2* were exclusively found in the known recurring hotspots in Arg132 and Arg140^{2,12}. Similarly, *SRSF2* mutations were confined to the well-known Pro95 hotspot^{13,14}. *TP53* mutations were primarily in the DNA-binding, transactivation and oligomerization domains^{15,16}. The distribution of mutations within functional protein domains, as well as the type of mutations found at each location, is shown in the Supplementary Information (Supplementary Figs. 5–8, 13–15). We also identified relatively few non-canonical mutations, such as non-RING domain *CBL* mutations, non-kinase *JAK2* mutations, as well as mutations in *FLT1* and *CARD11*. These mutations were almost always associated with the presence of probable driver mutations in *DNMT3A* and/or *TET2* and were unique to particular study subjects. Notably, these mutations typically demonstrated persistence in the study participant when serial samples were available (Supplementary Fig. 11). Although these mutations are not likely to drive the observed clonal expansions, they are likely passenger events that are symptomatic of driver mutations and thus accurately reflect the premalignant state in cases with AML and clonal hematopoiesis when present in the controls.

We next sought to derive population-based estimates of the impact of mutations in high-risk genes on AML development using a pseudolikelihood-based approach¹⁷. Mutation-free participants in the high-risk genes (*DNMT3A*, *TET2*, *TP53*, *IDH1* or *IDH2*, spliceosome (*SRSF2*, *SF3B1* or *U2AF1*)) were estimated to develop AML with an incidence of 2.7 per 100,000 per year. Mutations increased this risk at varying rates depending on the gene: *TP53*, 13.9 per 100,000 per year; *IDH*, 12.6 per 100,000 per year; spliceosome, 9.2 per 100,000 per year; *DNMT3A*, 4.6 per 100,000 per year; and *TET2*, 5.5 per 100,000 per year (Supplementary Fig. 12).

Mutations are associated with an accelerated time to AML presentation

Cases with AML who had mutations at baseline experienced significantly shorter latency of disease than cases without baseline mutations. The presence of any mutation shifted the median time to AML diagnosis from 11.9 years (no mutations) to a median of 8.2 years after baseline assessment ($P = 9.8 \times 10^{-4}$, log-rank test; Fig. 3a). Univariable analysis of mutated genes demonstrated that median time to AML varied according to mutated gene (Fig. 3b): *DNMT3A* (7.4 compared to 10.5 years), *TP53* (4.9 compared to 10.1 years), spliceosome genes (6.7 compared to 9.9 years) and *RUNX1* (1.5 compared to 9.6 years). The presence of *RUNX1* mutations seemed to be associated with especially rapid development of AML within two years, but the number of participants in this subgroup was small ($n = 3$). Clonal complexity also affected the latency of disease as patients with one mutation in the high-risk genes developed AML in 9.1 years, but those with two or more mutations in the high-risk genes developed AML in only 6.9 years (Fig. 3c).

Multivariable analyses produced similar findings (Fig. 2c). Mutations in *TP53* were independently associated with AML occurring within five years of baseline (odds ratio, 5.2;

95% confidence interval, 1.9–14.5; $P = 0.001$) as well as an increased annual rate of AML development (hazard ratio, 3.01; 95% confidence interval, 1.8–5.0; $P = 2.8 \times 10^{-5}$) when adjusted for confounding comutations and age. *DNMT3A* mutations when present with spliceosome mutations were also associated with increased odds of AML development within five years (odds ratio, 14.8; 95% confidence interval, 1.6–136; $P = 0.02$). None of the other genes were associated with increased odds of earlier AML development when present alone or with other comutations. For *TP53* and *DNMT3A* mutations, there was no appreciable difference in time to development of AML by mutation subtype, for example, in Arg882 in *DNMT3A*. Finally, whereas all participants (15 out of 15) with IDH mutations were eventually diagnosed with AML, there was no increased risk of AML earlier than five years after baseline assessment, suggesting the possibility that further downstream mutational events are required for AML development in these cases.

