IDENTIFYING NOVEL GENOMIC VARIANTS IN HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS IN ADULTS

by

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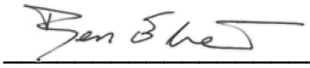

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TABLE OF CONTENTS

ABSTRACT 3
INTRODUCTION 4
METHODS 7
RESULTS 9
DISCUSSION 13
CONCLUSIONS 17
REFERENCES 18
TABLES AND FIGURES 20

ABSTRACT

Hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening disorder of immune system overactivation that occurs in familial (primary) and acquired (secondary) forms. While primary HLH is caused by inherited mutations in genes that regulate cellular immunity, secondary HLH usually occurs in the setting of infection, malignancy, or autoimmune disease and may be diagnosed at any age, including well into adulthood. Although these two forms of HLH have historically been regarded as distinct entities, evidence suggests that a subset of adults with HLH may in fact harbor an underlying genetic predisposition. In this study, a cohort of 88 adult patients with HLH were interrogated for germline genetic variants associated with primary HLH as well as somatic variants associated with clonal hematopoiesis (CH). Among the 17 germline genes sequenced, 7 variants in 18 patients were considered to be disruptive based on in silico model predictions. Among the 80 patients for whom somatic variant analysis was performed, CH with a variant allele frequency (VAF) greater than 0.02 was identified in 21 patients. The rate of disruptive germline variants and somatic variants associated with CH were found to be enriched relative to control populations. Overall, the results suggest that germline variants likely do not drive HLH in adults and that CH is more common in adults with HLH, although whether this represents a causal relationship remains unclear.

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is a rare, life-threatening disorder of immune system overactivation that occurs in familial (primary) as well as acquired (secondary) forms. Primary HLH is caused by inherited mutations in genes that regulate the cytotoxic function of T lymphocytes and natural killer (NK) cells, and is typically diagnosed early in childhood. By contrast, secondary HLH usually occurs in the setting of infection, malignancy, or autoimmune disease and may be diagnosed at any age, including well into adulthood. Although these two forms of HLH have historically been regarded as distinct entities, evidence suggests that a portion of adults who develop HLH may in fact harbor an underlying genetic predisposition.[1] Currently, the diagnostic criteria for HLH in adults is limited to a constellation of clinical symptoms and laboratory findings established by the HLH-2004 treatment protocol (Figure 1).[2] As a result, further investigation into the genetic basis of HLH in adults is warranted in order to identify those at increased risk, inform potential treatment decisions, and improve outcomes.

HLH was first described in two infant siblings with progressive cytopenias, hepatosplenomegaly, persistent fevers, and evidence of hemophagocytosis at autopsy.[3] These symptoms have been subsequently described in both pediatric and adult populations. While the familial form of HLH is well-described in the pediatric population, adult-onset HLH likely remains under-recognized, as many of the symptoms are nonspecific and overlap with other causes and manifestations of severe illness. Nevertheless, the number of reported cases of adult-onset HLH in the literature has increased considerably over the past decade. Today, it is estimated that adults may comprise up to 40% of all cases, with a median age at diagnosis of about 50 years.[4]

Left untreated, adult-onset HLH has a poor prognosis and is often fatal. Even in the presence of HLH-directed therapy, mortality approaches 70% with a median overall survival of only 4 months.[5] The current treatment algorithm of HLH in adults focuses on identifying and addressing the underlying trigger while simultaneously controlling the overactive immune system with immunosuppressive agents. Standard therapies are primarily derived from retrospective case series, and extrapolated from guidelines for the treatment of primary HLH in the pediatric population. Therapy is typically comprised of a combination of steroids, chemotherapy, a calcineurin inhibitor, and/or hematopoietic stem cell transplantation (HSCT).

