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Somatic Mutations in Aging, Paroxysmal Nocturnal Hemoglobinuria, and Myeloid Neoplasms

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By

Tho B. Tran, Class of 2024

ABSTRACT

Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal blood disorder frequently associated with bone marrow failure that in rare instances can progress to leukemia. PNH clones of varying sizes occasionally present even in patients with hematologic malignancies and no known history of classical PNH, particularly in myelodysplastic neoplasms (MDS). Curiously, somatic driver mutations considered to be pathogenic and likely pathogenic in myeloid leukemias can be found in phenotypically normal, elderly individuals, as well as those with clonal disorders such as PNH, clonal hematopoiesis of indeterminate potential (CHIP), and age-related clonal hematopoiesis (ARCH). These mutations may be the critical link between PNH and the development of cancer. We suspect that PNH clones, derived from mutations in *PIGA*, may reflect a disordered bone marrow prone to additional genetic hits involved in tumorigenesis. It is uncertain, however, whether leukemic cells directly arise from PNH+ progenitors with additional genetic variants or if they are in fact distinct populations. Moreover, the specific driver mutations that underlie both PNH and myeloid neoplasms remains an area of active investigation. Our study aims to characterize the prevalence of myeloid neoplasm-associated somatic mutations in older adults and in patients who develop myeloid malignancies (MN+ patients), stratified by whether they also harbor PNH clones. We retrospectively examined the initial genetic evaluation by next-generation sequencing (NGS) of 197 individuals at Yale-New Haven Hospital and elicited the most common driver mutations and their association with age and PNH clonal presence. We demonstrated that pathogenic and likely pathogenic somatic mutations increased with age (average age of patients with one or more mutations was 69.8 years compared to 58.0

years in those with no mutations of interest, p< 0.0001). Variants in *SF3B1* (average age= 74.4 years, p= 0.0015), *TP53* (average age= 72.8 years, p= 0.0057), *SRSF2* (average age= 72.4 years, p= 0.0024), *DNMT3A* (average age= 71.9 years, p= 0.0059), *TET2* (average age= 70.8 years, p= 0.0032), *ASXL1* (average age= 70 years, p= 0.0052), and *U2AF1* (average age= 69.7 years, p= 0.0082) were most commonly present in adults who were significantly older than those with no relevant mutations.

Those with a diagnosis of a myeloid neoplasm were drastically more likely to harbor driver mutations (81.1% with at least one variant of interest vs. 23.5% among those without a myeloid neoplasm, p< 0.0001), and had a greater number of mutations on average (1.95 vs. 0.38 mutations per patient, p< 0.0001). Interestingly, MN+ patients who had a PNH clone ≥0.01% were significantly more likely to possess a neoplasm-associated mutation than those with no PNH clones (91.9% vs. 60.6%, p= 0.0005), indicating that the presence of PNH clones augments the odds of possessing cancer-related genetic lesions as early as the time of the initial evaluation. Specifically, patients who were both MN+ and PNH+ were more likely than their PNH- counterparts to have one or two pathogenic and likely pathogenic variants (58.1% vs. 27.3%), though they were equally likely to possess three or more mutations (33.9% vs. 33.3%). Furthermore, among all patients in the cohort ≥70 years, PNH presence significantly increased the overall likelihood of discovering relevant gene variants (78.9% vs. 48.7%, χ^2 p= 0.0027).

Among all patients, PNH clone presence was correlated with mutations in *SF3B1* (85.7% of cases with *SF3B1* variants had PNH clones compared 56.8% of cases with no mutations had PNH clones, Fisher's exact test, p= 0.0402), and to a lesser degree, with *RUNX1* (83.3% PNH+ cases, p= 0.0686) and *DNMT3A* (80.0% PNH+ cases, p= 0.0914).

Finally, we qualitatively described that mutations in *ASXL1, TET2,* and *SRSF2* tended to occur together; there were also concomitant mutations in *TET2* with *EZH2* and in *SF3B1* with *RUNX1*.

Therefore, we recommend early genetic screening of all elderly patients ≥70 years who present with PNH clones of any size in the peripheral blood as these patients have a higher likelihood of harboring pathogenic and likely pathogenic driver mutations. We demonstrated that certain neoplasm-associated mutations are common in elderly patients, while others correlated with PNH clone presence, and that some variants tend to co-occur. Future studies should address the molecular mechanisms of these lesions in leukemogenesis. The observation that PNH clone presence is significantly correlated with somatic mutations in MN+ disease suggests an important relationship between PNH clones and cancer, either as direct tumor precursor populations or as an incidental consequence of high genetic mutability in a vulnerable bone marrow.

ACKNOWLEDGEMENTS

I would like to sincerely thank my thesis advisor and mentor, Dr. Alexa Siddon, who has provided truly excellent guidance, generous support, and kind encouragement during my medical school journey. I would also like to acknowledge the clinical and research mentors, former lab mates, and colleagues who have taught me throughout the years. Lastly, my deepest gratitude to my family– mom, dad, Helen, Calvin, and Trevor– and friends for bringing so much joy into my life.

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INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a benign clonal disorder of the bone marrow. It is characterized by acquired mutations in the *phosphatidylinositol glycan class A* (*PIGA*) gene, an X-linked gene involved in the production of glycophosphatidylinositol (GPI) anchors. These GPI anchors are essential for the appropriate attachment of anchored proteins (GPI-AP) including clusters of differentiation and other cell surface molecules required for intercellular interactions, interfacing with the immune system, and signaling. Classically, mutations in *PIGA* and the subsequent loss of GPI-AP such as CD55 (DAF) and CD59 (MIRL) on erythrocytes can trigger uninhibited complement activation, leading to chronic complement-mediated destruction of circulating blood cells and heightened activation of the innate immune system and platelets. When PNH clones are of sufficiently large size, the disorder can present as "classical PNH," a triad of intravascular hemolytic anemia, additional cytopenias, and increased risk of thromboembolism, although not every patient will develop all symptoms. Additionally, intravascular hemolysis can result in hemoglobinuria and smooth muscle dysfunction via nitric oxide depletion by excess free hemoglobin: symptoms of dystonia include abdominal pain, esophageal spasm, dysphagia, pulmonary hypertension, and erectile dysfunction. Patients with severe PNH also have a significantly higher risk than the general population of developing chronic kidney disease due to thrombosis-induced renal tubular damage.[1-3] Rarely, *PIGA* mutations may be inherited in the germline, causing craniofacial abnormalities, central nervous system malformations, seizures, and in some cases, can be embryearsnically lethal.[4]

Historically, PNH was associated with a high risk of mortality, frequently from serious thromboembolic events, with median survivals of 10 to 30 years left untreated. However, since the advent and more routine use of complement inhibitors, eculizumab and more recently, ravulizumab, the prognosis in PNH has significantly improved.[5] Nevertheless, as acquired *PIGA* variants afflict hematopoietic stem and progenitor cell (HSPC) populations, the only curative course remains bone marrow transplantation to fully eradicate PNH clones.[6]

The mainstay of diagnosis in PNH is flow cytometry, with early assays detecting loss of CD55 and CD59 in peripheral blood specimens. This technique replaced earlier methods such as the Ham test, which relied on lysing abnormal cells to demonstrate osmotic fragility in PNH populations. In the late 2000s, a newer flow cytometry assay was developed that utilizes aerolysin/ proaerolysin, a bacterial product that binds with fairly high specificity to GPI. Proaerolysin is conjugated to a fluorochrome (fluorescent aerolysin or "FLAER"), introduced to the patient sample, and attaches to GPI. In *PIGA* mutated cells that are deficient or lower in cell-surface GPI, the subsequent fluorescent signal by flow would be less intense than phenotypically normal cells and will appear as a distinct population.[7] FLAER-based flow cytometry has enabled significantly improved sensitivity and specificity in PNH testing, with current assays able to detect even small (<10% PNH+ cells) and very small (<1%) clone presence.[8,9]

As PNH is a relatively rare disease, it can be difficult to determine which patients merit testing. The International Clinical Cytometry Society (ICCS) Consensus Guidelines recommends PNH screening for patients with laboratory indications of intravascular hemolysis (elevated lactate dehydrogenase (LDH), free haptoglobin, and reticulocyte

index), unexplained thromboembolic events (especially in yearsunger patients or at an unusual site such as abdominal or cerebral vessels), and in certain hematologic conditions. Among these conditions are aplastic anemia (AA), which like PNH is a bone marrow failure syndrome, and myelodysplastic neoplasm (MDS), a myeloid malignancy.[10,11]

Notably, improved testing methods have led to the recognition of PNH clones in AA and MDS, particularly small and very small clones, though the clinical significance of these clones remains unclear. AA has the highest prevalence of PNH+ cases, as well as the largest clone sizes, whereas MDS is associated with a lower prevalence of PNH positivity and smaller clones.[9,12-15] Upwards of 46% of MDS cases display some PNH clonality (though most studies suggest a prevalence of <20%); the prevalence also varies across MDS subtypes and when assessed by different diagnostic methodology.[12,13,15] Thus, PNH can be roughly subdivided into three categories based on the size of the PNH clones and clinical presentation: classical PNH, with large clones (typically ≥50%) and usual PNH clinical features, PNH in the setting of other disorders of the bone marrow, and subclinical PNH, wherein there is indisputable flow cytometry evidence of PNH clones, though these clones are often small (<10%), and with variable or no overt symptoms.[16]

The nuance that PNH clones can be found in myeloid diseases is not entirely surprising. An uncommon but very grave complication of PNH (and AA) is the evolution to leukemia. Patients with PNH are at an increased risk of developing hematologic cancers, with reported incidences of up to 10% over a lifetime.[17] Most commonly, these patients present with myeloid disorders such as acute myeloid leukemia (AML), MDS,

myeloproliferative neoplasms (MPN), MDS/MPN such as chronic monomyelocytic leukemia (CMML), and chronic myeloid leukemia (CML).[2,6,17] Furthermore, patients with PNH clones have been shown to harbor somatic driver mutations commonly associated with myeloid tumors such as *ASXL1, NPM1, TET2,* and *U2AF1*.[17]

These observations raise the question of whether there is a direct pathologic relationship between PNH and myeloid neoplasms. Why do hematologic cancers like MDS occasionally present with PNH clones and do PNH and leukemic cells arise from the same progenitor populations? The presence of somatic driver mutations may provide a link between PNH and malignancy; however, the identification of these variants of interest in healthy individuals and in those with any clonal disorder remains an area of active investigation.