Mutations at any VAF are associated with increased AML risk

When we considered only mutations in the genes that are associated with development of AML (high-risk genes), as shown in Fig. 4a, mutations present at VAF > 10% were found more commonly among cases compared to controls (odds ratio, 14.8; 95% confidence interval, 5.8–48.8; $P = 1.5 \times 10^{-13}$). As demonstrated in the histograms, mutations in *DNMT3A* and *TET2* at lower VAFs were notably more distributed among cases and controls. By contrast, mutations present at higher VAFs in *DNMT3A* and *TET2* were almost exclusively seen in cases with AML, suggesting that mutations in *DNMT3A* and *TET2* are less specific to cases with AML at lower VAFs. In a multivariable model evaluating high (> 10%) and low VAFs (<10%) in the high-risk genes and adjusting for age and other comutations, high VAFs in *DNMT3A* (high VAF: odds ratio, 4.8; 95% confidence interval, 1.6–14.8; $P = 0.004$; low VAF: odds ratio, 2.5; 95% confidence interval, 1.4–4.4; $P = 0.002$), *TET2* (high VAF: odds ratio, 20.4; 95% confidence interval, 3.5–120.1; $P = 0.002$; low VAF: odds ratio, 3.6; 95% confidence interval, 1.5–8.9; $P = 0.004$) and spliceosome genes (high VAF: odds ratio, 14.1; 95% confidence interval, 0.8–260.9; $P = 0.019$, low VAF: odds ratio, 3.4; 95% confidence interval, 0.7–16.8; $P = 0.105$) were associated with higher odds of developing AML (Supplementary Fig. 22). For *TP53* and IDH mutations, the odds of AML development did not significantly change between lower and higher VAFs as all of these subjects developed AML. Next, we further examined the specificity of mutations in genes that were significantly associated with cases with AML (Fig. 1a) by determining their frequency in controls. The true-positive and false-positive rate of mutations at varying VAF cutoffs was visualized using receiver operating characteristic analysis (Fig. 4b). Individually, mutations in *TP53*, *SRSF2*, *U2AF1*, *SF3B1* and *IDH2* produced a <1% rate of false-positive detection at VAF cutoffs ranging from 1 to 2%. Although not exactly replicating the false-positive rate in the general population, these data provide a starting point for future studies to assess the false-positive rate in the general population. Tabulated results are available in Supplementary Fig. 9. For the subset of participants for whom serial samples were available, changes in VAF were assessed from baseline to one or three or years. We evaluated whether the time to AML development was influenced by the fold increase in VAF in participants who had demonstrated a statistically significant increase in VAF upon serial testing (Fig. 4c). The rate of increase in VAF for *IDH2* mutations ($R^2 = 0.67$; slope, -3.3 ; 95%

confidence interval, -21.5 to -3.7 ; $P = 0.02$) and *TP53* mutations ($R^2 = 0.87$; slope, -3.0 ; 95% confidence interval, -14.0 to -5.5 ; $P = 0.009$) was significantly associated with a shorter time to AML development in a linear regression analysis. Although this relationship was similar for mutations in *DNMT3A*, it did not achieve statistical significance. Overall, $>95\%$ of mutations persisted when evaluated longitudinally at one year or three years after baseline. However, there were fewer changes from baseline allelic fraction across all genes in year 1 compared to year 3 (Supplementary Figs. 10, 11).

Association between non-AML cancer history and mutations

We examined the mutational patterns of the 21 cases and 19 controls that had a history of malignancy at baseline, including breast, lung, bladder, endometrial, ovarian and colon cancer. Treatment history for the cancer was not known. In total, 42% of cases ($n = 9$ out of 21) and 26% of controls ($n = 5$ out of 19) with a prior history of cancer at baseline were found to have mutations (not significant). Although the absolute number of cases and controls with a prior cancer history was low, we did note that 3 out of 10 cases with a prior history of cancer had *IDH2* mutations at baseline evaluation and that all three of these cases had prior breast cancer. None of the cases or controls with a prior history of cancer had *TP53* gene mutations. Overall, because of the low number of cases and controls with a prior history of cancer, we did not have sufficient power to study this effect.

Progression to AML from baseline mutations

Despite having selected for participants who eventually developed AML, we noted the absence of *NPM1* and *FLT3* mutations, which are among the two most frequently recurring driver mutations in AML^{1,18}. This finding is consistent with other reports of their absence in clonal hematopoiesis, and suggests that they may have a cooperative role in AML pathogenesis^{4,5}. We identified a single case for whom follow-up at year 1 preceded AML diagnosis by <30 days (Fig. 5, case A) and compared mutations at baseline and year 1 (<30 days prior to AML diagnosis). The depth of sequencing coverage at *NPM1* insertion sites was similar at both time points (around 450x). The participant had an *IDH2* mutation (8% VAF) at baseline and after one year had acquired a new *NPM1* type-A mutation (14% VAF), along with an increased VAF of a mutation in *IDH2* (13%). AML was diagnosed less than 30 days later. The rapid development of AML after the acquisition of an *NPM1* mutation suggests cooperation with the pre-existing *IDH2* clone given the similarity of their allelic fractions.

Other patterns of clonal evolution and expansion are demonstrated in representative cases B, C and D (Fig. 5). Mutations in genes that are typically associated with clonal hematopoiesis, such as *DNMT3A* and *TET2*, were shown to generally have stable or minimally increased VAFs in follow-up. Progression to AML was often preceded by the acquisition of new mutations or by the expansion of mutations in other genes, such as *RUNX1* or *TP53* (Fig. 5). With the exception of case B, who had a large *DNMT3A*-mutated clone with multiple subclones, comutations in the same cell formally require single cell analysis.