In its familial form, HLH is caused by inherited, autosomal recessive mutations in genes responsible for the cytotoxic function of T lymphocytes and NK cells, including the gene that encodes perforin (PRF1) and several genes responsible for the regulation vesicle priming and fusion (UNC13D, STX11, and STXBP2/UNC18B), also known as the degranulation pathway. Loss-of-function alterations in these genes result in ineffective target cell lysis, aberrant macrophage activation, and the overproduction of proinflammatory cytokines. Notably, primary HLH can also manifest as a consequence of rare inherited immunodeficiency syndromes, including Chediak-Higashi syndrome (LYST), Griscelli syndrome (RAB27A), and X-linked Lymphoproliferative syndrome (SH2D1A and XIAP). By contrast, adult-onset HLH has been defined not by genetic alterations but by clinical presentation. While the disease is often triggered by an infection, underlying malignancy, or autoimmune disease, nearly a quarter cases in adults have no discernable trigger, leading some to speculate that less virulent germline variants in these same genes may contribute to development of HLH at an older age.[6]

At the same time, little attention has been paid to the potential role of alterations in other inflammatory pathways in the development of adult-onset HLH. As some patients do not manifest the disease until the seventh or eighth decade of life, it may be hypothesized that somatic alterations, particularly those associated with aging and hyperinflammatory states, may also play a role. Clonal hematopoiesis (CH) is a well-described phenomenon in which hematopoietic stem cells acquire defined somatic mutations that confer a clonal proliferative advantage.[7] While these somatic alterations are rare in healthy people younger than 40 years of age, they increase in frequency with age and have been linked to a number of inflammatory diseases.[8] Sensitive sequencing technologies have both confirmed CH as a widespread process inexorably linked with aging and also allowed for the detection of mutant clones with variant allele frequencies (VAFs) as small as 0.1%.[9]

The increasing incidence and poor prognosis of HLH in adults mandates further investigations to attempt to identify both germline and somatic alterations that drive the disease and, importantly, distinguish it from HLH in the pediatric population. An improved understanding of HLH in adults would allow for the *a priori* identification of adults who may be at high risk for developing HLH in the context of a known trigger, inform decisions regarding suitable transplant donors, and develop novel treatment strategies unique to the adult population. In this study, we sought to identify novel genetic variants, both germline and somatic, that may explain the etiology of HLH in the adult population.

METHODS

After obtaining institutional review board approval, bone marrow aspirate samples were obtained from adults treated at Brigham and Women's Hospital (Boston, MA) and Dana-Farber Cancer Center (Boston, MA) between 2001 and 2018. Each of these patients met HLH-2004 criteria, although some patients did not have complete information for each of the criteria on chart review. Genomic DNA was isolated from these samples using the DNEasy Kit (Qiagen, Hilden, Germany) according to the manufacturer protocol.

For the germline variant analysis, sequencing was performed using a custom SureSelect system (Agilent Technologies, Santa Clara, CA) targeting the exons of 17 genes implicated in familial HLH. This collection of genes occurs in the context of other inherited immune disorders and have either been described in the literature as associating with HLH or are tested on commercial HLH panels (AP3B1, BLOC1S6, BTK, CD27, IL2RG, ITK, LYST, MAGTI, PRF1, RAB27A, SH2D1A, SLC7A7, STX3, STX11, STXBP2, UNC13D, and XIAP). Sequencing was performed on the Illumina platform (Illumina, San Diego, CA). Germline variant calling was done using the GATK Haplotype caller pipeline. Common variants were excluded by removing alterations with a genome aggregation database (gnomAD) frequency of >0.05.

The effect of variants on protein function were predicted using several in silico tools (PON-P2, PROVEAN, FATHMM, M-CAP, and REVEL). Variants were classified as likely to be disruptive to protein function if at least 2 of these tools suggested the variant was disruptive and none of the tools classified the variant as nondisruptive. If a variant was classified as nondisruptive by a single tool, the variant was still considered

disruptive if 3 or more tools classified the variant as likely disruptive. The effect of splice site variants was predicted as previously described.[10]

For the somatic variant analysis, sequencing was performed using a separate custom SureSelect system that targeted exons from 98 genes implicated in clonal hematopoiesis. Sequencing was similarly performed on the Illumina platform. Variants were identified using Varscan 2.2.3 and annotated using Annovar. Variants were scored based on allele fraction, strand bias differential, noise and mapping quality, and frequency in germline polymorphism databases. Variants with a VAF > 0.02 were considered to be consistent with clonal hematopoiesis. Identified variants were manually inspected in Integrated Genome Viewer (Broad Institute, Cambridge, MA).