STATEMENT OF PURPOSE

This retrospective study aims to characterize the myeloid neoplasm-associated somatic mutations that are present in healthy individuals, those with paroxysmal nocturnal hemoglobinuria (PNH) clones, and patients who ultimately develop a myeloid leukemia using genetic data obtained by next-generation sequencing (NGS) and through identification of GPI-AP deficient clonal populations by FLAER-based flow cytometry.

METHODS

Author Contributions

- Tho B. Tran, BS formulated the study, performed retrospective chart review and statistical analysis, drafted the manuscript, and provided critical revisions.
- Alexa J. Siddon, MD formulated the study and provided critical revisions.
- All laboratory tests described in this study were performed by Yale-New Haven Hospital, Yale School of Medicine (YSM) Laboratory Medicine, YSM Molecular Diagnostics Laboratory, YSM DNA Laboratory (New Haven, CT), and/ or Yale Center of Genomic Analysis (West Haven, CT).

Ethics & Human Subjects Research Statement

This study was submitted to the Institutional Review Board at Yale University and granted an exemption with a waiver of HIPAA authorization due to its retrospective nature (IRB Protocol ID: 2000033407). Research was conducted in line with the responsible and ethical code of conduct of research as outlined by the university. All efforts were made to maintain patient confidentiality and to minimize any potential harm to patients.

FLAER-based Flow Cytometry

FLAER-based flow cytometry takes advantage of enzymatically inactive proaerolysin, a bacterial product capable of binding to all GPI-AP on cell surfaces of white blood cells (WBC).[7,18,19] Peripheral blood specimens were stained with the appropriate cocktail of fluorophore-conjugated antibodies to clusters of differentiation

(CD) depending on the target cell population (neutrophils, monocytes, erythrocytes). The neutrophil-specific antibody panel included FLAER (Alexa 488, measured in the FITC channel), CD24 (PE), CD15 (APC) (BD Pharmingen), CD16 (PerCP-Cy5.5), and CD45 (PE-Cy7) (all conjugated antibodies from BD Biosciences, BD Pharmingen, Beckman Coulter, Cedarlane, Life Technologies, or Sysmex). The monocyte-specific antibody panel included FLAER (Alexa 488), CD64 (PE-Cy7), CD14 (APC-H7), and CD45 (PerCP-Cy5.5). As FLAER does not build to RBC, the RBC flow panel consisted of permutations of CD59 (PE) and CD235a (FITC). All stained specimens were run on the BD FACS Canto Instrument with the FACS Diva Acquisition and Analysis Software with standardized voltages and compensations. Briefly, flow cell sorting and analysis was performed first with a forward scatter (FSC) by side scatter (SSC) gate for single cells of the correct size and density of the target populations, with subsequent gating by cell surface markers.

PNH clones in WBC were interpreted as cells that were dim in both the corresponding CD marker and FLAER, with CD16 and CD24 roughly representative of neutrophils/ granulocytes and CD14 of monocytes. These may be compared to phenotypically normal cells with bright expression of the population-specific CD and FLAER. Meanwhile, RBC considered PNH+ were low in CD59 expression and were further subclassified as Type 1 (normal CD59 expression), Type 2 (partial loss of CD59), or Type 3 (complete loss of CD59) based on fluorescence. The sensitivity in identifying PNH+ RBC was limited by recent RBC transfusions (influx of normal donor RBC, which may present as a false negative) and the presence of cold agglutinin.

Next Generation Sequencing

Genomic DNA extracted from patient peripheral blood and/or bone marrow aspirate were quantified by spectrophotometry at 260 nm (A260) and barcoded for quality control tracing. A library of the DNA fragments (without molecular barcodes) was generated and quantified by real-time polymerase chain reaction (RT-PCR) and using ligated Illumina adapter sequences (San Diego, CA). The library was enriched for the whole exome and for 525 genes associated with myeloid neoplasms with capture probes developed by the Genomics Organization for Academic Laboratories (GOAL).

Next-generation sequencing (NGS) and bioinformatic analysis was performed on the Illumina NovaSeq. For 51 genes commonly associated with myeloid neoplasms and that have established clinical relevance with diagnostic, prognostic, and/or therapeutic potential, whole exome sequencing and gene annotation were performed with a limit of detection for single nucleotide variants (SNV) at 5% of coverage of 200X. Mutations outside of the exons and those at coverage below 100X may be missed in this method. Testing was performed at the Yale School of Medicine (YSM) Molecular Diagnostics Laboratory, YSM DNA Laboratory (New Haven, CT), and Yale Center of Genomic Analysis (West Haven, CT).

Among the genes of interest interrogated by the NGS panel were: *ABL1, ALK, ASXL1, ATRX, BCOR, BCORL1, BRAF, BRCC3, CALR, CBL, CEBPA, CSF3R, DDX41, DNMT3A, EED, EP300, ETV6, EZH2, FLT3, GATA1, GATA2, IDH1, IDH2, JAK2, KIT, KRAS, MPL, MYC, NF1, NPM1, PDGFRA, PDS5B, PHF6, PPM1D, PRPF8, PTPN11, RAD21, RAS, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG1, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2*. Gene variants were compared to those reported in cancer

specimens in the COSMIC (cancer.sanger.ac.uk/) and ClinVar (www.ncbi.nlm.nih.gov/clinvar/) databases. Predictions for the effects of specific mutations on protein function were determined by software including Sorting Intolerant from Tolerant (SIFT), PolyPhen-2, and by Grantham score.[20,21] Herein, the targeted genes are labeled as "pathogenic and likely pathogenic somatic mutations" (abbreviated in figures and tables as "somatic mutations" or "SM"), though of note, some variants may be of germline origin.

Statistical Analysis

Most results presented in this study were provided with descriptive statistics such as means of ages and the mean number of mutational variants. Proportions of the whole, such as the prevalence of particular mutations or PNH clones within disorders, were frequently given as percentages with or without the absolute value (n). All statistical analyses were performed with GraphPad Prism software with a significance threshold set at $p < 0.05$. Continuous variables including patient ages, prevalence of somatic driver mutations, and number of mutations per case were analyzed using Student's t-tests or one-way ANOVA. Categorical variables including presence or absence of PNH clones and of somatic mutations were analyzed using Chi-squared tests (if $n \geq 50$) or with Fisher's exact test (if $n < 50$) in the case of PNH clones in specific mutations. Confidence intervals at the 95% significance level were calculated by hybrid Wilson/Brown statistics in the case of comparing the number of somatic mutations by disorder (PNH+/- and MN+/).[22,23]

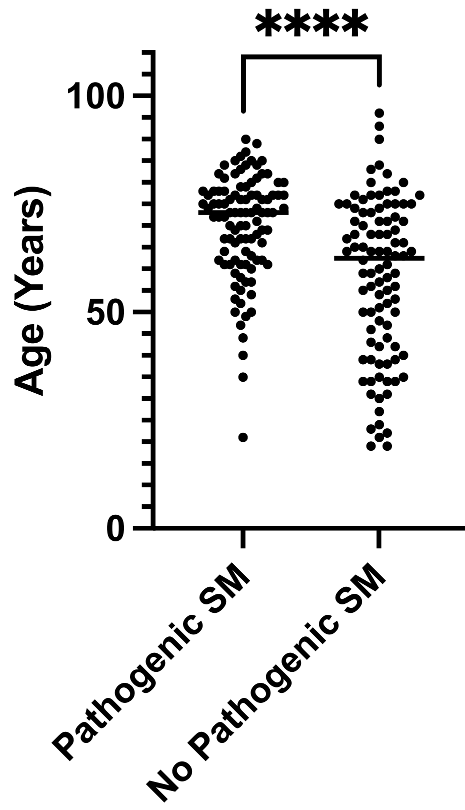
RESULTS

Study Population

Our study compromised 197 adults with NGS panels and FLAER-based flow cytometry assessments within the last decade at Yale-New Haven Hospital. Of the patients, 114 (57.9%) were men and 83 (42.1%) were women. Nearly half of the patients had a diagnosis of a myeloid neoplasm (n= 95 (48.2%)), with a male predominance (n= 62 (65.3%)). The average age in the study was 63.0 years (range 19-96 years). Patients who had a myeloid malignancy were significantly older (average 67.2 years) than those without (59.1 years, p= 0.0005).

Prevalence of Somatic Mutations Increased with Age

The prevalence of pathogenic and likely pathogenic somatic mutations in hematologic cells is significantly greater with advanced age. In the initial NGS evaluation of 197 patients, 101 (51.3%) have one or more neoplasm-associated mutation(s) and an average age of 69.8 years (range 21-90 years) compared to 58.0 years (19-96 years) among those without any such mutations (p< 0.0001) (Figure 1A). Specifically, patients with variants in *SF3B1* (74.4 years, n= 14, p= 0.0015), *TP53* (72.8 years, n= 13, p= 0.0057), *SRSF2* (72.4 years, n= 17, 0.0024), *DNMT3A* (71.9 years, n= 15, p= 0.0059), *TET2* (70.8 years, n= 20, p= 0.0032), *ASXL1* (70 years, n= 21, p= 0.0052), and *U2AF1* (69.7 years, n= 19, p= 0.0082) were significantly older than those with no pertinent somatic mutations (Figure 1B).