Discussion

Peripheral blood samples collected years before AML diagnosis enabled a unique and unprecedented opportunity to establish the existence of a genomically defined premalignant state of AML years before the development of overt disease and to determine the specific risk and time to AML development that are associated with various mutations, comutations and clone sizes. We found that study participants who eventually developed AML had higher mutational complexity at WHI baseline evaluation than age-matched controls. The presence of a mutation at baseline was associated with increased odds of developing AML. Individually, the most-significant mutations associated with increased odds of AML included those in *TP53*, *IDH1* and *IDH2*, spliceosome (*SRSF2*, *SF3B1* and *U2AF1*), *TET2* and *DNMT3A*. As mutations in *DNMT3A* and *TET2* were common in controls as well, it is important to note that both higher VAFs (>10%) and the presence of a higher number of variants (two or more) in these genes were associated with higher risk of AML. The median time to development of AML was shorter in subjects with mutations present at baseline WHI evaluation and those participants with baseline *TP53* mutations and *DNMT3A* comutated with spliceosome genes were more likely to develop AML within the following five years. Participants with mutations in the *RUNX1* gene all developed AML within two years after baseline, but the number of cases was too low to make statistical inferences. The time to develop AML was inversely correlated with an increasing VAF for somatic mutations in *TP53* and *IDH2*. Although having a mutation in any gene from our panel at baseline increased the risk of developing AML, we identified a set of genes with high AML penetrance, including *TP53*, *IDH1* and *IDH2*, *SRSF2*, *SF3B1* and *U2AF1*. Any detectable VAF in the high-penetrance genes at a sensitivity cutoff of 1% was associated with increased risk of AML. When serial samples were available, we found that a more rapid rise in the allelic fraction of *IDH2* or *TP53* mutations was significantly associated with a shorter time to AML development. As expected, serial samples also revealed the stepwise acquisition of mutations leading to AML. Mutations in genes commonly associated with clonal hematopoiesis, such as *DNMT3A* and *TET2*, were maintained over time, while new dominant subclones arose in genes such as *NPM1*, *TP53*, and *SRSF2* preceding the development of AML. We note that the absolute rate of clonal hematopoiesis in our study was on the higher end of the reported range for controls^{4-6,19,20}, likely in part because our study consisted of participants older than 50 years of age and was performed at relatively high non-duplicate sequencing depth with a lower 1% allelic fraction cutoff. Additionally, while there is increasing consensus around the biological definition of clonal hematopoiesis, there is no universally accepted technical definition. In general, studies have varied with respect to both the demographics of the study population and technical factors, among which are the sequencing techniques used (whole-genome, whole-exome or targeted sequencing), analytical approaches, in-solution capture versus amplicon sequencing, sequencer technology, depth of coverage and allelic fraction cutoff. For example, early seminal studies that helped to establish our general understanding of the prevalence of clonal hematopoiesis mutations were performed with relatively low-coverage whole-exome sequencing with lesser sensitivity for mutations present at low allelic fractions, as well as suboptimal coverage of genes recurrently mutated in clonal hematopoiesis (for example, mutations in *TET2* for which there was essentially no coverage of coding exons outside of exon 3)^{4,5}. Our study

finds that >70% of *TET2* mutations in both our case and control groups outside exon 3, suggesting possibly >3-fold underestimation of clonal hematopoiesis mutations in *TET2* by previous work. Moreover, recent data²⁰ using high-depth error-corrected sequencing demonstrated nearly ubiquitous clonal hematopoiesis dominated by mutations in *DNMT3A* and *TET2*. Irrespective of these differences, we observe in our study significantly increased mutations in cases with AML relative to clonal hematopoiesis in the control population, a significantly shorter latency period to AML development when mutations were detected, and mutations in genes of known significance among the cases with AML, such as *TP53* and *IDH2*, that are not found among the clonal hematopoiesis controls.

A major strength of our study is having a cohort of normal controls that was reliably followed over 10 years for outcomes, including detailed baseline demographics, medical history and laboratory data, thus providing a solid scientific base for a matched case-control analyses at the population level. A limitation to our study is that we could not perform sequencing studies of blood or bone marrow taken at the time of AML diagnosis. However, for participants with serial samples, we noted that the vast majority of mutations persist at one or three years after baseline, indicating their stability and often increasing clone size (Supplementary Fig. 11). Another limitation is that because our study included only women, there is also likely to be underestimation of mutations in X-linked genes with male predominance, such as *PHF6* and *ZRSR2*^{21,22}, as well as possible overrepresentation of mutations in *DNMT3A*, as has been reported previously⁴. Several commonly mutated genes in AML were absent from our study, notably *FLT3* and *NPM1*, suggesting that the acquisition of these mutations may be later events in AML ontogeny. Although pre-existing mutations in *TP53* and spliceosome genes were strongly associated with the development of AML in this study, it is well-known that these mutations are not exclusive to AML and are also associated with other hematological malignancies and as a rare clonal hematopoiesis event in normal populations²³. However, these data provide a strong rationale for following participants with *TP53* mutations. By contrast, *IDH2* Arg140 mutations are relatively more specific to AML. While it is possible that some of the participants in our study developed myelodysplastic syndrome after baseline and prior to developing AML, it should be noted that all of our cases and controls had normal baseline hematological parameters. Thus, further prospective monitoring studies will be required to confirm the specificity, or lack thereof, of these mutations in predicting AML versus other hematological disorders. Within our study, we determined that for specific genes (that is, *TP53*, *IDH2*, *SF3B1*, *SRSF2* and *U2AF1*), a VAF cutoff correlating with a falsepositive fraction of less than 1% could be achieved. These estimates would be a starting point for future studies to estimate the real falsepositive fraction in a general population.