Statistical comparisons for the enrichment of germline variants in the HLH cohort versus control population was performed using a Fisher's exact test with $P < 0.05$ considered significant. Statistical comparisons for differences in clinical characteristics were performed using a two-tailed t-test. All analyses were performed using Stata 16.0 (Statacorp, College Station, Texas).

RESULTS

Cohort description

The cohort investigated in this study included 88 adult patients with HLH (Table 1). The median age at diagnosis was 54 years old (range, 18-81 years old) and the majority were male (55%). All patients met HLH-2004 criteria. An underlying etiology was identified in most cases (77%). A portion of patients had multiple etiologies (16%). The most common etiologies were malignancy (49%), infection (30%), and autoimmune disease (17%). Thirty-eight patients (43%) were diagnosed with a lymphoid malignancy. Although clonal hematopoiesis of indeterminate potential (CHIP) is defined by the absence of a hematologic malignancy, patients with a lymphoid malignancy were included in the analysis of somatic variants associated with CH. Eight patients (9%) were diagnosed with a myeloid malignancy, including 3 patients who had diagnoses of both lymphoid and myeloid malignancies. Due to the significant overlap between mutations identified in myeloid malignancy and those that characterize CH, patients with a myeloid malignancy were excluded from the analysis of somatic variants.

Germline variant analysis

Among the 17 germline genes sequenced, a total of 42 variants with a gnomAD frequency of <0.05 were identified in 45 patients. Of these, 7 variants in 18 patients were considered to be disruptive based on in silico model predictions (Figure 2A). Fourteen patients harbored one disruptive variant, while 4 patients harbored multiple disruptive variants. The most common disruptive variant was PRF1 A91V (n = 12; 14%),

which is a commonly reported alteration in adult HLH that has been shown to impair lymphocyte cytotoxicity.[11,12] Three patients had SLC7A7 A91V alterations. The remaining disruptive variants were limited to individual subjects and included PRF1 H222Q, ITK R581Q, LYST R2624W, SH2D1A R55Q, and STX3 splice site variants. Two patients harbored multiple disruptive lesions, both of which included one PRF1 A91V variant; the second variants were PRF1 H222Q and SLC7A7 A91V. One patient had biallelic PRF1 A91V variants. No disruptive variants were identified in other genes implicated in familial HLH, including UNC13D, STX11, or STXBP2 (Figure 2B).

In order to determine whether the germline variants identified in our cohort were enriched relative to the general population, whole-exome data from 2504 patients in the 1000 Genomes Project (TGP) were interrogated in a similar fashion using the same 17 genes of interest. The specific variants identified in our cohort were significantly less common in the TGP (20% vs. 5%; $P < 0.001$). When this analysis was liberalized to include any variant in the TGP dataset that was predicted to be disruptive, our cohort still had a significantly higher frequency of disruptive variants compared with the TGP (20% vs. 9%; $P < 0.01$). Next, we limited our cohort based on ancestry, as “white” or “Caucasian” ancestry comprised the most common ancestry in our cohort (n = 68, 77%). When the frequency of identified variants in the cohort were comparted with only those of European ancestry in the TGP, the result was no longer significant (20% vs. 9%, $P = 0.13$). Furthermore, when patients with a PRF1 A91V variant were excluded, there was no significant difference between the frequency of variants identified in our cohort and those identified in the TGP (4.4% in both cohorts) (Figure 2C).