A.
Somatic Mutations by Age
****
Age (Years)
100
50
0
Pathogenic SM
No Pathogenic SM

B. **Age of Patients with Somatic Mutations**

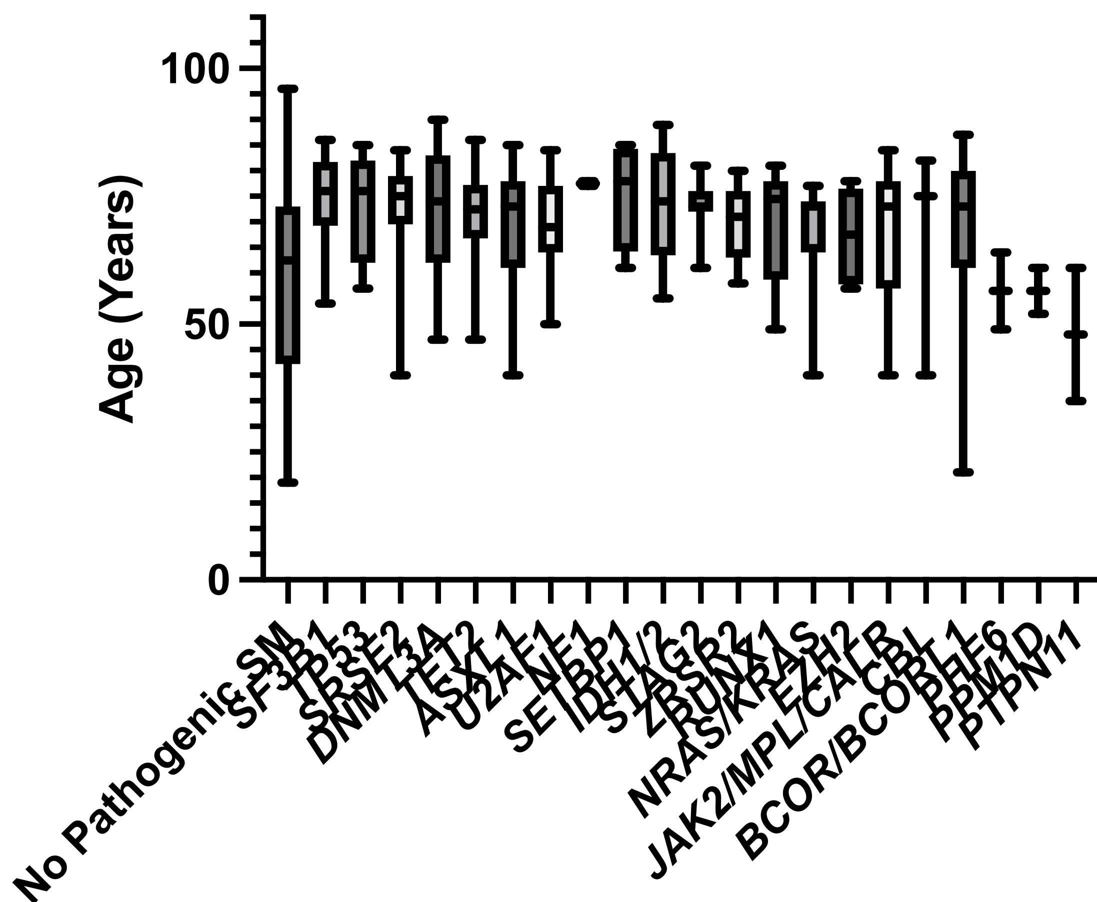


Figure 1. Patients with any pathogenic or likely pathogenic somatic mutation (SM) are significantly older. (A) The average age of patients with any clinically relevant mutation was 69.8 years and was 58.0 years in those without any relevant mutation ($p< 0.0001$). **(B)** Patients with mutations in *SF3B1* (74.4 years, n= 14, p= 0.0015), *TP53* (72.8 years, n= 13, p= 0.0057), *SRSF2* (72.4 years, n= 17, p= 0.0024), *DNMT3A* (71.9 years, n= 15, p= 0.0059), *TET2* (70.8 years, n= 20, p= 0.0032), *ASXL1* (70 years, n= 21, p=

0.0052), and *U2AF1* (69.7 years, n= 19, p= 0.0082) were significantly older than those with no relevant mutations. While the average age of patients with the remaining mutations other than *PPM1D* (56.5 years), *PHF6* (56.5 years), and *PTPN11* (48 years) were also qualitatively higher than those with no mutations, they were not statistically significant.

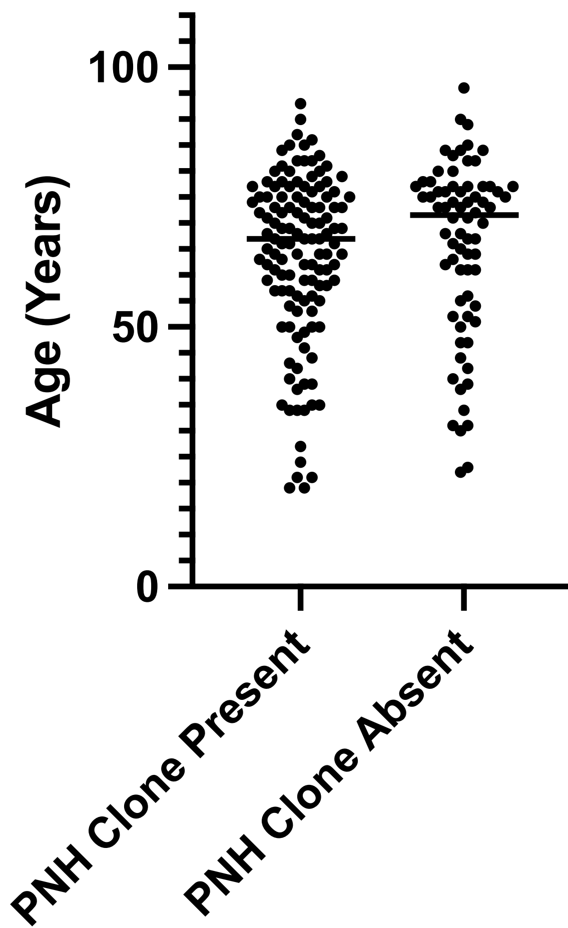
PNH Clone is Associated with Somatic Mutations in Elderly Individuals

While patients who possessed PNH clones (63.2 years, n= 125) were of a similar age to those who did not (65.5 years, n= 72), among elderly patients, the presence of PNH clones was strongly correlated with possessing pathogenic and likely pathogenic somatic mutations (Figure 2A). In patients ≥70 years, 78.9% (n= 41) of those with PNH clones had at least one neoplasm-associated mutation compared to only 48.7% (n= 11) of those without any clones (χ^2(1, n= 91) = 9.006, p= 0.0027). However, in patients <70 years, there was no significant association between PNH and neoplasm-associated genetic variants (43.8% of PNH+ patients (n= 32) vs. 27.3% of PNH- patients (n= 9) had at least one mutation) (Figure 2B).

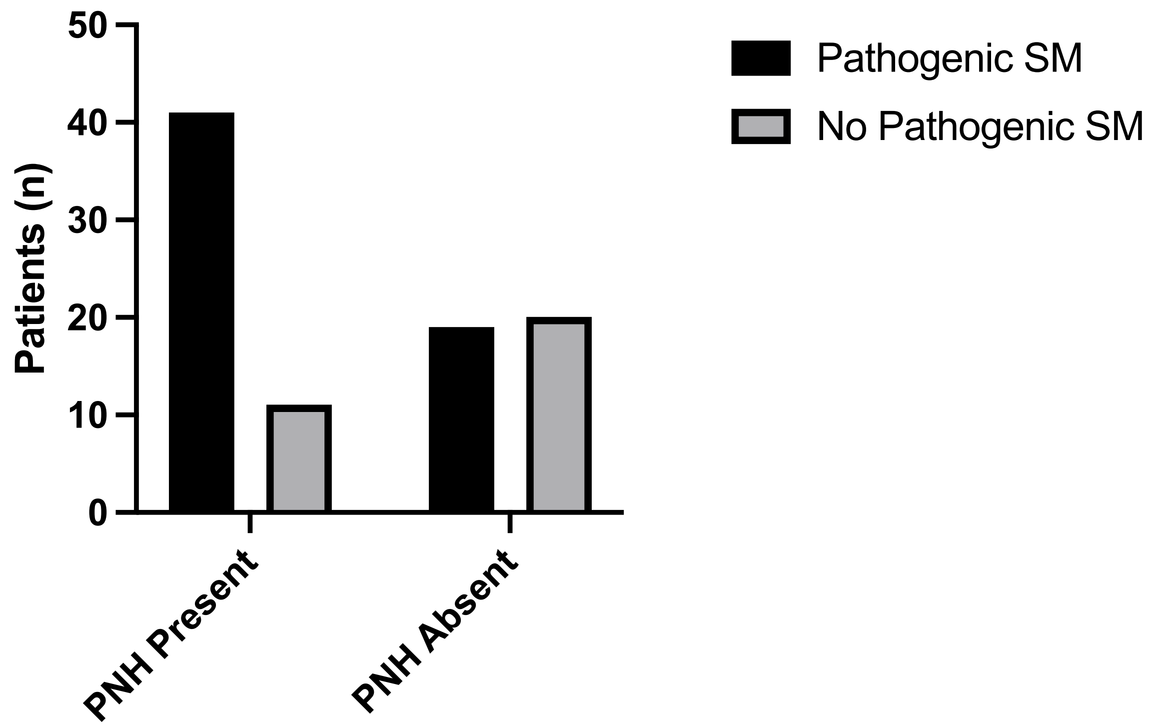
Among the most frequently occurring somatic mutations in our cohort including *SF3B1, RUNX1* (n= 12), *DNMT3A, ASXL1, TET2, U2AF1, SRSF2, TP53, BCOR/BCORL1* (n= 7), *STAG2* (n= 7), *NRAS/KRAS* (n= 6), *ZSRS2* (n= 7), and *IDH1/2* (n= 5), we found that only *SF3B1* was correlated with PNH presence. Of the patients with an *SF3B1* mutation, 85.7% also harbored PNH clones compared to 54.2% of patients with no pathogenic variants (p= 0.0396). Patients with *RUNX1* and *DNMT3A* mutations

also had a higher prevalence of PNH clones (83.3%, p= 0.0671 and 80.0%, p= 0.0904 of patients with each mutation, respectively), though not significant at the 95% confidence interval compared to those with no variants (Figure 3).

A.



B.



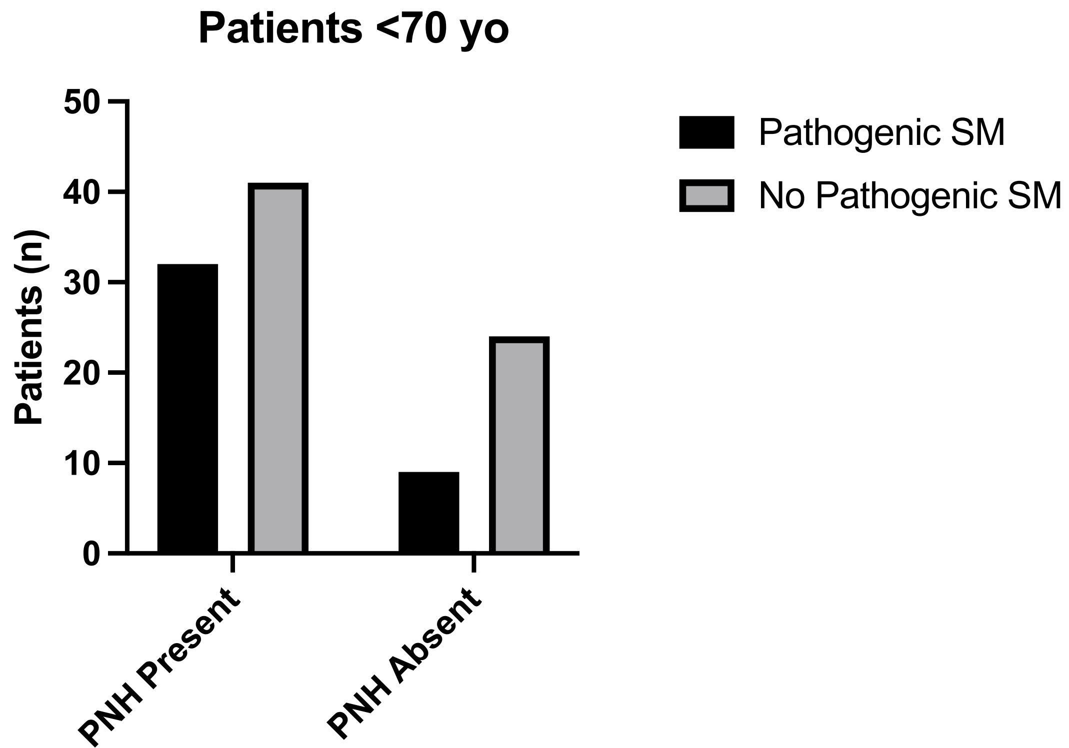


Figure 2. Presence of PNH clone is associated with pathogenic and likely pathogenic somatic mutations in elderly patients. (A) Patients with and without PNH clones in the peripheral blood were of similar ages (63.2 years and 65.5 years, respectively). **(B)** However, in patients ≥70 years, 78.9% of those with PNH clones had at least one clinically relevant somatic mutation, whereas only 48.7% of those without PNH clones possessed a relevant variant ($\chi^2(1, N= 91) = 9.006$, p= 0.0027). In individuals <70 years, there was no significant difference in the prevalence of somatic mutations in those who were PNH+ and those who were PNH- (43.8% vs. 27.3%, respectively).