In conclusion, our study establishes the premalignant landscape of mutations preceding overt AML that is present in peripheral blood at a median of 9.6 years prior to the diagnosis of AML. In our study, all participants with *TP53* and IDH mutations eventually developed AML. In comparison to the mutational landscape of de novo AML^{24,25}, our premalignant AML 'case' group demonstrated a similar frequency of mutations in genes that have high AML penetrance, especially *IDH2* and *TP53*. *DNMT3A* and *TET2* are accordingly overrepresented in our case cohort compared to de novo AML since most instances of these mutations do not lead to AML on a population level. Our results showing an association of

high allelic fractions in *DNMT3A* and *TET2* along with clonal complexity with a higher risk of AML is important in discriminating who would likely progress to AML. The ability to detect and identify high-risk mutations suggests that monitoring strategies for patients, as well as clinical trials of potentially preventative or disease-intercepting interventions should be considered. Indeed, molecularly targeted therapy is already available for *IDH2* mutations²⁶ and is under development for mutations in other candidate genes found in this study including *IDH1*, *TP53* and spliceosome genes^{27–29}. Moreover, preclinical data suggest that vitamin C, in clinically achievable doses, may reverse leukemogenesis mediated by *TET2* deficiency³⁰. Thus, as the cost of detecting mutations declines and its use in cancer surveillance increases, the feasibility of monitoring becomes more reasonable, especially when associated with a low false-positive rate and the potential availability of therapies with favorable toxicity profiles. We propose that patients at increased risk of developing AML should be followed longitudinally in long-term monitoring studies using next-generation sequencing to further evaluate the prognostic and predictive importance of a premalignant state in this disease. Data from these studies will provide a robust rationale for clinical trials of preventative intervention strategies in populations at high risk of developing AML.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at <https://doi.org/10.1038/s41591-018-0081-z>.

Methods

De-identified samples and data were obtained from the WHI and collection of these samples and data was performed with approval from the WHI Ancillary Committee and the WHI Extension Study Steering Committee. Informed consent from participants was obtained at the onset of the WHI trial conducted by participating centers (IRB approval reference number 3467-EXT). All ethical regulations were followed. Genomic DNA was provided by WHI in a blinded manner, in which case-control status and clinical covariates were revealed only after variant calling was completed. Library generation and amplification were performed using a low-error rate Hi-Fi DNA polymerase according to the Kapa HyperPrep protocol (Kapa Biosystems). Dual sample indexing, rather than single indexing, of libraries was performed to minimize signal spread errors arising from misidentification of multiplexed samples³¹. Targeted sequencing of genes recurrently mutated in hematological malignancies was performed using custom capture probes (Nimblegen) to a median coverage of 2,000× for both cases with AML and controls (Supplementary Fig. 1). Reads were aligned to the 1000 Genomes Phase 2 human reference genome and decoy contigs (hs37d5) using BWA MEM³². Variants were detected using VarDictJava³³ using a 1% VAF cutoff and filtered for artifacts as described previously³⁴. Additional method details can be found in the Supplementary Information.

Reporting Summary.

Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Data availability.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge all our donors to Leukemia Fighters, without whom this work would not have been possible; the women who generously participated in the WHI study. We thank J. Z. Xiang and the Weill Cornell Genomics Core Facility as well as J. Catalano of the Englander Institute for Precision Medicine for assistance in sequencing; L.-B. Yan for technical assistance. The WHI program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, US Department of Health and Human Services through contracts N01WH22110, 24152, 32100–2, 32105–6, 32108–9, 32111–13, 32115, 32118–32119, 32122, 42107–26, 42129–32, and 44221, and the Cancer Center Support Grant NIH:NCI P30CA022453. We acknowledge the dedicated efforts of investigators and staff at the WHI clinical centers, the WHI Clinical Coordinating Center, and the National Heart, Lung and Blood program office (listing available at <http://www.whi.org>). We are additionally grateful for funding from the Sandra and Edward Meyer Cancer Center, which partially supported this study (D.C.H.).