Finally, we sought to determine whether the presence of a disruptive variant was associated with any clinical features identified in the cohort. Disruptive variants were not associated with age (mean age, 53 vs 51 years; P = 0.64), sex (33% vs 49% female; P = 0.22), or etiology including malignancy (19% vs 22%; P = 0.79). There was no difference in the frequency of variants in patients meeting 5 to 6 HLH criteria (20%) and those meeting 7 to 8 criteria (21%). Finally, no differences were identified in patients with or without PRF A91V variants.

Somatic variant analysis

Patients were omitted from the analysis of somatic variants if they had a history of a myeloid neoplasm. Among the remaining 80 patients for whom somatic variant analysis was performed, CH with a VAF > 0.02 was identified in 21 patients (26%) (Figure 3A). Patients with clonal hematopoiesis were significantly older (median age, 58 vs 48 years; P = 0.025). The prevalence of clonal hematopoiesis was not significantly different between those who had or had not received prior radiation (30% vs 25%, P = 0.77). DNMT3A and TET2 were the most commonly mutated genes.

Given the large number of patients in our cohort with a history of lymphoid malignancy (44%), a similar analysis was performed after controlling for the presence of a lymphoid malignancy. While patients with a lymphoid malignancy were significantly older than those without a lymphoid malignancy (median age, 63 vs 43 years old, P < 0.01), there was a similar prevalence of clonal hematopoiesis in both groups (34% vs 20%, P = 0.20).

In order to determine if clonal hematopoiesis was more common in HLH than in healthy, age-matched controls, we compared our cohort to prior published reports of clonal hematopoiesis (Figure 3B).[9,13,14] Two of these reports used targeted panels in a large number of health controls and one used whole genome sequencing in the Trans-Omics for Precision Medicine (TOPMed) Program. In the targeted sequencing cohorts, the prevalence of CH with a VAF > 0.02 was approximately 2- to 15-fold higher in the HLH cohort. Due to the lower sequencing dept in the TOPMed cohort, a VAF > 0.1 was used. Using this threshold, CH was approximately 3-fold more prevalent in our HLH cohort. These data indicate that the prevalence of CH in patients with HLH is higher than the prevalence of CH in the general population, and is unlikely to be driven by the age of the cohort alone.

DISCUSSION

In this study, a cohort of patients with clinically diagnosed adult-onset HLH were interrogated for both germline and somatic genetic variants hypothesized to be related to their disease. Specifically, genes assessed for germline variants were a comprehensive set previously implicated in in familial forms of HLH, while the genes assessed for somatic variants are those known to be implicated in clonal hematopoiesis. Overall, our results suggest two findings. First, in contrast to familial HLH, germline variants alone are unlikely to be responsible for driving disease in the adult population. Second, clonal hematopoiesis is likely more prevalent in adults with HLH compared to the general population.

Assessing the contribution of germline variants in adults with HLH is difficult, as the clinical presentation of HLH in the relatively well-studied pediatric population in which germline defects are frequently identified is already diverse and dictated not only by the mutated gene but also by the type of mutation or the combination of multiple autosomal recessive mutations. For example, patients who carry the canonical loss-of-function mutation in PRF1 present earlier and with greater defects in NK cell activity than patients who carry a missense mutation in the same gene.[15] Similarly, patients with a splice-site mutation in STXBP2 present later than patients with nonsense mutations.[16] Finally, patients who are compound heterozygotes for 2 mutations in the degranulation pathway tend to present around the same age as patients who are homozygous for biallelic mutations while patients who have a single heterozygous mutation or are compound heterozygotes for a mutation in the degranulation pathway and a mutation in

perforin tend to present later.[17] Studies have demonstrated that the accumulation of monoallelic mutations in HLH-causing genes impairs lymphocyte cytotoxicity and contributes to HLH pathology in mice.[18] Thus, at least in patients with familial HLH, the type and combination of mutations appears to play an important role in dictating the age of onset and severity of disease.