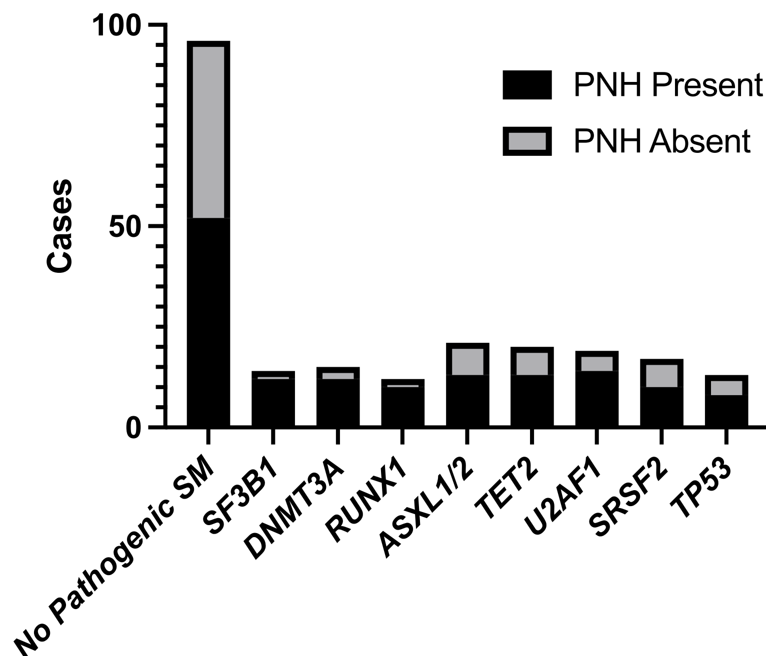


Figure 3. *SF3B1, RUNX1*, and *DNMT3A* variants demonstrate greater prevalence of PNH clones than in cases of no pathogenic somatic mutations. Of patients with *SF3B1, RUNX1,* and *DNMT3A* mutations, PNH clones were present in 85.7% (p= 0.0396), 83.3% (p= 0.0671), and 80.0% (p= 0.0904) of patients, much higher than what was noted in patients with no pathogenic somatic mutations, of whom 54.2% harbored at least one PNH clone.

Somatic Mutations are Abundant in Myeloid Neoplasms and Correlates with Presence of PNH Clones

To interrogate the association between somatic mutations targeted by our NGS panel and clinically overt leukemia and myeloproliferative disorders, we grouped patients

by whether they have ever been diagnosed, whether prior, after, or at the time of genetic evaluation, with a myeloid neoplasm. We included in our category of myeloid neoplasm (MN+) patients those who were diagnosed with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), myelodysplastic neoplasm (MDS) of any subtype, myeloproliferative proliferative neoplasms (MPN) such as polycythemia vera (PV), primary myelofibrosis (PMF), and essential thrombocythemia (ET), and chronic myelomonocytic leukemia (CMML), the prototypic and most common form of myelodysplastic/ myeloproliferative neoplasms (MDS/MPN). Patients diagnosed with lymphoid neoplasms such as B or T-cell lymphomas and multiple myeloma (MM) were categorized into the non-myeloid neoplasm (MN-) group. Additionally, patients with clonal hematopoiesis (CH), clonal hematopoiesis of indeterminate potential (CHIP), and clonal cytopenia of undetermined significance (CCUS), which may derive from mutated multipotent and progenitor cells and may be a precursor of myeloid disease, were also defined as MN-. In total, 95 (48.2%) patients fell into the category of MN+ and 102 (51.8%) were classified as MN-.

Somatic mutations were significantly more common in MN+ patients, with 81.1% (n= 77) of these patients displaying at least one gene variant in the initial NGS evaluation compared to only 23.5% (n= 24) of MN- patients ($p< 0.0001$). We further investigated whether the presence or absence of PNH clones modified the odds of having one or more somatic mutation in myeloid disease. Patients were sorted into one of four groups based on the presence of any size PNH clone above the limit of detection by FLAER-based flow cytometry and diagnosis of myeloid neoplasm. In our cohort, 62 (31.5%) patients were PNH+/MN+, 63 (32.0%) were PNH+/MN-, 33 (16.8%) were PNH/MN+, and 39

(19.8%) were PNH-/MN-. PNH+/MN+ patients had the highest rates of harboring one or more pathogenic and likely pathogenic somatic mutation (91.9%, n= 57), followed by PNH-/MN+ patients (60.6%, n= 20), PNH+/MN- patients (25.4%, n= 16), and PNH-/MN- patients (20.5%, n= 8). Notably, the presence of PNH clones significantly increased the odds of having any somatic mutation in MN+ patients ($p = 0.0005$), but not in MN- patients (Table 1).

Myeloid Neoplasms are Associated with Higher Numbers of Somatic Mutations, Irrespective of PNH Clone Presence

On average, patients who were diagnosed with a myeloid neoplasm at any point tended to have a greater number of mutations than those who do not (1.95 vs. 0.38 mutations per patient, $p < 0.0001$). This remained true even when considering only MN+ and MN- patients who had *any* somatic mutation of interest, wherein MN+ patients possessed on average 2.40 mutations per patient compared to 1.63 in MN- ($p = 0.0138$). When further subdivided into whether the patient had PNH clones, patients who were PNH+/MN+ had the highest average number of variants (2.03 mutations per patient), followed by PNH-/MN+ (1.79), PNH+/MN- (0.43), and PNH-/MN- (0.31).

Again, patients who were MN+, regardless of PNH status had significantly higher average somatic mutations than those who were MN- (among MN+ patients, there was no significant difference in average mutations between those with and without PNH clones). Furthermore, amongst patients harboring *any* gene variant of interest, PNH-/MN+ patients displayed the highest average number of mutations (2.95), similar to PNH+/MN+

patients (2.21), but significantly more than what was observed in patients who were PNH+/MN- (1.69, p= 0.0249) and PNH-/MN- (1.5, p= 0.0456).

The percentage of PNH+/MN+ patients harboring a single pathogenic and likely pathogenic mutation was significantly higher (30.6% (Wilson/Brown 95% CI 20.6-43.0%)) than those with PNH+/MN- (15.9% (8.86-26.8%)) and PNH-/MN- (10.3% (4.06-23.6%)), and trended toward higher compared to PNH-/MN+ (18.2% (8.61-34.4%)). Similarly, a significantly greater proportion of PNH+/MN+ patients had two somatic mutations (27.4% (17.9-39.6%)) than PNH+/MN- (6.35% (2.50-15.2%)) and PNH-/MN- (10.3% (4.06-23.6%)), and PNH-/MN+ (9.09% (3.14-23.6%)). Finally, within the MN+ groups, there was a similar percentage of patients with three or more somatic mutations in both PNH+ and PNH- subsets (33.9% (23.3-46.3%) and 33.3% (19.8-50.4%)); both of these groups had significantly more patients with three or more variants than those who are PNH+ and PNH-/MN- (3.18% (0.56-10.9%) and 0% (0-8.97%)) (Table 1).

Number of Somatic Mutations (SM) by Disorder				
	PNH+/MN+	PNH+/MN-	PNH-/MN+	PNH-/MN-
No Pathogenic SM	5 (8.06%)	47 (74.6%)	13 (39.4%)	31 (79.5%)
One Pathogenic SM	19 (30.6%)	10 (15.9%)	6 (18.2%)	4 (10.3%)

Two Pathogenic SM	17 (27.4%)	4 (6.35%)	3 (9.10%)	4 (10.3%)
≥Three Pathogenic SM	21 (33.9%)	2 (3.17%)	11 (33.3%)	0 (0%)
Avg. Number of SM (Overall)	2.03	0.43	1.79	0.31
Avg. Number of SM (Pts with ≥1 SM)	2.21	1.69	2.95	1.5

Table 1. Number of Unique Somatic Mutations (SM) by Disorder. Patients who developed a myeloid neoplasm were significantly more likely to harbor a somatic mutation than those without a MN diagnosis (81.1% vs. 23.5%, $p < 0.0001$), regardless of the presence or absence of PNH clones. Within the subset of MN+ patients, those with PNH clones were also more likely to have at least one mutation than those without any PNH clones (91.9% (95% CI 82.471 – 96.506%) vs. 60.6% (43.683 – 75.317%)).