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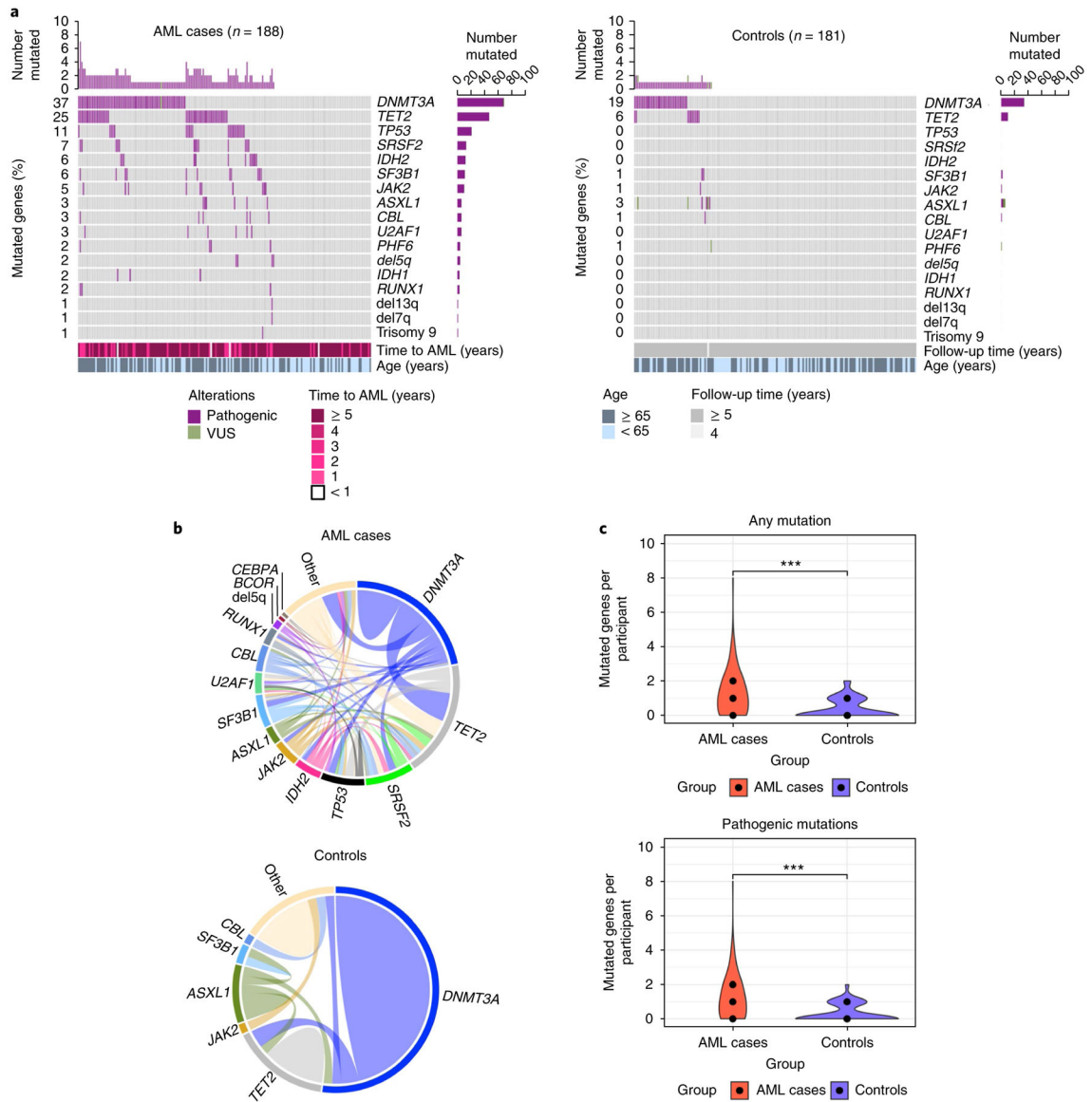


Fig. 1 | Spectrum of mutations seen at baseline years prior to the diagnosis of AML alongside matched controls.

a, Mutated genes in cases with AML ($n = 188$; left) compared to controls ($n = 181$; right). For each gene (rows), pathogenic mutations (purple) and variants of unknown significance (VUS; green) are indicated. Side bar plots indicate the number of participants in whom the gene is mutated in each group. Top bar plots indicate the number of mutated genes per participant. In cases with AML, time to AML and age are shown. For controls, the time of follow-up and age are shown. **b**, Comutations between genes in cases with AML ($n = 129$) and controls ($n = 56$). ‘Other’ indicates other genes. **c**, Violin plot indicating the number of mutated genes per participant in cases with AML (red) compared to controls (blue). Median number of mutated genes, first and third quantile are shown for each group (middle, lower and upper black dots, respectively). Left plot indicates the number of mutated genes per participant in cases with AML (median, 1; range, 0–8; $n = 129$ out of 188 mutated) versus controls (median, 0; first quantile, 0; range 0–2; $n = 56$ out of 181 mutated) ($P < 2.2 \times 10^{-16}$,

two-tailed Mann–Whitney U test). The right plot indicates the number of pathogenic mutations in cases with AML (median, 1; range, 0–8; $n = 127$ out of 188 mutated) versus controls (median, 0; first quantile, 0; range, 0–2; $n = 53$ out of 181 mutated). $P < 2.2 \times 10^{-16}$, two-tailed Mann–Whitney U test). *** $P < 0.001$, Mann–Whitney U test.