This proposed phenomenon may help to explain why variants in the familial HLH genes are being identified with increasing frequency in patients who manifest the disease during adulthood. Zhang, et al. identified hypomorphic missense and splice-site variants in 14% of 175 adult patients who were suspected of having HLH.[19] The majority of these variants were in perforin and in silico analyses of the structural effects of the missense variants ranged from “benign” to “probably damaging”. Wang, et al. identified mutations in 6 HLH-associated genes in 7.1% of 252 Chinese adults with perforin variants again being the most common.[20] However, a high-throughput sequencing panel of 12 HLH-associated genes was unable to arrive at a genetic diagnosis for any of the 8 patients studied who were diagnosed at 18 years of age or older.[21] Moreover, this study determined that, with the exception of the PRF1 A91V variant, rare and putatively pathogenic monoallelic variants in HLH-associated genes are not enriched compared to healthy individuals. By contrast, a report of 112 adults with HLH in China concluded that germline variants were indeed enriched in this population (43%), however the variants identified were primarily missense and monoallelic.[22] To date, there has been significant variability across studies which can be attributed to differences in ancestral composition, and methods for predicting disruption.

In our study, alterations including deletions, nonsense, and frameshift mutations were very rare. While the disruptive variants that we identified were enriched compared to a cohort of healthy individuals, this association disappeared when controlling for ancestry, particularly when considering non-*PRF1* A91V variants. Additionally, there were no significant clinical differences observed between patients with and without disruptive variants. Moreover, the relatively high frequency of disruptive variants (9%) observed in the control population suggests either that the in silico model overestimates the severity of the disruption or that the variants identified are not significant drivers of disease. Overall, our analysis does not strongly suggest that all individuals with adult-onset HLH should be tested for potential germline variants. This does not preclude the consideration of testing for variants in cases in which familial HLH is thought to be particularly likely, such as cases with a young age-of-onset or strong family history, as understanding the genetic landscape may influence the decision to select donor for allogeneic stem cell transplant.

The role of somatic variants in driving HLH, on the other hand, remains poorly understood. In addition to being associated with the development of malignant myeloid neoplasms, clonal hematopoiesis has been shown to be associated with diseases of immune system hyperactivation, including rheumatoid arthritis.[23] Most notably, CH has been shown to confer an increased risk of atherosclerotic cardiovascular disease and insulin resistance.[24,25] In mice, TET2 loss-of-function mutations in myeloid cells promoted atherosclerosis and proinflammatory cytokine production.[26]

In our study, we found that clonal hematopoiesis is indeed more prevalent in adults with HLH. Multiple potential explanations exist for this association. The first is

causal, suggesting that clonal hematopoiesis does indeed drive the development of HLH in adults. Alternatively, it is possible that the increased prevalence of clonal hematopoiesis represents a natural response to aberrant cytokine signaling and bone marrow stimulation associated with HLH.

Functional studies have been performed to address this question of causality. In one study, bone marrow-derived macrophages bearing a common mutation associated with clonal hematopoiesis were challenged with a Toll-like receptor (TLR) agonist implicated in adult-onset HLH and found these macrophages exhibited an exaggerated inflammatory response to TLR stimulation.[27] While preliminary, this work suggests that clonal hematopoiesis can enhance the hyperinflammatory state associated with adult-onset HLH.

This study has several limitations. Given the rarity of HLH, our cohort is limited in size. Despite obtaining good yields from the DNA extraction process, we were unable to extract DNA from several biospecimens. Unlike prior studies that were limited by cohort size, our study includes strict diagnostic inclusion criteria, a higher median age, an expanded gene panel for the germline analysis. Additionally, our study incorporated ancestry which proved important when performing the germline analysis. Another limitation was the use of in silico algorithms to predict the effect of mutations on protein function. Future studies to better define disruptive variants in larger numbers of genes, including whole-exome sequencing, as well as larger cohorts of adult patients with HLH will help to further elucidate the genetic basis of the disease. Additional functional studies may help to reveal whether or not there is a causal relationship between CH and development of HLH.