ASXL1 and *TET2* are Commonly Mutated in Myeloid Neoplasms

We explored whether certain myeloid malignancy-associated mutations were more common in our cohort of patients. In those with any history or eventual diagnosis of a myeloid neoplasm, the two most frequent variants were *ASXL1* (n= 20, 21.1%) and *TET2* (18, 18.9%). Following these, the next most prevalent somatic mutations in all MN+ cases were: *SRSF2* (16, 16.8%), *U2AF1* (15, 15.8%), *SF3B1* (12, 12.6%), and *RUNX1* (11, 11.6%). Of these genes, only *SRSF2* mutations were present in ≥10% of

both PNH+/MN+ and PNH-/MN+ cases, whereas the remainder of the most frequently occurring variants differed between the two PNH subsets. In PNH+/MN+ patients, the order of the most prevalent mutations was: *ASXL1* (12, 19.4%); *TET2* (12, 19.4%); *U2AF1* (11, 17.7%); *SF3B1, RUNX1, SRSF2* (each with 10 cases, representing 16.1% of PNH+/MN+ patients); and *TP53, DNMT3A,* and *ZRSR2* (6, 9.68% each). PNH-/MN+ cases were enriched with mutations in: *ASXL1* (8, 24.2%); *TET2* and *SRSF2* (6, 18.2% each); and *DNMT3A, EZH2, IDH1/2, JAK2, TP53*, and *U2AF1* (3, 9.10% each). While most MN- patients had no somatic mutations at the time of the initial NGS evaluation, *DNMT3A* was mutated in 6 cases (5.88%), *U2AF1* in 5 (4.90%), and *TP53* in 4 (3.92%), representing the most common variants among these patients (Table 2).

Pathogenic and Likely Pathogenic Somatic Mutations Frequently Occur Together

We investigated whether the most common somatic mutations in MN+ cases occur in isolation or in concert with other pathogenic and likely pathogenic variants. We defined co-occurring mutations as any variant of a target gene (for example, a patient with a single *TP53* and single *ASXL1* mutation and a patient with biallelic *TP53* would both be considered as having one co-occurring variant to *TP53*). Genes with fewer than three cases each were excluded in the analysis for insufficient data. In both PNH+/MN+ and PNH-/MN+ groups, almost all mutations were observed with at least one co-occurring variant: in particular, in all MN+ cases, *TET2* and *SRSF2*, possessed co-mutations 100% of the time. Among PNH+/MN+ patients, *ZRSR2, STAG2, NRAS/KRAS*, and *EZH2* also always had co-mutation(s); conversely, *BCOR/BCORL1* and *TP53* mutations only appeared with other mutation(s) in 40% and 50% of cases, respectively.

Meanwhile, in PNH-/MN+ patients, *TET2, SRSF2, DNMT3A,* and *JAK2* co-occurred with other variants in 100% of cases; the lowest rate of co-occurring mutations in this group was 66.7% of cases with *EZH2, IDH1/2,* or *TP53*.

In addition to always presenting with at least one other mutation, variants of *TET2* also had the highest number of unique co-occurring mutations in MN+ disease. Furthermore, patients who were PNH-/MN+ had a significantly greater number of *TET2* co-mutations (average 3.83) than PNH+/MN+ (2.42, p= 0.0402). Of the other somatic mutations that possessed co-occurring variants in ≥50% cases in PNH+ and PNH-/MN+, the number of additional variants was significantly higher in PNH- patients with *U2AF1* (2.67 vs. 0.91, p= 0.0177) and *DNMT3A* (3.0 vs. 1.17, p= 0.0342). This difference in the number of additional variants was not observed between PNH- and PNH+/MN+ patients with mutations in *ASXL1* (2.88 vs. 1.92), *SRSF2* (3.0 vs. 2.1), or EZH2 (3.33 vs. 3.33) (Table 2).

Certain Somatic Driver Mutations Tend to Co-Occur

We observed that certain pathogenic and likely pathogenic somatic mutations clustered together more frequently than others in our cohort of MN+ patients. In order to qualitatively describe these clusters, for each somatic mutation ("A"), we identified unique, co-occurring variants ("B") that were present in ≥25% cases. We then examined if the reciprocal relationship between the two unique mutations was also ≥25% (*i.e.* if ≥25% patients with "B" also possessed "A"), representing a prevalent co-mutation.

Most notably, mutations in *ASXL1, TET2,* and *SRSF2* were frequently present together. In patients with *ASXL1*, 30% had an additional *TET2* and 30% had an *SRSF2* mutation. Reciprocally, 33.3% and 44.4% of *TET2* mutations occurred with *ASXL1* and *SRSF2*, respectively. Further, 37.5% and 50% of SRSF2 occurred with *ASXL1* and *TET2*. Another frequent pairing was *TET2* and *EZH2*, wherein *EZH2* represented 27.8% of *TET2* cases and *TET2* appeared in 83.3% of *EZH2* cases. While *ASXL*1 was also highly prevalent in *EZH2* mutated cases (50%), *EZH2* was only present in 15% of *ASXL1* mutated cases. Finally, *SF3B1* and *RUNX1* also tended to cluster together: *RUNX1* was present in 25% of *SF3B1* and *SF3B1* in 27.3% of *RUNX1* cases (Table 2).

PNH+/MN+				
Somatic Mutation	Number of Patients (Frequency)	Freq. of Co-Occurring SM	Avg. Number of Co-Occurring SM (Range)	Co-Occurring Unique SM (n of patients)
ASXL1	12 (19.4%)	75%	1.92 (0 – 4)	*SRSF2* (3), *TET2* (3), *EZH2* (2), *NRAS/KRAS* (2), *RUNX1* (2), *SF3B1* (2), *U2AF1* (2), *ZRSR2* (2), *CALR* (1), *CBL* (1), *EP300* (1), *JAK2* (1), *STAG2* (1)

TET2	12 (19.4%)	100%	2.42 (1 – 4)	*SRSF2* (5), *ASXL1* (3), *EZH2* (3), *ZRSR2* (3), *SF3B1* (2), *CBL* (1), *DNMT3A* (1), *IDH1/2* (1), *JAK2* (1), *RUNX1* (1), *STAG2* (1), *U2AF1* (1)
U2AF1	11 (17.7%)	63.6%	0.91 (0 – 2)	*ASXL1* (2), *BCOR/BCORL1* (1), *JAK2* (1), *KIT* (1), *NF1* (1), *NRAS/KRAS* (1), *RUNX1* (1), *TET2* (1)
SF3B1	10 (16.1%)	70%	1.7 (0 – 4)	*RUNX1* (3), *ASXL1* (2), *DNMT3A* (2), *STAG2* (2), *TET2* (2), *EZH2* (1), *MPL* (1), *NF1* (1), *ZRSR2* (1)
RUNX1	10 (16.1%)	90%	1.9 (0 – 4)	*SF3B1* (3), *ASXL1* (2), *BCOR/BCORL1* (1), *EZH2* (1), *IDH1/2* (1), *MPL* (1), *NF1* (1), *PHF6* (1), *SRSF2* (1), *STAG2* (2), *TET2* (1), *U2AF1* (1), *ZRSR2* (1)
SRSF2	10 (16.1%)	100%	2.1 (1 – 4)	*TET2* (5), *ASXL1* (3), *CBL* (2), *NRAS/KRAS* (2), *CALR* (1), *IDH1/2* (1), *RUNX1* (1), *ZRSR2* (1)
TP53	6 (9.68%)	50%	0.83 (0 – 2)	*DNMT3A* (2), *SRSF2* (1)

DNMT3A	6 (9.68%)	83.3%	1.17 (0 – 3)	*SF3B1* (2), *TP53* (2), *SETBP1* (1), *TET2* (1)
ZRSR2	6 (9.68%)	100%	2.17 (1 – 4)	*TET2* (3), *ASXL1* (2), *EZH2* (2), *GATA* (1), *RUNX1* (1), *SF3B1* (1), *SMC3* (1), *SRSF2* (1)
BCOR/BCORL1	5 (8.06%)	40%	0.6 (0 – 2)	*RUNX1* (1), *STAG2* (1), *U2AF1* (1)
No Pathogenic SM	5 (8.06%)	-	-	-
STAG2	4 (6.45%)	100%	2.25 (2 – 3)	*RUNX1* (2), *SF3B1* (2), *ASXL1* (1), *BCOR/BCORL1* (1), *IDH1/2* (1), *TET2* (1)
NRAS/KRAS	3 (4.84%)	100%	2.33 (1 – 4)	*ASXL1* (2), *SRSF2* (2), *CBL* (1), *CALR* (1), *U2AF1* (1)
EZH2	3 (4.84%)	100%	3.33 (2 – 4)	*TET2* (3), *ASXL1* (2), *ZRSR2* (2), *JAK2* (1), *RUNX1* (1), *SF3B1* (1)
CBL, IDH1/2, JAK2, MPL, NF1 each with 2 patients (3.23%) with the SM.				
CALR, EP300, GATA, KIT, PHF6, PTPN11, SETBP1, SMC3 each with 1 patient (1.61%) with the SM.				
PNH+/MN-				

Somatic Mutation	Number of Patients (Frequency)	Freq. of Co-Occurring SM	Avg. Number of Co-Occurring SM (Range)	Co-Occurring Unique SM (n of patients)
No Pathogenic SM	47 (74.6%)	-	-	-
DNMT3A	6 (9.52%)	16.7%	0.83 (0 – 4)	*SETBP1* (1), *TP53* (1)
U2AF1	3 (4.76%)	100%	1.67 (1 – 3)	*CSF3R* (1), *JAK2* (1), *NRAS/KRAS* (1), *STAG2* (1)
JAK2, SF3B1, TP53 each with 2 patients (3.23%) with the SM.				
ASXL1, BRCC3, CALR, CSF3R, NRAS/KRAS, SETBP1, STAG2, TET2 each with 1 patient (1.61%) with the SM.				
PNH-/MN+				
No Pathogenic SM	13 (39.4%)	-	-	-
ASXL1	8 (24.2%)	87.5%	2.88 (0 – 6)	*SRSF2* (3), *TET2* (3), *U2AF1* (2), *EZH2* (1), *IDH1/2* (1), *JAK2* (2), *NRAS/KRAS* (1), *PHF6* (1),

				PTPN11 (1), *RUNX1* (1), *SETBP1* (1), *SF3B1* (1)
TET2	6 (18.2%)	100%	3.83 (2 – 6)	*ASXL1* (3), *SRSF2* (3), *DNMT3A* (2), *EZH2* (2), *IDH1/2* (1), *JAK2* (2), *NPM1* (1), *NRAS/KRAS* (2), *PTPN11* (1), *SETBP1* (1)
SRSF2	6 (18.2%)	100%	3 (1 – 6)	*ASXL1* (3), *TET2* (3), *IDH1/2* (2), *JAK2* (2), *DNMT3A* (1), *EZH2* (1), *NRAS/KRAS* (1), *SETBP1* (1), *TP53* (1)
DNMT3A	3 (9.10%)	100%	3 (2 – 4)	*TET2* (2), *BCOR/BCORL1* (1), *EZH2* (1), *JAK2* (1), *U2AF1* (1), *SRSF2* (1), *STAG2* (1)
EZH2	3 (9.10%)	66.7%	3.33 (0 – 6)	*TET2* (2), *ASXL1* (1), *DNMT3A* (1), *PTPN11* (1), *SETBP1* (1), *SRSF2* (1)
IDH1/2	3 (9.10%)	66.7%	1.33 (0 – 3)	*SRSF2* (2), *ASXL1* (1), *TET2* (1)
JAK2	3 (9.10%)	100%	3.33 (2 – 6)	*ASXL1* (2), *SRSF2* (2), *TET2* (2), *DNMT3A* (1), *NRAS/KRAS* (1)

TP53	3 (9.10%)	66.7%	1.33 (0 – 2)	*PPM1D* (1), *SETBP1* (1), *SRSF2* (1)
U2AF1	3 (9.10%)	100%	2.67 (1 – 4)	*ASXL1* (2), *DNMT3A* (1), *BCOR/BCORL1* (1), *PHF6* (1), *RUNX1* (1), *STAG2* (1)
NRAS/KRAS, SETBP1, SF3B1 each with 2 patients (6.06%) with the SM.				
BCOR/BCORL1, CBL, PHF6, PPM1D, PTPN11, NPM1, RUNX1, STAG2 each with 1 patient (3.03%) with the SM.				
PNH-/MN-				
No Pathogenic SM	31 (79.5%)	-	-	-
TP53, U2AF1 each with 2 patients (5.13%) with the SM.				
BCOR/BCORL1, PDGFRA, PPM1D, RUNX1, SRSF2, STAG2, TET2, ZRSR2 each with 1 patient (2.56%) with the SM.				