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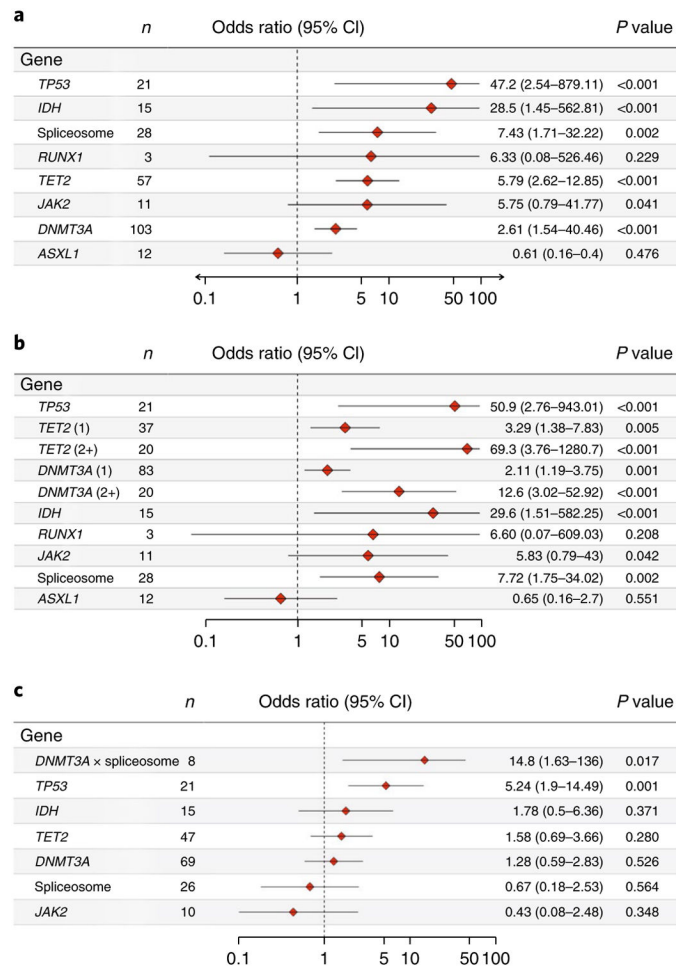


Fig. 2 |. Multivariable analysis of the risk to develop AML associated with the presence of mutated genes.

a. Forest plot indicating the odds ratio of mutations in each gene occurring in the cases with AML compared to controls. Genes or gene categories significantly associated with AML include *TP53* ($P = 5.5 \times 10^{-6}$), *IDH* ($P = 3.0 \times 10^{-4}$), spliceosome, *TET2* ($P = 2.4 \times 10^{-6}$) and *DNMT3A* ($P = 3.4 \times 10^{-4}$). **b.** Forest plot indicating odds ratio of mutations in each gene including number of mutations in *DNMT3A* and *TET2* occurring in the cases with AML compared to controls when one mutation in each gene is present per participant (1) compared to two or more mutations in present in each gene per participant (2+). $P < 0.001$: *TP53* ($P = 3.2 \times 10^{-6}$); *TET2* (2) ($P = 1.8 \times 10^{-7}$); *DNMT3A* (2) ($P = 1.9 \times 10^{-5}$) and *IDH* ($P = 2.8 \times 10^{-4}$). **c.** Mutations in *TP53* and *DNMT3A* are significantly associated with rapid development of AML. Odds ratios per gene were adjusted by age (years) as a continuous variable. *IDH* category includes *IDH1* and *IDH2*. The spliceosome category includes *SRSF2*, *SF3B1* and *U2AF1*. Interaction between *DNMT3A* and spliceosome is indicated (*DNMT3A* × spliceosome). CI, confidence interval; *N*, number of affected cases. *P* values are shown for penalized likelihood multivariable logistic regression.