CONCLUSIONS

Our analysis of germline genetic variants suggests that the pathophysiology of adult-onset HLH likely distinct from the familial form of the disease. In addition, while clonal hematopoiesis is enriched in adults with HLH, the causality of this association is unclear. In this setting, the role of clonal hematopoiesis in HLH may be best understood as a potential risk factor that may place an individual at increased risk of developing the hyper-inflammatory state associated with HLH in the adult population.

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TABLES AND FIGURES

Figure 1. HLH-2004 Criteria.

The diagnosis of HLH can be established if one of either 1 or 2 is fulfilled:

(1) A molecular diagnosis consistent with HLH

(2) At least 5 of 8 diagnostic criteria:

- Fever (38.5°C for > 7 days)
- Splenomegaly (3 cm below costal margin)
- Cytopenias (2+ lineages)
  - Hemoglobin < 9 g/dL
  - Platelets < 100 x 10^9 / L
  - Neutrophils < 1.0 x 10^9 / L
- Hypertriglyceridemia and/or hypofibrinogenemia
  - Fasting triglycerides ≥ 265 mg/dL
  - Fibrinogen ≤ 1.5 g/L
- Hemophagocytosis in bone marrow, spleen, or lymph nodes
- Low or absent NK-cell activity
- Ferritin ≥ 500 ug/L
- Soluble CD25 (i.e., soluble IL-2 receptor) ≥ 2,400 U/mL

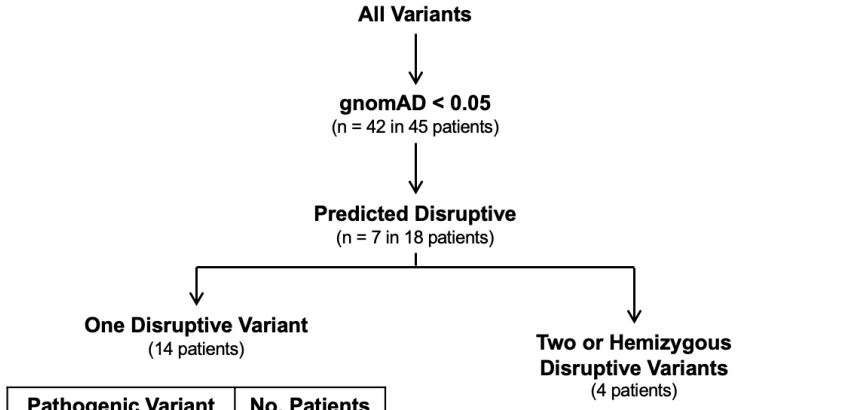
Table 1. Clinical Characteristics of Patients with Adult-Onset HLH.

Total Patients	**N = 88**
Median age (range)	54 (18-81)
Median age (n, %)	
<40	23 (28)
40-59	33 (38)
>60	32 (36)
Sex (n, %)	
Male	48 (55)
Female	40 (45)
Self-reported ethnicity (n, %)	
White or Caucasian	68 (77)
Hispanic or Latino	5 (6)
Asian	5 (6)
Black or African-American	2 (2)
South Asian or Indian	1 (1)
Unknown/not reported	7 (8)
Precipitating etiology (n, %)	
Malignancy	43 (49)
Infection	26 (30)
Autoimmune disease	15 (17)
Multiple	14 (16)
Idiopathic	20 (23)
HLH-2004 criteria (n, %)	
5*	26 (30)
6	38 (43)
7	21 (24)
8	3 (3)

*Three patients were diagnosed by the treating clinician as meeting at least 5 HLH-2004 criteria but had only 4 documented criteria based on incomplete information in the clinical database

Figure 2. Germline Variant Analysis. (A) Schematic of germline variant frequency within cohort among 17 HLH-associated genes sequenced. (B) Frequency of total and predicted disruptive germline variants observed in familial HLH genes in cohort (C) Percentage of individuals in HLH and the 1000 Genomes Project (TGP) cohort with variants in HLH-associated genes.

A.