Table 2. Somatic Mutations Associated with Myeloid Neoplasm and with the Presence of PNH Clones. Among patients with any history or eventual diagnosis of a myeloid neoplasm, the most commonly appearing somatic mutations identified in the initial NGS evaluation were *ASXL1* (n= 20, 21.1%) and *TET2* (18, 18.9%). In patients with PNH clones present and those without, the next most frequently occurring somatic mutations differ. PNH+/MN+ cases were enriched for variants in *ASXL1, TET2, U2AF1, SF3B1, RUNX1,* and *SRSF2*, all present in at least ≥10% of patients. PNH-/MN+ cases

most commonly had variants in *ASXL1, TET2,* and *SRSF2,* occurring in ≥10% of patients; the remaining mutations occurred <10% of the time. The most prevalent somatic mutation identified in MN- patients was *DNMT3A.* Most pathogenic and likely pathogenic somatic mutations in MN+ disease were accompanied by other variants, with some genes such as *TET2* possessing at least one co-mutation in 100% of cases. Conversely, mutations in MN- disease tended to occur more often in isolation; *DNMT3A,* the most prevalent somatic mutation in MN- patients harbored one or more additional variants in only 16.7% of cases. Mutations in *ASXL1, TET2,* and *SRSF2* tended to co-occur with each other. *TET2* also frequently appeared in conjunction with *EZH2* variants. A third pairing of *SF3B1* and *RUNX1* also were frequently observed together in MN+ disease.

Variant Allele Frequency (VAF) Does Not Significantly Differ Across Disease Status

We found that the variant allele frequency (VAF) at the initial NGS evaluation of several common somatic mutations in our cohort to be statistically similar regardless of PNH clone presence and even diagnosis of myeloid neoplasm. Interestingly, the average VAF of *TP53* in PNH+/MN+ (average 34.9%, range 12-45.4%) and PNH-/MN+ (33.4%, 6.5-45%) were only mildly increased from PNH+/MN- (22.4%, 3.2-38.6%) and PNH-/MN- (28%, 14-42%) cases. On the other hand, the VAF of *U2AF1* appeared slightly lower in patients with myeloid neoplasms (26.5%, 3-46.4% in PNH+/MN+ and 25.7%, 13-35$% in PNH-/MN+) than those without (39.7%, 37-43.2% in PNH+/MN- and 31.1% in PNH-/MN-), though not significantly so. Additionally, though the VAF of *SF3B1*

appeared to be greater in PNH+ (33.3%, 2-46%) than PNH- (22.5%, 3-42%) patients with myeloid malignancies, this was also not statistically significant (Table 3).

Average Variant Allele Frequency (VAF) by Disorder.				
	PNH+/MN+	PNH+/MN-	PNH-/MN+	PNH-/MN-
TP53	34.9% (12 – 45.4%)	22.4% (3.2 – 38.6%)	33.4% (6.5 – 45%)	28% (14 – 42%)
ASXL1	29.5% (11 – 42.3%)	4.2%	23.2% (2 – 38%)	-
TET2	35.7% (3 – 75.9%)	5.6%	39.6% (22 – 51.8%)	41%
SF3B1	33.3% (2 – 46%)	27.8% (14.6 – 41%)	22.5% (3 – 42%)	-
RUNX1	26.3% (7.1 – 43.6%)	-	19% (18 – 20%)	13.7%
SRSF2	33.4% (6.1 – 56%)	-	45.3% (40 – 51.5%)	47.2%
DNMT3A	30.1% (3.8 – 47.5%)	15.7% (3 – 31%)	32.7% (26.2 – 42.9%)	-
U2AF1	26.5% (3 – 46.4%)	39.7% (37 – 43.2%)	25.7% (13 – 35%)	31.1% (19 – 43.1%)

Table 3. Average Variant Allele Frequency (VAF) of Common Somatic Mutations by Disorder. Among the most common somatic mutations identified in our cohort in their initial NGS evaluations, the variant allele frequencies (VAF) were similar across disorders, both between patients with and without a myeloid neoplasm diagnosis, as well as patients with and without PNH clones. While the average VAF of *TP53* in PNH+/MN+ (34.9%) and PNH-/MN+ (33.4%) were mildly greater than in MN- patients (22.4% and 28% in PNH+ and PNH-, respectively), this was not statistically significant. Conversely, the VAF of *U2AF1* was higher in MN- patients (39.7% and 31.1% in PNH+ and PNH-)

compared to those who were MN+ (26.5% and 25.7%), though this also did not reach significance.

DISCUSSION

A critical component in the laboratory assessment of hematologic malignancies is evaluating for driver somatic mutations and chromosomal aberrations that have diagnostic, prognostic, or therapeutic significance. This can be performed using molecular techniques such as G-band karyearstyping, targeted fluorescent in situ hybridization (FISH), conventional PCR, next-generation sequencing (NGS), whole exome sequencing (WES), and whole genome sequencing (WGS). However, not all patients with myeloid-associated genetic changes develop clinical disease. Indeed, in our cohort, almost one-quarter (23.8%) of patients found to have one or more pathogenic and likely pathogenic somatic mutation were never diagnosed with a myeloid malignancy (AML, CML, MDS, MPN, and CMML) at any point prior or following NGS testing.

As patients age, the likelihood of discovering a somatic mutation in blood and bone marrow cells drastically increases. However, it remains unclear whether these mutations are truly clinically significant or are an inevitable accumulation of benign genetic variants over the course of a lifetime. Nevertheless, elderly patients are at greater risk of developing pre-malignant hematopoietic clones and leukemia than their yearsunger counterparts, so it stands to reason that the appearance of certain mutations that confer growth or selection advantages, or are involved in DNA mutagenesis, may be an early herald of cancer. In our cohort of 197 adults (ages ranging from 19-96 years) who received genetic evaluations for any cause– commonly, cytopenias of unknown etiology and histories of bone marrow failure syndromes– about half of the patients presented with at least one myeloid neoplasm-associated somatic mutation in their initial NGS panels. These patients were significantly older (average 69.8 years) than those who

had no pathogenic mutations in their initial testing (58.0 years). This is in agreement with data from large trials demonstrating that genetic mutations are rare in yearsung patients, especially those <40 years, but increasingly frequent with age: in one study, approximately 10% of those ≥65 years had one or more mutations.[24] In another, compromising 17,182 individuals, mutations were identified in 9.5% of those 70-79 years, 11.7% of 80-89 years, and 18.4% of those ≥90 years.[25] Furthermore, older patients tended to have a higher mutational burden.[24,25]

We found that the most common genes associated with advanced age were *SF3B1* (74.4 years), *TP53* (72.8 years), *SRSF2* (72.4 years), *DNMT3A* (71.9 years), *TET2* (70.8 years), *ASXL1* (70 years), and *U2AF1* (69.7 years). Among these, *DNMT3A, TET2,* and *ASXL1* have also been noted by many others to be prevalent in elderly populations, with unclear prognostic implications.[24-30] While these genes are all prevalent in AML and MDS, they also represent a large subset of patients with non-malignant clonal states: clonal hematopoiesis of indeterminate potential (CHIP) and aging-related clonal hematopoiesis (ARCH). In one common definition, ARCH and CHIP are differentiated by the degree of clonality of the mutated gene(s), with variant allele frequency (VAF) <0.1% in ARCH and VAF ≥2% in CHIP.[31-33] Analogous to the relationship of monoclonal gammopathy of unknown significance (MGUS) and lymphomas and myelomas, CHIP and ARCH denotes patients with clonal, acquired mutations but without significant hematologic signs or symptoms. They are, however, at mildly enhanced risk for progression to leukemia, and may represent a "pre-leukemic" state or cells at a very early stage of leukemogenesis.[25,33-35] Interestingly, patients with CHIP and ARCH also appear to have increased risks of overall mortality, coronary heart disease, and ischemic

stroke, suggesting a possible interaction of these myeloid driver mutations with dysfunctional monocyte and macrophage activity in the cardiovascular system.[25,27]

One of the most well-studied of these genes, *DNMT3A* encodes a DNA methyltransferase, which in mouse models has been implicated in hematopoietic stem and progenitor cell (HSPC) differentiation, and therefore may lend to unchecked self-renewal of immature cells when mutated.[36-38] It is found in 15-22% of AML and 8% of MDS, wherein variants correlate with worse outcomes such as shorter overall survival (OS) in AML (mOS 12.3-14.1 mo vs. 21.3-41.1 mo in non-mutated cases) and greater risk of progression to AML in MDS (25.8% vs. 1.7%).[39-42] However, in many healthy individuals, *DNMT3A* alone appears to have little to no clinical manifestations such as severe cytopenias.[28,30] Moreover, *DNMT3A-R882* transcript can persist at relatively stable levels in patients with AML even after consolidation chemotherapy and poorly correlates with tumor burden, suggesting that the relationship of *DNMT3A* variants and leukemia is perhaps neither direct nor very strong.[43]