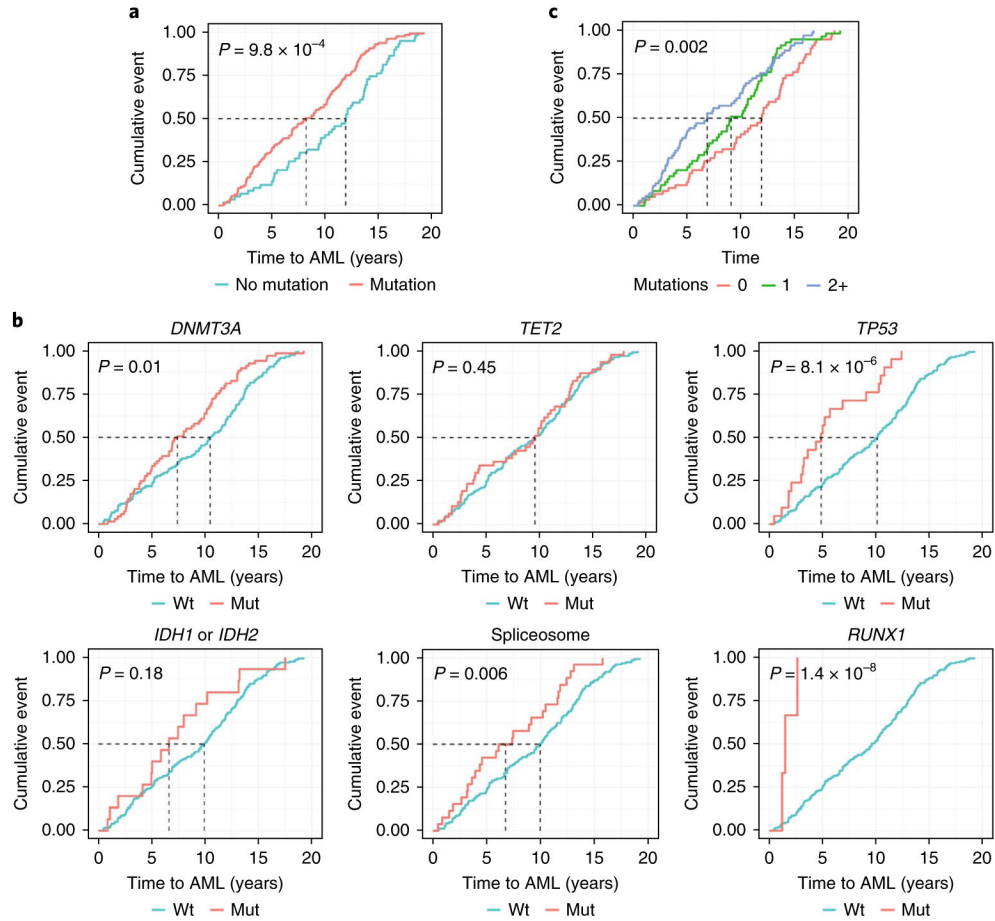


Fig. 3 | Time to AML diagnosis is influenced by mutation status.

Cumulative incidence of AML diagnoses (cumulative event; y axis) as a function of time (years to AML diagnosis) is shown. **a**, Participants include cases with AML only at baseline ($n = 188$) with any mutated gene ($n = 129$ out of 188) versus no mutations ($n = 59$ out of 188). **b**, Participants with mutations in genes associated with AML versus participants with no mutations in these genes (*DNMT3A*, $n = 69$ out of 188; *TET2*, $n = 47$ out of 188; *TP53*, $n = 21$ out of 188; *IDH1* or *IDH2*, $n = 15$ out of 188; spliceosome, $n = 26$ out of 188) in addition to *RUNX1* ($n = 3$ out of 188). Data on *RUNX1* are provided because all participants with a *RUNX1* ($n = 3$) mutation rapidly developed AML (< 2 years) although significance was not achieved due to the few participants who had mutations in *RUNX1* within the cohort. **c**, Cases with AML who had no mutated genes ($n = 86$ out of 188), one mutated gene ($n = 56$ out of 188), or two or more mutated genes (2+) ($n = 46$ out of 188) in significant high-risk genes associated with development of AML. Two-sided P values are shown for the log-rank test.

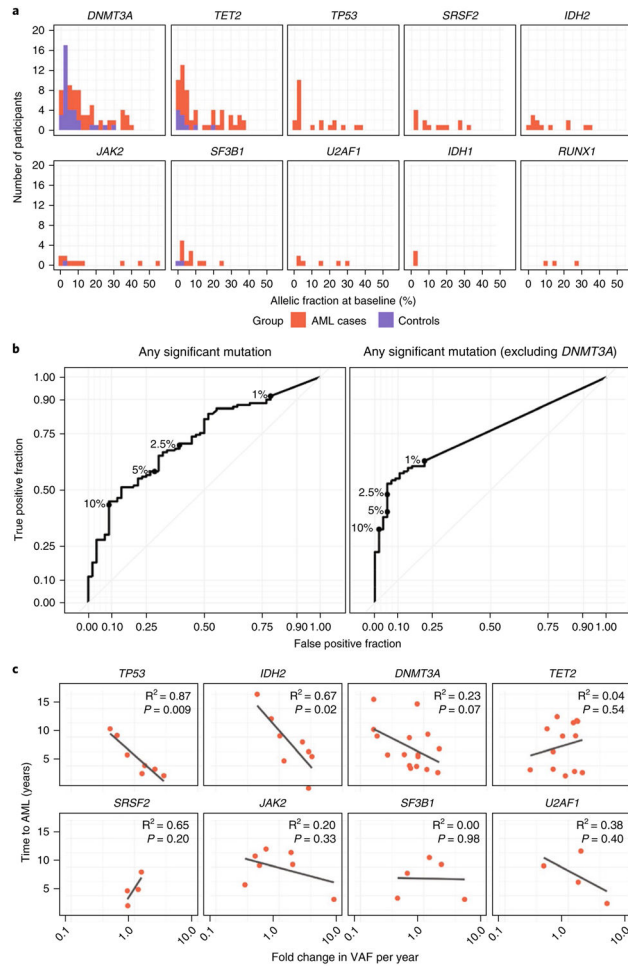


Fig. 4 | Mutations pose AML risk irrespective of the variant allele fraction.

a, Maximum allelic fraction for baseline mutations per gene: *DNMT3A*, $n = 69$ cases, 34 controls; *TET2*, $n = 47$ cases, 10 controls; *TP53*, $n = 21$ cases; *SRSF2*, $n = 13$ cases; *IDH2*, $n = 12$ cases; *JAK2*, $n = 10$ cases, 1 control; *SF3B1*, $n = 11$ cases, 2 controls; *U2AF1*, $n = 6$ cases; *IDH1*, $n = 3$ cases; *RUNX1*, $n = 3$ cases. Proportion of cases with AML (red) and controls (blue) is shown in each bin (width, 2.5%). **b**, Receiver operating characteristic curves indicating the percentage of true-positive rates compared to the percentage of false-positive rates for detecting cases with AML. Performance is shown for mutations in any gene significantly associated with cases with AML (left; *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *SRSF2*, *SF3B1*, *U2AF1*, *TP53*; $n = 164$ (118 cases with AML, 44 controls)) or the same set of genes excluding *DNMT3A*; $n = 94$ (81 cases with AML, 12 controls) (right). **c**, Annual rate of VAF change per year influences kinetics of AML diagnosis for *TP53* ($n = 7$) and *IDH2* ($n = 8$). Time to AML (years) is plotted against fold change in VAF at year 1 or year 3 compared to baseline. Regression line is indicated. R^2 and P values, linear regression. *DNMT3A*, $n = 16$; *TET2*, $n = 13$; *SRSF2*, $n = 4$; *JAK2*, $n = 7$; *SF3B1*, $n = 5$; *U2AF1*, $n = 4$.