Pathogenic Variant	No. Patients
PRF A91V	9
SLC7A7 A91V	2
ITK R581Q	1
LYST R2624W	1
STX3 Splicing	1

Pathogenic Variants	No. Patients
PRF A91V + *SLC7A7* A91V	1
PRF A91V + *PRF* H222Q	1
PRF A91V + *PRF* A91V	1
SH2D1A R55Q*	1

B.

Gene	Patients with Variant (gnomAD < 0.05)	Patients with Predicted Disruptive Variant
PRF1	14	12
UNC13D	13	0
STX11	4	0
STXBP2	6	0

C.

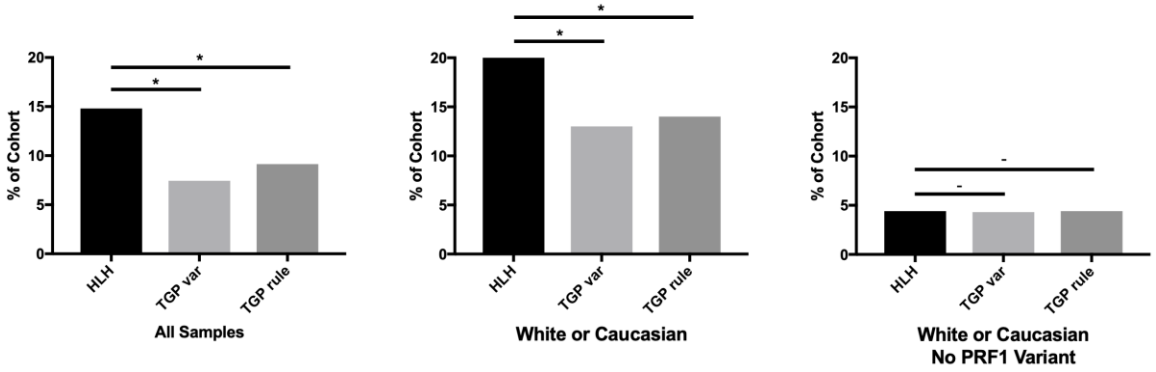


Figure 3. Somatic Variant Analysis. (A) Characteristics of patients in the cohort included for somatic variant analysis (i.e., those without a known myeloid malignancy), stratified by the presence of absence of clonal hematopoiesis (CH). Statistical comparisons were performed using either Fisher's exact or Mann-Whitney tests. "Cytotoxic Therapy" refers to treatment directed at the malignancy associated with the HLH diagnosis, where applicable. (B) Frequency of CH with a variant allele frequency > 0.02 identified in the HLH cohort and healthy individuals from cohorts utilizing targeted sequencing of 71 genes (Acuna-Hidalgo et al.) or 111 genes (Abelson et al.), or frequency of CH with a variant allele frequency > 0.01 using whole-genome sequencing (Bick et al.).

A.

Characteristics	Total	CH	No CH	
Patients (n, %)	80	21 (26)	59 (74)	
Age (median, IQR)	52 (36-83)	58 (51-70)	48 (32-60)	P = 0.025
Gender (n, %)				P > 0.99
Male	43 (54)	11 (26)	32 (74)	
Female	37 (46)	10 (27)	27 (73)	
Cytotoxic Therapy (n, %)				P > 0.77
Received	20 (25)	6 (30)	14 (70)	
Never Received	60 (75)	15 (25)	45 (75)	
Specimen Source (n, %)				P > 0.75
Bone Marrow	64 (80)	16 (25)	48 (75)	
Peripheral Blood	16 (20)	5 (31)	11 (69)	
Lymphoid Malignancy (n, %)				P > 0.20
Present	35 (44)	12 (34)	23 (66)	
Absent	45 (56)	9 (20)	36 (80)	

B.

Cohort	All	<60 years	> 60 years
Adult HLH	26%	22%	36%
Acuna-Hidalgo, et al. (2017)	2%	1%	5%
Abelson, et al. (2018)	11%	7%	14%
Bick, et al. (2020)	3%	N/A	N/A