Like *DNMT3A*, *TET2* is also an epigenetic modifier gene involved in DNA methylation, occurs in up to 27.4% of AML, 26% of MDS, and 7-13% of MPN, and is highly age-correlated.[28,44-46] In our study, both *DNMT3A* and *TET2* mutations almost always occurred in conjunction with at least one other somatic driver mutation: in patients who develop a myeloid neoplasm, *DNMT3A* co-occurred with other mutation(s) in 88.9% of cases and *TET2* in 100%. *TET2* also presented with an elevated number of additional acquired mutations (average 2.89, with a maximum of 6 co-mutations per patient). Our findings support the hypothesis that *DNMT3A* and *TET2* sporadically mutate in HSPC, the risks of which augment over the course of a lifetime and therefore

such variants are significantly more prevalent in older adults. Although these mutations in isolation are insufficient for leukemogenesis, they provide a minor self-renewal or proliferation advantage to HSPC, enabling them to continue to accrue other driver variants and grow in number. Clones that acquire additional pathogenic and likely pathogenic variants may eventually become cancer, while those that do not remain as CHIP and ARCH. This mechanism may also explain the high frequency of mutations in elderly populations of *ASXL1*, which also plays a role in epigenetic regulation via chromatin modification and harbored additional mutations in 75% of cases of patients with myeloid neoplasms in our study. Driver mutations such as *DNMT3A, TET2,* and *ASXL1* in ARCH may be important for sustaining grossly normal levels of hematopoiesis, resulting in overall stable and normal peripheral blood counts, in otherwise aging HSPC, endowing the cells with a slight growth advantage.[29,47]

We also reported a correlation of older age with mutated *SF3B1, SRSF2, U2AF1*, and *TP53,* in concordance with the literature.[24,31] The former three mutations are all involved in spliceosome regulation, with strong associations with MDS with ring sideroblasts (*SF3B1*) and CMML, a subtype of MDS/MPN (*SRSF2* and *U2AF1*).[48] MDS is classically considered a disease of the elderly, most commonly affecting patients in the seventh and eighth decades of life, so it is not surprising that these prototypical variants also presented in the older patients of our cohort. *SRSF2* may be similar to genes such as *TET2*, necessitating cooperating mutations to manifest overt leukemia– among the patients with any diagnosis of myeloid neoplasms, 100% of those with *SRSF2* mutations had one or more co-occurring mutation (with an average of 2.44 additional variants per patient).[49]

Interestingly, we demonstrated that *TET2, ASXL1,* and *SRSF2* frequently presented concomitantly in myeloid disease. Amongst patients with each of these mutations, 30-50% harbored additional mutations in the other two genes. This triad has been described in the literature as prevalent in CMML, with one or more of the mutations appearing in at least of half of CMML cases.[50-52] In our study, four patients were diagnosed with CMML: one patient had the trifecta of *TET2, ASXL1,* and *SRSF2*, and two patients had both *TET2* and *SRSF2*. Additionally, two patients found to have all three genes in their initial NGS panel were diagnosed with MDS. Other permutations of this triad were also associated with myeloid disorders. All five patients with concurrent *TET2* and *SRSF2* variants had a myeloid neoplasm: two with CMML (as previously noted), two with MDS, and one with AML. Three patients were found to harbor both *TET2* and *ASXL1* variants: one with AML/MDS and two with MDS alone. Finally, of the three patients with mutated *ASXL1* and *SRSF2*, two were diagnosed with MPN and one with MDS.

It is curious why *TET2, ASXL1,* and *SRSF2* appear to have a particular affinity for developing MPN and MDS/MPN– of all nine patients in our cohort with a diagnosis of MPN (PV, ET, and PMF) or CMML, *TET2* (44.4% of cases), *ASXL1* (55.6%), and *SRSF2* (55.6%) were as prevalent as *JAK2* (44.4%) and more so than *CALR* (11.1%), the canonical molecular markers of MPN. Unlike mutations such as *JAK2-V617F* that directly upregulate the JAK/STAT pathway, thereby promoting clonal proliferation, the genes of this triad likely drive leukemogenesis in a multistep fashion. In mouse and *in vitro* studies, Abdel-Wahab *et al.* demonstrated that loss of *ASXL1* alone resulted in craniofacial and skeletal defects, cytopenias, and multilineage cellular dysplasia (the

latter two being highly characteristic of MDS). However, these *ASXL1* knockout HSPC had deficiencies in self-renewal capacity and therefore in isolation could not significantly expand. With the additional loss of *TET2*, which enhanced cellular renewal and compensated for the decrease in bone marrow cellularity from the knockout *ASXL1*, the mice displayed worse dysplasia and overall disease severity.[53]

In short, *ASXL1* and *TET2* can cooperate in tandem to induce both myelodysplasia and the proliferation of dysplastic clones, thus producing an overt tumor phenotype, though each gene in isolation may be insufficient to induce malignancy. *SRSF2* may be an additional genetic hit enhancing neoplasm development or tumor burden.[50,52,54-56] Furthermore, in diseases deriving from JAK/STAT overactivity such as MPN, *ASXL1 in vitro* may also augment fibrocyte differentiation via upregulation of *EGFR1* and *TNFA*, leading to the characteristic scarred marrow morphology seen in myelofibrosis.[57] Appreciating the contributions of these somatic driver mutations to oncogenesis in MPN and MDS/MPN may increase therapeutic targets in diseases for which the primary treatment remains hematopoietic stem cell transplant (HSCT) and the use of JAK1/2 inhibitors such as Ruxolitinib. If dysregulated methylation by *TET2* variants is necessary as a cooperating step in the pathway from aberrant clone to clinical disease, for example, then hypomethylating agents and TET2 inhibitors may stall tumor evolution.[58]

The remaining three somatic mutations that we identified as prevalent in older individuals, *U2AF1, SF3B1,* and *TP53* have important roles in transcription regulation and DNA repair. Both *U2AF1* and *SF3B1* are splicesomal genes. Like *SRSF2*, *U2AF1* is commonly associated with CMML, while *SF3B1* is so overrepresented in MDS, specifically low-risk MDS with ring sideroblasts, that in the fifth edition of the WHO

classification of myeloid disorders, MDS-*SF3B1* is a unique subgroup associated with favorable prognosis.[34,49,59,60] Unlike most of the other age-related variants we have described, *ASXL1, U2AF1,* and *TP53* mutations tend to carry a poor prognosis in AML and MDS.[34,60,61] Oddly, however, in our study, *U2AF1* and *TP53* were among the more common gene variants identified in patients without a diagnosis of a myeloid neoplasm. This may be skewed because of the very low number of MN- patients with any pathogenic and likely pathogenic mutation (most MN- patients had no mutations at all). Also, two patients with mutated *TP53* developed lymphoid malignancies, which is unsurprising as *TP53* is not a myeloid-specific tumor suppressor, but is widely prevalent across all cancers. It is highly possible that the remainder of the MN- patients at the time of this writing may still ultimately develop leukemia in the future.

We wondered if, perhaps, patients with a higher VAF of a pathogenic and likely pathogenic mutations in the initial genetic evaluation would more likely develop a hematologic malignancy. This may reflect larger clones that are outcompeting genetically normal progenitors, displaying growth and/ or survival advantages due to the mutations that would enable malignant transformation. Weeks *et al.* published an association between VAF ≥ 0.2 and incidence of myeloid neoplasm in their biobank of healthy adults.[62] It has also been demonstrated across somatic mutations that VAF is strongly correlated with tumor burden, disease severity, and/ or outcomes in AML and MDS, particularly in *TP53*, where the presence of biallelic *TP53* mutations is a strong predictor of abysmal prognosis.[59,60] In our study, however, there was no significant relationship between VAF of the most common mutations and the diagnosis of a myeloid neoplasm. In fact, patients who had a history or developed leukemia had qualitatively lower VAF of

U2AF1 than those with no myeloid neoplasm diagnosis. With larger sample sizes, we would be able to validate if this is statistically significant, though the mechanism driving this observation would seem counterintuitive to the mutation's generally adverse role in established cancers. It would also be interesting to determine if the VAF increases at a faster rate in those who develop a hematologic malignancy– that despite a similar VAF in the initial NGS panel to patients who do not develop cancer, perhaps MN+ patients show increasing or erratic VAF in subsequent genetic testing, whereas those who are MN- have VAF that remain stable over time.

We also investigated if there is an interplay between the development of PNH clones, myeloid neoplasm-associated somatic mutations, and leukemia. Similar to the relationship between age-related sporadic mutations and cancer, acquired mutations in *PIGA*, the pathognomonic X-linked gene in PNH, may not always result in classical PNH. *PIGA* mutations can be detected in hematologically normal adults; in murine models wherein *PIGA* expression is disrupted in embryearsnic stem cells, GPI-AP-negative cells remained stably low and without significant proliferation for up to a year after introducing the variant gene.[63-66] It has therefore been proposed that additional factors are needed for the expansion of PNH clones and clinical presentation of symptoms: chiefly, that there are autoimmune processes eliminating non-PNH competitors in the bone marrow and via the accumulation of additional driver mutations.[67,68]

For instance, *TET2*, as previously discussed may be a second-hit that provides a proliferation advantage for not only pre-leukemic cells, but also for PNH clones.[67] In mixed PNH/MPN syndromes, *PIGA* mutations occurred concomitantly with *JAK2*.[69]

Patients with PNH clones have also been shown to harbor karyearstypic abnormalities in up to 24% of cases, though these chromosomal aberrations fluctuated over time.[70] Moreover, in a larger study examining 114-gene NGS panels in PNH patients, 30.6% of patients had additional non-*PIGA* variants. The most common included *DNMT3A, ASXL1, U2AF1, SETBP1, RUNX1, PHF6,* and *BCOR/BCORL1*, all of which also have pathogenic and likely pathogenic roles in leukemia. In one of the cases with a concurrent *FLT3* mutation, the patient developed AML.[71] Others revealed concomitant mutations in *TET2, SUZ12, BCR-ABL1, NRAS, CALR*, *BRAF,* and *WT1*, though it remains unclear which mutations underlie disease progression and which are mere passenger mutations.[72-76]

Additionally, PNH and other bone marrow failure syndromes such as AA have an increased risk of developing hematologic malignancies, with a cumulative incidence of 11.6% development of myeloid neoplasms over ten years.[32,33] The risks are more profound in AA (15-20% over ten years) than in PNH (2-6%). More broadly, any patient with a clonal hematopoiesis has a greater lifetime probability of developing cancer than hematologically unaffected individuals (HR 2.4-12.9 across multiple studies).[47]