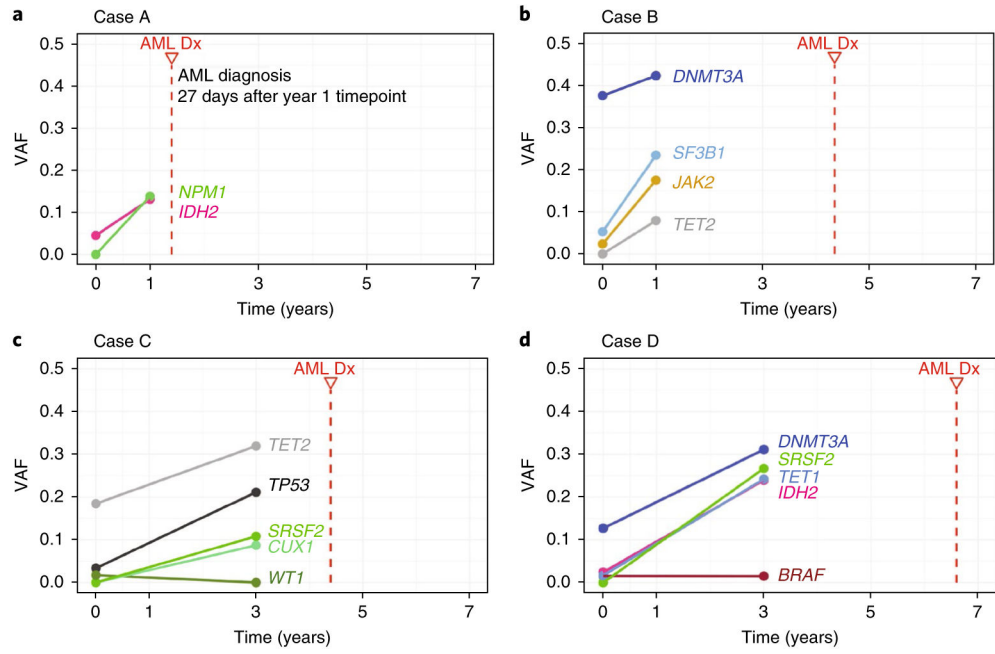


Fig. 5 | Clonal evolution towards AML in selected patients.

Clonal composition and evolution are shown for four selected examples of participants who were evaluable serially (cases A, B, C and D). Peripheral blood was sampled at baseline and years 1 or 3. The x axis indicates time (years). The y axis indicates the VAF where the maximum possible VAF is 1 (100%). Mutated genes are shown at each time point as indicated on the line chart. Time of AML diagnosis (AML Dx) relative to baseline is indicated by the red vertical dotted line. **a**, Case A, an *IDH2* mutation (8% VAF) is present at baseline at lower VAF and persists at year 1 at 13% VAF with an acquired *NPM1* type-A mutation at 14% VAF. AML diagnosis occurs <30 days after the year 1 sample. **b**, Case B: a *DNMT3A* mutation remained stable from baseline to year 1 follow-up. VAFs of *JAK2* and *SF3B1* increased from 5% to 24% before AML diagnosis at 4.3 years after baseline. **c**, Case C: clonal expansion of *TP53* from 3% to 21% with acquired *SRSF2* and *CUX1* mutations between baseline and year 3 follow-up. *TET2* remains relatively stable. AML diagnosed at 4.4 years after baseline. **d**, Case D: low VAF mutations in *IDH2* and *TET1* expand by year 3 along with acquisition of *SRSF2* in the presence of a relatively stable *DNMT3A* mutation. AML diagnosis occurs 6.6 years after baseline.

Mutation frequencies and odds ratios of AML

Table 1 |

Age	Cases with AML (<i>n</i> = 188)		Controls (<i>n</i> = 181)		Odds ratio Odds ratio (95% confidence interval)	<i>P</i> value
	Number of cases harboring any mutations (%)	Number of non-mutated cases (%)	Number of controls harboring any mutations (%)	Number of controls with no mutations (%)		
<65	43 (53.75)	37 (46.25)	16 (20.78)	61 (79.22)	4.39 (2.08–9.61)	3.1×10^{-5}
65	86 (79.63)	22 (20.37)	40 (38.46)	64 (61.54)	6.19 (3.25–12.14)	1.0×10^{-9}
Total	129 (68.62)	59 (31.38)	56 (30.94)	125 (69.06)	4.86 (3.07–7.77)	3.8×10^{-13}

Number and frequency of mutations in cases with AML versus controls overall and for participants younger than 65 or at least 65 years of age.