Certain mutations are associated with the development of secondary AML and MDS after bone marrow failure syndromes. For example, Negoro *et al.* demonstrated that patients who progressed from AA to secondary MDS were more likely to have myeloid neoplasm-associated mutations than those whose disease remained benign (50% vs. 8%). These mutations included *ASXL1, U2AF1,* and *JAK2*. Furthermore, compared to primary MDS, secondary MDS patients more frequently displayed loss of chromosome 7 or del(7q) and variants in *RUNX1* (and to a lesser degree, *TET2* and *SETBP1*).[77] In other

studies, AA patients with variants in *ASXL1, DNMT3A,* and monosomy 7 persisting for at least six months were more likely to develop MDS.[32,33,78,79] Conversely, mutations in *BCOR/BCORL1* appears to prognosticate lower risk of secondary leukemia following AA.[33,77,78,80] The data on somatic mutations in post-PNH myeloid neoplasms is more sparse, though patients have been identified to have an increased prevalence in, again, *ASXL1, RUNX1,* monosomy 7, as well as *NPM1, TET2, U2AF1,* and *WT1*.[17,32,33] Finally, several case reports described the persistence of cells including blasts, erythrocytes, granulocytes, and monocytes that were deficient in CD59 in patients with AML and MDS who had known histories of PNH, suggesting that PNH clones can directly transform into leukemic cells.[81-84]

Several possible hypotheses could unite all of these observations (the heightened risk of leukemia in patients with benign clonal disorders, the discovery in AA and PNH of driver mutations commonly associated with myeloid neoplasms, and the occasional malignant cells with CD55/59 deficiency). One compelling explanation is a combination of both autoimmune escape and spontaneous mutations in HSPC over time. A progenitor cell may acquire a *PIGA* variant, either alone or in conjunction with additional somatic mutations, particularly in an aging or failing bone marrow. This clone may exist at low levels, but may not necessarily have a sufficient growth advantage to become the dominant hematopoietic population. Over time, the clone may continue to accumulate additional passenger or driver mutations, particularly in epigenetic modifiers and spliceosomal regulators (which in turn could increase likelihood of mutagenesis). In a very small subset of patients, a "shock" may occur either through T-cell mediated attack and depletion of normal cells not deficient in GPI-AP and/or via the acquisition of a

sufficiently leukemogenic mutation (such as biallelic *TP53*) that transforms the once-benign clone into leukemia.

If this proposed mechanism is true, we might expect that the presence of PNH clones increases the odds of possessing leukemia-associated somatic mutations in MN+ patients. Indeed, in our study, we found that patients with PNH clones who had a diagnosis of a malignant myeloid disorder either preceding or following the initial genetic evaluation were significantly more likely to possess at least one pathogenic mutation in the initial NGS panel than those lacking PNH clones. Moreover, among patients without myeloid neoplasm diagnoses, those with PNH clones were modestly more likely to harbor genetic variants of interest, though this was not statistically significant. As may be expected, the number of patients with any malignancy-associated mutations in the MN-group was quite low; perhaps with larger sample sizes, we might be able to elucidate a positive correlation between PNH presence and somatic mutations even among the MN-patients.

Specifically, we demonstrated that in older patients ≥70 years, those with PNH clones are significantly more likely to harbor one or more pathogenic and likely pathogenic somatic mutation in their initial genetic evaluation. Meanwhile, in yearsunger patients, there is no significant association between the presence of PNH clones and of genetic variants. Based on these findings, we would recommend molecular testing for myeloid neoplasm-associated mutations in elderly patients ≥70 years who present with any size PNH clone by FLAER-based flow cytometry. Early detection and subsequent close surveillance of both PNH clones and possible leukemogenic genetic variants may allow for improved detection of hematologic cancers, differentiation of malignant and

benign states (for example, between AA and hypoplastic MDS, which can appear morphologically similar), prognosis, and choice of treatment. Although the presence of PNH clones correlated with a higher likelihood of discovering pathogenic and likely pathogenic mutations in patients who develop myeloid neoplasms, it was not associated with an increased number of mutations, at least in the initial NGS panel. Further studies are needed to characterize if the number of somatic mutations increase over time to determine if patients with PNH clones acquire additional variants more frequently than those without this clonal disorder.

We investigated if particular mutations were associated with PNH clone presence and found that patients with variants in *SF3B1* were significantly more likely to also have a PNH clone than patients with no pathogenic and likely pathogenic mutations. To a lesser, non-statistically significant extent, those with *DNMT3A* and *RUNX1* mutations also more frequently presented with PNH clones than patients with no relevant mutations. To our knowledge, *SF3B1* has not previously been demonstrated to be associated with the pathogenesis of PNH nor the progression to a myeloid malignancy, whereas *DNMT3A* and *RUNX1* mutations have been identified in PNH and AA patients who later developed leukemia. Nearly all *SF3B1* mutated cases (85.7%) developed into MDS, with one patient transforming to AML. However, in another study we found that there was no significant increase in the prevalence or size of PNH clones in MDS-*SF3B1* compared to other subtypes (unpublished data). It would seem, then, that *SF3B1* does not necessarily endow PNH clones with a competitive advantage over non-mutated HSPC, and may be considered more of a passenger or cooperating mutation on the pathway to MDS.

Our NGS findings in leukemia and PNH offer several clinical implications. Foremost, we recommend that elderly patients ≥70 years who have PNH clones of any size should be evaluated for myeloid neoplasm-associated somatic mutations as they are more likely to harbor a variant of interest. Overall, while AML, MDS, MPN, and their overlap disorders, already frequently present with malignancy-related variants, possessing PNH clones further augments the likelihood of identifying a pathogenic or likely pathogenic somatic mutation in the initial genetic evaluation. This observation aligns well with the mildly increased risk of developing leukemia in patients with clinical PNH.

We hypothesize two possible mechanisms that underlie the association between PNH clones, somatic driver mutations, and hematologic malignancies. First, PNH mutations may actively aid in leukemogenesis: HSPC with *PIGA* mutations acquire additional somatic mutations, such as those found commonly in myeloid disorders or aging hematopoiesis, and manage to evade immune detection due to their GPI-AP deficiency. With time, either through the acquisition of driver mutations that offer a sufficiently large proliferation advantage over non-mutated HSPC, or because competing cells in the bone marrow are depleted via T-cell mediated attack, these PNH clones become the dominant population. The additionally acquired pathogenic mutations allow the transformation of these previously benign clones to malignant cells. Alternatively, the *PIGA* variant is an independent and incidental marker for genetic stress in the (often aging) bone marrow that would have developed leukemia even without the PNH population. In this case, *PIGA* acts as a passenger mutation and could be an early indication of impending neoplasm.

We also demonstrated that several somatic mutations commonly found in myeloid cancers (namely, *DNMT3A, TET2, ASXL1, U2AF1, SF3B1, SRSF2,* and *TP53*) are highly correlated with older age, in concordance with the literature. Of these, the trifecta of *TET2, ASXL1,* and *SRSF2* often appear in conjunction and are likely associated with a myeloproliferative phenotype. Additionally, we noted that *EZH2* and *ASXL1*, as well as *SF3B1* and *RUNX1* tend to cluster together, especially in patients who develop leukemia. Similar to the trifecta, concomitant *EZH2* and *ASXL1* have been described to be associated with CMML and myelofibrosis.[53] That *ASXL1* is present in both of these gene clusters associated with MPN and MDS/MPN is perhaps unsurprising as murine knockout and *in vitro* models have implicated the mutation in impaired HSPC differentiation.[85] We are uncertain if *SF3B1* and *RUNX1* are truly concomitant with increased frequency in hematologic conditions; we would require larger sample sizes to validate this observation. Of note, *RUNX1* variants may worsen the prognosis in MDS with *SF3B1* mutations, which otherwise usually portends more favorable outcomes.[35,86]

There remains much work to be done to differentiate benign, non-pathogenic instances of leukemia-associated mutations presenting as ARCH or CHIP from those who will ultimately progress to malignancy. As we and others have observed, the presence of somatic driver mutations alone is sometimes insufficient to drive disease. Nevertheless, molecular testing for acquired variants of interest remains, and should remain, an integral part of care for patients with hematologic cancers as they can guide prognostication and therapeutic choice in a field with burgeoning targeted treatments. The identification of a *FLT3* mutation in an AML patient may prompt the use of FLT3 inhibitors such as

midostaurin or gliteritinib, for example, or caution against the use of hypomethylating agents as the mainstay for those with *TP53*.[35,60]

CHALLENGES & LIMITATIONS

This study possesses several critical limitations. Due to the retrospective nature of the research, it is not possible to identify causality in the associations between PNH clone presence, hematologic disorders, and myeloid neoplasm-associated somatic mutations. For instance, we are curious if certain genetic variants commonly found in patients with both PNH clones and myeloid neoplasms actively participate in the pathogenesis of PNH and/or malignant transformation, or if they are merely incidentally acquired in aging progenitors. This knowledge would aid in better differentiating benign age-related genetic changes and true pathogenic aberrations. In particular, we identified that many somatic driver mutations prevalent in our cohort of patients who developed cancer occurred concomitantly. Murine and *in vitro* models would provide help characterize the molecular mechanisms of these mutations in hematopoiesis and how they might function (or dysfunction) synergistically (which combinations of mutations increase an HSPC's neoplastic growth?).

Importantly, we also were not able to determine if leukemic cells directly arose from GPI-AP deficient progenitors, which would provide supporting evidence that PNH clones possess increased leukemogenicity. With biobanked blood and bone marrow cells derived at different time points (from grossly hematologically normal to cancer diagnosis), we would be able to track the progression of clonal cells as they accumulate mutations and morphologic dysplasia over time. Alternatively, targeted deep-sequencing of biospecimens of mutations including *PIGA* and their VAF in cancer patients could be applied to identify if tumor cells originated as PNH clones and to infer the sequence of events from clone appearance to malignancy with gene ontogeny algorithms.[28,71]

As we interrogated PNH clone size with FLAER-based flow cytometry and acquired genetic variants with NGS panels obtained during the initial evaluation, we may be only obtaining a snapshot of the ever-changing bone marrow dynamics, rather than a comprehensive view over the course of disease. For example, we did not find any significant differences in the VAF of oncogenes and tumor suppressor genes in our initial NGS panels between non-leukemic and leukemic patients, despite VAF likely being ultimately greater in those with neoplasms.[47] In a subset of the patients in our cohort for whom we have multiple molecular studies, we will track the changes in PNH clones and mutations to identify, for instance, if those with PNH clones have a significantly greater increase in VAF or number of driver mutations than those without. However, not every patient has multiple studies; specifically, patients who had no somatic mutations or very small GPI-AP deficient clones in their initial evaluation may be underrepresented as clinicians are much less likely to follow these patients over time. Finally, although we described several trends in common somatic mutations, we would need larger sample sizes, especially of the rarer driver mutations discovered in MN- patients, to draw more definitive conclusions.

DISSEMINATION

The work presented in this thesis has not yet been shared publicly with the scientific or general community, though the authors plan to do so in the near future.

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